THE EFFECT OF THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS ON THE PRODUCTION OF PHYTOCHEMICALS IN BASIL

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Thesis submitted for the degree of

Doctor of Philosophy

Faculty of Sciences

Discipline of Soil and Land Systems

School of Earth and Environmental Sciences

The University of Adelaide

May 2008

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Summary

The overall objective of this thesis was to investigate how the arbuscular mycorrhizal (AM) symbiosis can affect the production of phytochemicals (antioxidants; rosmarinic and caffeic acid, RA & CA) in the shoots of basil (*Ocimum basilicum* L.). As a result of an increasing interest in natural/herbal medicines, more effort is now needed to produce herbal products of better quality, *i.e.* higher and standardised phytochemical concentrations. Thus, it was hypothesised that the naturally occurring AM fungi (AMF) could play an important role in improving the growth and phytochemical concentrations in medicinal herbs such as basil, as organic methods of cultivation are increasingly sought after to grow such plants.

Despite a reasonable amount of information available in the literature on the changes of phytochemical concentrations in the roots of host plants following AM colonisation, very little is known about such processes in the aerial part of such plants. Furthermore, basil has hardly been studied as a host plant in AM research, and very little is known of its responsiveness to AM colonisation. As AMF are well known to improve phosphorus (P) uptake in their host plant, the first objective of this work was to obtain AM and non-mycorrhizal (NM) plants matched for tissue P concentrations and growth rates. Only under such conditions would it then be possible to separate benefits derived from improved plant P uptake from non-nutritional benefits.

It was found that basil is highly responsive to P, and that under low or little P supply it is quite dependent on the AM symbiosis in order to grow. However,

growth depressions were observed when growing basil in winter with *Glomus intraradices*, suggesting that the fungal symbiont can act as a strong sink of carbon (C) under such conditions. Thus, in order to obtain AM and NM plants with matched growth rates and tissue P concentrations, it was found that basil needed to be cultivated in summer in a soil/sand mixture with a ratio of 1:3 (w/w), along with 0.2 g/kg $CaHPO_4$ and 25% of AM inoculum (AM plants). Under these conditions, AM plants grew as well as NM plants and *G. caledonium* and *G. mosseae* were shown to increase the concentrations of RA and CA in the shoots of basil, but not in roots. Such results were not an indirect effect of improved P uptake.

In order to understand the mechanisms by which AMF increased RA and CA concentrations in basil, further experiments were set up to investigate the effect of 1) AM developmental stages, 2) nitrogen (N) supply and 3) phytohormone changes on the production of RA and CA in the shoots. None of these factors was found to contribute to increases in antioxidants in basil under AM symbiosis. Therefore, the mechanisms by which AMF affect RA and CA concentrations in basil still remain unknown. A final experiment was carried out to investigate the potential of an AM fungus to improve the growth of basil when challenged with a specific pathogen *Fusarium oxysporum* f.sp. *basilici* (*Fob*), which causes significant production losses. The results showed that inoculation of basil with *G. mosseae* not only improved plant growth compared to NM plants, but also conferred a protective effect against *Fob*. However, shoot antioxidant concentrations (RA, CA, total phenolics and essential oils) were not increased in

AM plants compared to NM plants, and the mechanism of protection against *Fob* could not be elucidated.

Due to the high variability of RA and CA concentrations obtained in AM plants in different experiments, it cannot be concluded that AMF confer an absolute advantage over uninoculated plants if the main concern is to obtain standardised concentrations of phytochemical in basil. On the other hand, the key results presented in this thesis do indicate that inoculating basil with AMF can be beneficial to improve its growth as well as antioxidant concentrations, compared to NM plants grown under similar conditions. Such results could be of potential interest to basil growers who wish to cultivate this medicinal herb organically (*i.e.* low P supply and no chemical fertilisers added).

Publications and presentations from the thesis

Journal papers

Toussaint JP, Kraml M, Nell M, Smith SE, Smith FA, Steinkellner S, Schmiderer C, Vierheilig H and Novak J. **2008**. Effect of *Glomus mosseae* on the concentrations of rosmarinic and caffeic acids and essential oil compounds in basil inoculated with *Fusarium oxysporum* f.sp. *basilici*. Accepted in *Plant Pathology*.

Toussaint JP. **2007**. Investigating physiological changes in the aerial parts of AM plants: what do we know and where should we be heading? *Mycorrhiza* **17**: 349-353.

Toussaint JP, Smith FA and Smith SE. **2007**. Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. *Mycorrhiza* **14**: 291-297.

Conference talks and posters

Toussaint JP, Smith FA, Smith SE. **2006**. The influence of AM on the production of phytochemicals in sweet basil*. *5th International Conference On Mycorrhizas* (ICOM5), Granada, Spain.
*BIOTISA student award for best presentation in biotechnology
http://www.mycorrhizas.org/files/NEWSLETTER_2006_NOVEMBER.PDF

Toussaint JP, Smith FA, Smith SE. **2006**. Investigating the effects of AM fungi on phytochemical production in sweet basil. *Centre for Soil and Plant Interaction workshop* (CSPI), Adelaide, Australia.

Toussaint JP, Smith FA, Smith SE. **2005**. Improving the yield of active compounds in medicinal plants through the arbuscular mycorrhizal symbiosis. *CSPI workshop*, Adelaide, Australia.

Toussaint JP, Smith FA, Smith SE. **2005**. Can the AM symbiosis improve the yield of active compounds in medicinal plants? *Australian Society for Biochemistry and Molecular Biology meeting* (COMBIO), Adelaide, Australia. (poster)

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University Library.

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Jean-Patrick Toussaint

May 2008

Acknowledgments

Doing a Ph.D. is not an easy dish to prepare. Although the recipe might vary from one person to the next, I would say that it takes 1 cup of commitment, 1 cup of perseverance and approximately ½ cup of wits. To this, you can add some fun-times, according to your taste…though not too much, otherwise the whole thing might turn sour. However, one key ingredient that does not always appear in the cook book is the people that you meet during this journey. They are the "secret ingredient" that will make the difference in creating a "Chef-d'oeuvre" (or so to speak)! Therefore, I would like to take this opportunity to acknowledge and thank all of those who made this thesis possible – I would not dare say it is a "Chef-d'oeuvre", but hopefully good enough to be a "Main dish".

First, I would like to deeply thank Professors Sally and Andrew Smith, my principal supervisors. Their knowledge, wisdom, dedication and support have been outstanding and of great inspiration to me. They are the reason that made me decide to come to Adelaide to pursue my Ph.D., and, without a doubt, I would have gone astray many times during my project if not for them. They have always respected my opinions, and my "stubbornness" at times, to lead this project to completion. Thank you for all your tremendous help and friendship. I am also very grateful to Dr Horst Vierheilig who became a co-supervisor during the course of my candidature. Dr Vierheilig has been very inspiring, as well as being open-minded to the ideas that were tossed his way, which led to research projects that we conducted together. He has also been truly generous of his time as well as being supportive. Thank you!

I am grateful to everyone in Vienna, who welcomed me during my short stay in Dr Vierheilig's lab and made my stay even more enjoyable. I would also like to thank Dr Eloise Foo and Dr John Ross from the University of Tasmania who have helped me tremendously during the last part of my project.

I am thankful to the University of Adelaide for providing me with financial support during my candidature through an International Postgraduate Research Scholarship (IPRS).

Many thanks have to go to the people with whom I've worked and who have helped me countless times in the lab here in Adelaide, namely: Debbie Miller, Colin Rivers, Rebecca Stonor and Michael Karkkainen. I couldn't have done all this work without your generous help and knowledge. You guys are simply the best! I am also very appreciative of the whole Soil-Biology group, with whom I have interacted countless times from lab meetings, to simple one-on-one discussion. To all of you, thank you. I could not have asked for a better working environment!

Of course, building up towards a Ph.D. is not an easy task in itself. Doing it overseas, away from most of your bearings and familiar faces can greatly add to the challenge. I was lucky enough to make up a whole new family here in Adelaide, who has sustained and carried me through some of the most difficult times of the Ph.D. Laurence, Em, Clayton, Gabe, Kris and Pete, I love you all, and thank you so much for all that you have brought me. You are definitely that "secret ingredient" that made my Ph.D. a wonderful experience!

I also wish to thank Ryan Farquharson: you were a great flat-mate, friend and inspiration to me; Dr Patrick O'Connor: for allowing me to explore other avenues of research and keeping me "afloat" financially speaking; and last but not least, Jodie Duffield: I cannot thank you enough for all that you brought me: you added colour, spice and creativity to my life. Thank you for all your support during those last months of the thesis and for helping me keeping up with Bill Gates complicated tools. I love you very much and wish nothing but the best for you… keep those Kung-Fu moves grooving!

Finally, I cannot finish without paying my deepest regards to my parents, Claire and Jacques. You have always been and still are a great source of inspiration, perseverance and generosity to me. I cannot put into words how much I love you – and missed you during my time away. Thank you for your support, encouragement, patience and faith in me. I wish that one day I become as wise and inspiring to others as you were to me.

Le Nord est devant toi…

General introduction

Research in the field of arbuscular mycorrhizal (AM) symbiosis has taken a giant leap in the past two decades, as demonstrated by the vast amount of literature being published every year. Much of the research efforts has been put towards the understanding of the mechanisms of this symbiosis. However, there are still many unknowns on the systemic effects of the AM symbiosis on plants and our understanding of non-nutritional effects on the physiological changes occurring in the aerial parts of the host plant is still quite limited.

In this thesis I illustrate the importance and relevance of focusing on the physiological changes occurring in the shoots of an AM plant. This topic is often neglected in AM research, except for obvious effects on growth and nutrient concentrations. More specifically, I address the question of whether there are any changes in the secondary metabolic activities that are triggered by AM fungi in sweet basil (*Ocimum basilicum* L.; from now on, referred to as "basil"), an important medicinal herb widely used for its culinary and antioxidant properties. I report on the investigations of several factors that can alter the production of phytochemicals in basil through the AM symbiosis, such as: 1) the nutritional status of the plant (mainly phosphorus and nitrogen); 2) the influence of the identity of the fungal symbiont; 3) the developmental stage of the AM symbiosis, and 4) the influence of a pathogen specific to basil. These investigations led me to answer the following questions: a) does the AM symbiosis affect the production of phytochemicals in the shoots of basil (harvested product) and if so b) through which mechanisms (*i.e.* improved P content of AM plants or other)? This review of the literature covers the latter aspects but also addresses the cultural and

economic importance of medicinal herbs (*e.g.* basil), which have become part of a growing herbal medicine market.

1. Literature review

An historical overview of medicinal herbs

Plants have been (and still are) used over millennia in many ways and forms: as sources of food, building materials or tools. Amidst these applications of plants, one tradition that has been maintained in many cultures is the use of plants as medicines (made from leaves, roots or even bark). It is believed that medicinal herbs have been used since prehistoric times (Dragland *et al.* 2003) and that Neanderthals were among the first to use plants for healing purposes (Kleiner 1995, Winslow and Kroll 1998). According to Sumner (2000), pollen evidence indicates that medicinal herbs were buried in Palaeolithic graves, suggesting that knowledge of herbal medicine is at least sixty thousand years old. The use of plant material for healing purposes eventually gave rise to traditional medicine, or the use of medicinal herbs based on a long tradition (Leonti *et al.* 2003). Hence, traditional medicine has evolved in different parts of the globe, giving birth to a source of invaluable knowledge about plant uses. One of these traditions is, notably, the Ayurvedic system (meaning science of life), originating from the Indian culture, and the Chinese system, both of which are well-known examples of long-established harmonisation of traditional medicine that have evolved by systematic human experience (Wijesekera 1991).

Traditional medicine has evolved in a context of advances in knowledge. For instance, in the Middle Ages, it was believed that plants that were shaped like human organs had some medicinal properties for those particular organs. This was known as the "Doctrine of Signatures" (Gesler 1992). The shape, taste, texture and colour of the plant were used to reveal those signatures. Today, significant

relics of those ancient medicinal practices are still part of our culture as botanical gardens, which were created during the 16th century as places to grow and keep herbal medicines for teaching purposes in medical schools (Akerele 1993). Medical practices were intimately connected to the plant kingdom until the middle of last century and many herbs were used as conventional medicines until the first half of the 20th century (O'Hara *et al.* 1998, Dragland *et al.* 2003). Not only have plants been used for traditional medicines, but they have also provided raw material from which many chemical structures have been determined for the production of synthetic drugs. The derived plant chemicals now represent 25-30% of the known prescription drugs (Akerele 1993, Kleiner 1995, Cox 1998, O'Hara *et al.* 1998, Cox 2000).

Despite the tremendous scientific advances of the last decades, there is now a growing interest in plant compounds with medicinal activity, particularly in industrialised countries. In many developing countries, the use of alternative medicines is still the main or only source of medication (Akerele 1993) and the World Health Organisation (WHO) recognises that nearly 80 percent of the world population is dependent on traditional medicine for primary health care (Wijesekera 1991, Gesler 1992, Akerele 1993, Ernst 1998, Winslow and Kroll 1998). This information helps to understand why herbal medicines are still in demand.

1.1. The growing market for herbal medicines

Herbs are known as sources of phytochemicals (or active compounds) that have medicinal properties and are widely sought after. Nowadays, the growing interest

in herbal medicines stems from a greater number of people seeking natural or alternative medicine, considering those medicines as "healthier" than the conventional ones (Cox 1998, MacLennan *et al.* 2002, Duncan 2003). O'Hara *et al.* (1998) suggested that one of the best explanation for the enthusiasm towards natural plant products is that they are commonly perceived to be "healthier" than synthetically manufactured medicines. In the 1990s, the market for herbal medicine was considered as one of the most vital and high growth industries (Pubrick 1998) and between 1996 and 1998 the herbal industry was worth $1.2 - 1.5 billion/yr in the USA, and three times that size in European markets (Ernst 1998, Winslow and Kroll 1998). By the year 2000 this number was approaching $4 billion/yr in the USA (Ernst 2000), whereas in the United Kingdom and Germany this was estimated to be about £31 million and £1.3 billion respectively (Ernst 2003). Another reason for the increased interest in herbal medicines is probably that herbal remedies are easily available to the public and do not require any prescription by physicians (O'Hara *et al.* 1998), as well as often being less costly than prescribed drugs.

As a result of this movement towards herbal medicines, there is now a need to develop herb varieties with better growth and increased concentrations of phytochemicals (Rai *et al.* 2001). In order to obtain such herbal medicines, new perspectives must be adopted and new techniques developed. As suggested by Cox (1998), this could be achieved through ethnobotany (the study of the uses of plants by indigenous people), which was once the major source of new pharmaceuticals. However, as traditional knowledge is being lost in many cultures

(Akerele 1993, Cox 1998), new ways of improving the production and phytochemical concentrations of herbal medicines must be developed.

1.2. A new approach…

Formerly, the materials for drug production from medicinal herbs were collected from the wild, with a high variability in the concentration of phytochemicals of the raw material and potential damage to biodiversity of ecosystems. Today, development of production techniques to help reduce costs and increase capacity of industrial processing is more and more essential in the industry of herbal medicines (RIRDC 1997). Phytochemicals are often criticised for their lack of standardised concentrations from the natural plants (Israelsen 1993). Thus, large scale and sustainable cultivation of medicinal herbs offering consistent phytochemical concentrations is highly desirable.

An alternative way of producing medicinal herbs could reside in the exploitation of the arbuscular mycorrhizal (AM) symbiosis. As will be discussed below, the AM symbiosis is known to be beneficial for a majority of vascular plants in a number of ways. Hence, the association between AM fungi (AMF) and medicinal herbs could result in better plant growth as well as increases in phytochemical concentrations. The following paragraphs will outline significant features that may lead to the consideration of the AM symbiosis as a new approach to increase the biomass and concentration of phytochemicals in medicinal herbs in a way that minimises the use of inorganic fertilisers.

1.3. The arbuscular mycorrhizal (AM) symbiosis

One of the most successful strategies that terrestrial plants have evolved to withstand different edaphic conditions is the capacity of their root systems to establish close relationships with a variety of fungi living in their vicinity. The AM symbiosis is certainly one of the most prevalent symbiotic associations found in nature in a wide range of ecosystems. In fact, it is generally accepted that as few as 20% of vascular plant species belong to families that do not form such a symbiosis (Smith and Read 1997). Recently reclassified as part of a monophyletic phylum of fungi – the *Glomeromycota* (Schussler *et al.* 2001) – AMF are widespread in different ecosystems and very ancient, as fossil and molecular phylogeny evidence suggest (Nicolson 1975, Simon *et al.* 1993, Remy *et al.* 1994, Redecker *et al.* 2000). Arbuscular mycorrhizal fungi are known for a broad range of functions, but are characterised by two major aspects: 1) AMF colonise roots, improving plant nutrition by transferring poorly available nutrients, mainly phosphate (P), from the soil to the plant, whereas plants provide the fungi with essential carbohydrates in order for them to complete their life cycle (Smith and Read 1997). Other macronutrients such as nitrogen (N) can also be more easily acquired through the AMF (Johansen *et al.* 1992, 1994, Tobar *et al.* 1994, Toussaint *et al.* 2004). Generally (but not always) this results in positive growth responses of the AM plant, especially in nutrient-poor soils (Smith and Read 1997). This responsiveness of AM plants can vary, forming a continuum of growth effects ranging from mutualism to parasitism (see Johnson *et al.* (1997) for a general review); 2) Plants colonised by AMF may directly or indirectly acquire protection against pathogens (St-Arnaud *et al.* 1995, Cordier *et al.* 1996, Fillion *et al.* 1999). However, the mechanisms involved in bioprotection have not been

clearly identified (St-Arnaud *et al.* 1995, Azcón-Aguilar and Barea 1996, Cordier *et al.* 1998).

Other functions also well known to this symbiotic association and possibly related to an improved nutrition are drought resistance (Cui and Nobel 1992, Subramanian and Charest 1997, 1999, Ruiz-Lozano 2003), and soil aggregate stability (Tisdall and Oades 1979, Miller and Jastrow 1990, Hodge 2000). The biological potential of AMF to promote plant growth and nutrition in many disciplines of plant biology [*e.g.* agriculture, agroforestry, horticulture; see also Gianinazzi and Vosátka (2004)] could also be extended to the cultivation of medicinal herbs. The next sections aim to provide an understanding of how the AM symbiosis affects host plants in a way that could be related to the production of higher concentrations of phytochemicals in medicinal herbs.

1.3.1. AMF affect secondary compound production in host roots

The process of AM colonisation involves a series of different steps (Fig. 1.1), each characterised by specific structures as well as morphological and physiological changes in the host plant (Bonfante and Perotto 1995, Smith 1995, Gianinazzi-Pearson 1996, Harrier 2001). For instance, once AMF colonise the host roots, the production of secondary plant compounds has been shown to be altered. Those plant compounds include defence-related chemicals such as low molecular weight antimicrobial flavonoids, *e.g.* phytoalexins (VanEtten *et al.* 1994, Scharff *et al.* 1997) or pathogenesis-related proteins, *e.g.* chitinase, β-1,3-glucanase (Bonfante and Perotto 1995, Benhamou 1996, Dumas-Gaudot *et al.* 1996). It has been shown that the first steps of AM colonisation can induce the production of those

compounds in the host plant. However, this response is usually small in associations between highly compatible host plants and AMF (Spanu *et al.* 1989, Harrison and Dixon 1993, Vierheilig *et al.* 1994, Volpin *et al.* 1994). Overall, evidence suggests that the mechanisms associated with plant defence play a key role in the first stages of AM colonisation and compatibility with the host plants (Garcia-Garrido and Ocampo 2002).

Morandi *et al.* (1984) were among the first to study the accumulation pattern of secondary compounds in the roots of AM plants. Their results indicated that colonisation of soybean (*Glycine max* L.) roots with *Glomus mosseae* or *G. fasciculatum*, increased the concentrations of the isoflavonoids glyceollin, coumestrol and diadzein in host roots. Those three phytoalexins are known to have antimicrobial activities and to accumulate during pathogen attack (Morandi *et al.* 1984). These results prompted the authors to suggest that AM plants would be more able to respond quickly to a pathogenic attack. However, this study was not based on a time-course and could only demonstrate such patterns at the later stages of the symbiosis. Harrison and Dixon (1993) found a qualitative and quantitative change of secondary compounds in medic (*Medicago truncatula* L.) roots colonised by *G. versiforme* during the first stages of the symbiosis. These changes seemed to be transient, as the host defence response was subsequently suppressed. Volpin *et al.* (1995) also observed that defence-related gene transcripts were induced and then suppressed during early colonisation of alfalfa (*M. sativa* L.) roots by *G. intraradices*. These results were corroborated in a study that showed accumulation of cyclohexenone derivatives in barley

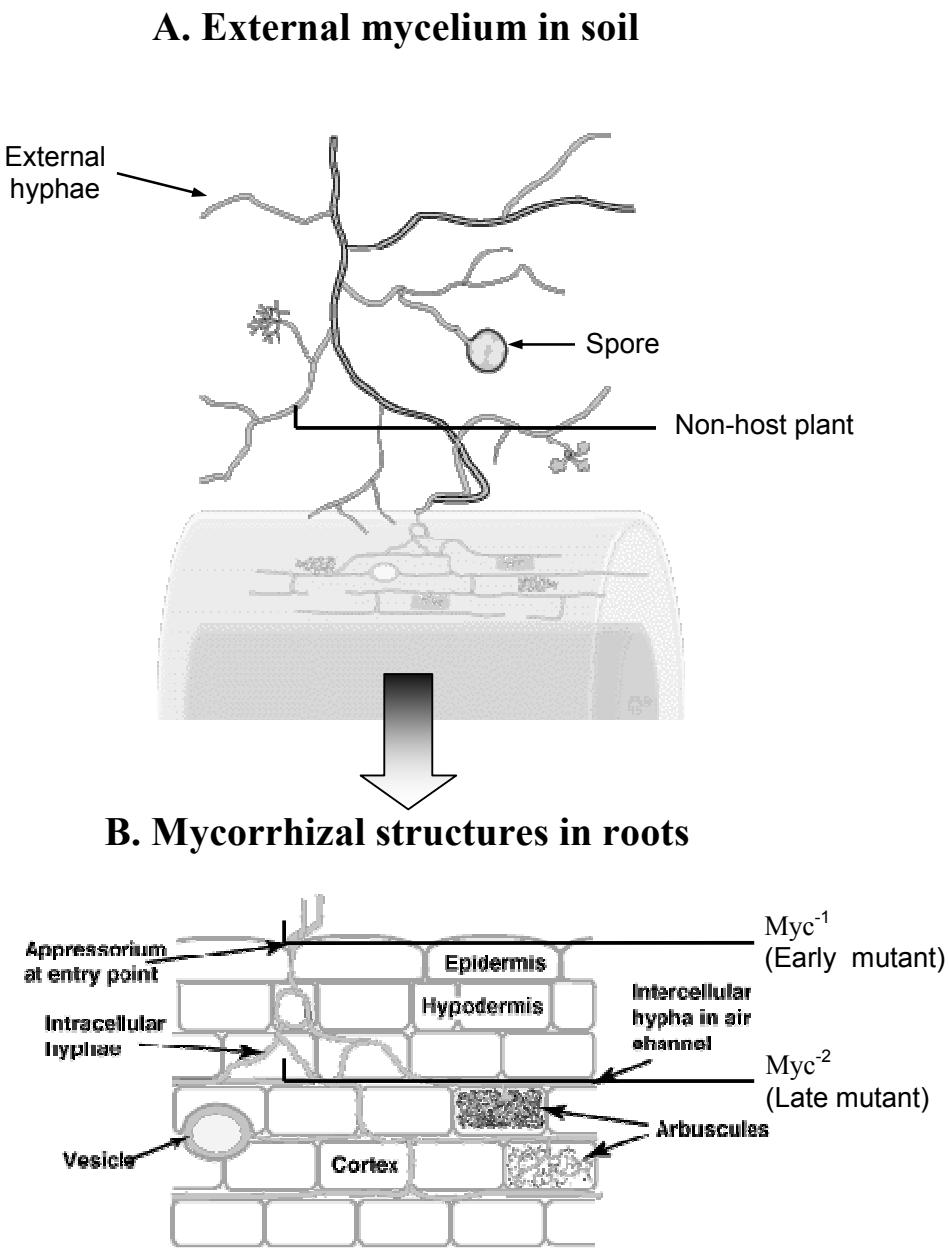

Figure 1.1. Components of the arbuscular mycorrhizal symbiosis [here Arum type, taken from Toussaint (2002), originally adapted from Brundrett *et al.* (1996)]. A) External hyphae and spores produced by mycorrhizal fungi in soil; B) structures formed during various steps of the colonisation of host-roots (appressoria, internal hyphae, arbuscules and vesicles). There are also non-host plants in which the colonisation by AMF is stopped at different steps. This is further represented by the Myc^{-1} and Myc^{-2} mutants (adapted from the review of Giovannetti and Sbrana (1998) on non-host plants of AMF).

(*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) roots colonised by *G. intraradices* (Fester *et al.* 1999).

A number of recent studies have also provided evidence of accumulation of alkaloids, flavonoids and other phenolic compounds in mycorrhizal roots of different plant species (Vierheilig *et al.* 2000, Devi and Reddy 2002, Rojas-Andrade *et al.* 2003, Yao *et al.* 2003). In addition, a transient accumulation of salicylic acid (SA), a signal molecule involved in the transduction pathway activated in plant-pathogen responses, was found during the early stages of colonisation of rice (*Oryza sativa* L.) with *G. mosseae* (Blilou *et al.* 2000). However, contrasting results were reported in which phytoalexins were not increased by colonisation of soybean roots with *G. mosseae* (Wyss *et al.* 1991).

Most of the findings from past research seem to indicate that the increases of the defence-mediated molecules are low in AM plants compared to pathogenic interactions, as further reviewed by Morandi (1996). Morandi (1996) also concluded that AMF are able to induce an accumulation of phenolics such as phytoalexins and associated flavonoids and isoflavonoids in the roots of their host plants. It is now commonly accepted that a weak, transient and uncoordinated activation of plant defence response is what characterises AM colonisation in host plants (as further reviewed by Bonfante and Perotto 1995, Azcón-Aguilar and Barea 1996, Garcia-Garrido and Ocampo 2002, Harrier and Watson 2004). From these studies two points stand out: 1) the production of secondary plant compounds can vary depending on the host plant and the fungal species; and 2) the investigations carried out did not take into account the changes in secondary

compounds that might occur in the shoot of the host plant following mycorrhizal colonisation. The latter point is of particular importance, as many phytochemicals in medicinal herbs are found in the aerial parts (the most usually harvested product). Hence, exploring the systemic effects of different fungal species on the production of phytochemicals in the host plant (especially the shoots) needs further attention.

Systemic effects of AMF in host plants have been partly addressed in the past. Using a split-root system, Cordier *et al.* (1998) demonstrated that *G. mosseae* can decrease the development of *Phytophthora parasitica* in tomato (*Solanum lycopersicum* L.) roots through localised and systemic resistance. Using a similar split-root system, Catford *et al.* (2006) showed that formononetin and ononin are systemically regulated by *G. mosseae* in alfalfa roots when one side of the root system is colonised and the other not. However, in both studies, the authors did not investigate the possible changes of phytochemical concentrations in the shoot of the host plants. Singh *et al.* (2004) recently demonstrated that *G. mosseae* can induce a systemic defence response in the leaves of pea (*Pisum sativum* L.) when infected by the aerial pathogen causing powdery mildew, *Erysiphe pisi* L. Finally, using differential mRNA display, Taylor and Harrier (2003) identified five differentially regulated cDNAs in leaf and root tissues of tomato plants colonised by *G. mosseae* compared to non-mycorrhizal (NM) plants. The authors suggested that these regulation patterns might have been the result of a difference in nutritional content in AM vs. NM plants, which was not tested in that study. These observations highlight the importance of considering the nutritional effect of AMF

on host plants when investigating systemic effects or the alteration of secondary compounds in AM plants.

1.3.2. Secondary compound production related to phosphorus

It is generally accepted that the AM symbiosis can improve P nutrition (Section 1.3), which often promotes plant growth through higher P content. Enhanced nutrient uptake in AM plants might lead to an indirect effect on the production of secondary compounds in host plants. It might then be difficult to separate benefits derived from improved plant P content and concentrations from non-nutritional benefits (O'Connor 2001). Hence, to discriminate any fungal non-nutritional effect on the production of secondary compounds in host plants from that in NM plants, it is necessary to be able to discount any indirect effect of improved nutritional status (Smith and Read 1997, Gao 2002). Morandi and Gianinazzi-Pearson (1986) demonstrated that isoflavonoid concentrations in soybean roots were not affected in NM plants supplied with potassium phosphate, whereas those of AM roots were greatly increased. Such results indicated that the response was not a result of changes in plant P uptake induced by the fungi, but rather a direct fungal effect. These results were corroborated by Harrison and Dixon (1993) who demonstrated that increases in flavonoid concentrations and their corresponding RNA transcripts in medic roots were a direct effect of AMF and not an indirect effect of P availability, as NM control plants supplied with additional P showed similar flavonoid content than NM plants grown without P. Similar results were obtained in alfalfa roots in which formononetin concentrations were higher in roots colonised by *G. intraradices* compared to NM controls supplied with a low or high P solution (Volpin *et al.* 1994). Although these studies have demonstrated

what seems to be a non-nutritional mycorrhizal effect, they did not specifically take into account the P concentration of AM plants versus that of NM plants, which might not be a direct reflection of the actual P availability that is being supplied to the plants. It is therefore important to obtain NM and AM plants that are matched for tissue P concentrations to properly discriminate the real contribution of AMF in the production of phytochemicals in host plants such as medicinal herbs. A recent study by Liu *et al.* (2007) demonstrated significant changes in gene expression in the shoots of medic colonised by *G. intraradices* compared to NM shoots that were matched for P concentrations. Furthermore, the authors showed that the transcriptional profile of AM shoots corresponded to a significant resistance to the bacterial pathogen *Xanthomonas campestris*, supporting the idea that the AM symbiosis leads to systemic resistance in the shoots of host plant following a pathogenic attack.

1.4. AMF and medicinal herbs

Despite numerous papers published in the last two decades on secondary metabolism in AM plants, it seems that very little work has been done on the accumulation of phytochemicals in AM medicinal herbs. There have been reports on the activity of antioxidants in AM plants, but mainly in response to abiotic stresses (Schutzendubel and Polle 2002, Porcel *et al.* 2003, Porcel and Ruiz-Lozano 2004). Other reports on medicinal herbs and AMF aimed to understand the effects of inoculation on growth responses of those plants. Camprubi *et al.* (1990) observed root and shoot dry weight increases in a range of medicinal herbs (basil – *O. basilicum* L.; sage - *Salvia officinalis* L.; tarragon - *Artemisia dracunculus* L.; and thyme – *Thymus vulgaris* L.; all of which, except tarragon,

are members of the Lamiaceae) inoculated with *G. mosseae* compared to NM plants. However, the authors did not investigate the production of phytochemicals or essential oils in those plants under AM conditions. In a similar study, Rai *et al.* (2001) evaluated the growth responses and morphological changes in *Spilanthes calva* L. and *Withania somnifera* L., two plants of medicinal importance used in Ayurvedic medicine, after inoculation with *Piriformospora indica*, a fungus belonging to the *Hymenomycetes* that is known to promote plant growth (Varma *et al.* 1999). Their results indicated a significant positive growth response of these two species colonised by the fungus, which suggested a commercial potential for large-scale cultivation. Growth stimulations were also observed in Asian ginseng (*Panax ginseng* L.) colonised by different AMF isolates (Zeuske and Weber 2000). The latter studies focused on growth stimulation but not on the increase of phytochemical concentrations in the symbiotically associated host plants.

Other studies involving medicinal herbs and AMF either focused on inter-specific root competition (Moora and Zobel 1998), colonisation patterns of the fungi (McGonigle *et al.* 1999, Armstrong and Peterson 2002), or mycorrhizal dependency (Azcón and Barea 1997). Sirohi and Singh (1983) showed greater oil concentrations in peppermint (*Mentha piperita* L.) when associated with *G. fasciculatus* compared to NM plants. The authors also suggested (but did not test) that the AM symbiosis might have increased P uptake efficiency, which would have resulted in greater synthesis of essential oils in peppermint. In another study, it was shown that a mixed inoculum of naturally occurring fungi (*G. mosseae* and *G. fasciculatum*) enhanced the growth biomass of host plants (onion - *Allium sativum* L; coriander - *Corriandrum sativum* L.; basil – *O. basilicum*, and sesame -

Sesamum indicum L.) (Basu and Srivastava 1998). The authors observed a noticeable increase of essential oil concentrations in those plants when mycorrhizal. However, these results were presented at a conference and have not been published yet to my knowledge.

It is only recently, during the course of my work, that a few studies were published relating to the investigation of the production of essential oils in basil, coriander and wormwood (*Artemisia annua* L.) under the AM symbiosis (Copetta *et al.* 2006, Khaosaad *et al.* 2006, Kapoor *et al.* 2007). In all cases, the AMF increased the essential oil concentrations of the medicinal plants, an effect that was not related to improved tissue P concentrations. Copetta *et al.* (2006) and Kapoor *et al.* (2007) hypothesised that increases in essential oil or artemisinin concentrations were related to increases in the number of trichomes in the leaves of basil and wormwood, which in turn could have been regulated by changes in hormonal balance in AM plants. The idea that AMF can induce changes in the hormonal balance of host plants has been proposed by several authors, but with conflicting results (Allen *et al.* 1980, 1982, Esch *et al.* 1994, Torelli *et al.* 2000, Fitze *et al.* 2005). While some authors have found increases in auxin concentrations in AM plants (Torelli *et al.* 2000, Fitze *et al.* 2005), others have observed the opposite in regards to abscisic acid (Allen *et al.* 1982). As phytohormones are important plant growth regulators (Roberts and Hooley 1988), they might greatly influence plant metabolism including that of phytochemicals. So far however, no clear mechanisms have been proposed to explain the increases in phytochemical concentrations in AM plants.

1.5. The selection of a medicinal herb

Among the wide variety of medicinal herbs to choose from, only a few were considered for inclusion in this project. They were: basil, sage, thyme, St. John's Wort (*Hypericum perforatum* L.) and echinacea (*Echinaceae purpurea* L.). These plants were evaluated on the following bases: 1) they have great medicinal and commercial value; 2) they form arbuscular mycorrhizas; 3) they are relatively easy to grow in terms of cultivation and, 4) the assay techniques used to determine their main phytochemicals are already established. Appendix 1 gives the information that was gathered when reviewing which plant(s) to use for the project and summarises some key points about them, *e.g.* their medicinal use, the specific parts that are used, their growth requirements as well as their key phytochemicals. It was originally intended to study all these plants. However, as this turned out to be too optimistic, the work was primarily done on basil.

1.5.1. Members of the Lamiaceae family

Among the various medicinal herbs that are being used worldwide, the plants belonging to the Lamiaceae family are probably the most popular. Members of this family include basil, mint (*Mentha* spp. L.), thyme, sage, rosemary (*Rosmarinus officinalis* L.) and oregano (*Origanum vulgare* L.). Many of the species from this family have been appreciated worldwide as a source of spices and flavouring (Hirasa and Takemasa 1998) as well as for their pharmaceutical properties (Bais *et al.* 2002). It is also generally accepted that the medicinal virtues of this family are due to their high contents of essential (aromatic) oils, which have strong antioxidant properties (Kahkonen *et al.* 1999). Other antioxidant properties of the Lamiaceae are due to components such as phenolic

compounds, including flavonoids and phenylpropanoids and also anthocyanins (Phippen and Simon 1998, 2000). Rosmarinic acid (RA), an ester of caffeic acid (CA), is produced by members of the Lamiaceae and has antioxidant activity as well as antiviral, antibacterial and anti-inflammatory (Petersen and Simmonds 2003). RA is also believed to serve as a constitutively accumulated and induced defence compound (Bais *et al.* 2002, Javanmardi *et al.* 2002). The antimicrobial activity of many plants is due to compounds synthesised in their secondary metabolism, often associated with defence responses (Nascimento *et al.* 2000). This leads to the assumption that RA could similarly be induced during mycorrhizal symbiosis in a host plant belonging to the Lamiaceae. The biochemical pathway of RA is shown in Fig. 1.2, in which both phenylalanine (Phe) and tyrosine (Tyr) are required to synthesise RA (*i.e.* Phe and Tyr do not independently lead to RA formation). CA is the immediate precursor of RA and also contributes to the antioxidant properties of members of the Lamiaceae.

Antioxidants are also quite important in the food processing industry and have been used for over 50 years to avoid loss in nutritional and gustative quality of foods (Cuvelier *et al.* 1994). Natural antioxidants have recently gained recognition among consumers as a result of major concerns about toxic side effects of synthetic antioxidants in food such as BHT and BHA (butylated hydroxytoluene and butylated hydroxyanisole) (Pizzale *et al.* 2002, Sacchetti *et al.* 2004). Therefore, the research and development of natural sources of antioxidants is of current interest (Cuvelier *et al.* 1994, Juliani and Simon 2002, Pizzale *et al.* 2002). This concern strongly supports the need to find new ways of improving the production of natural products such as those found in the Lamiaceae.

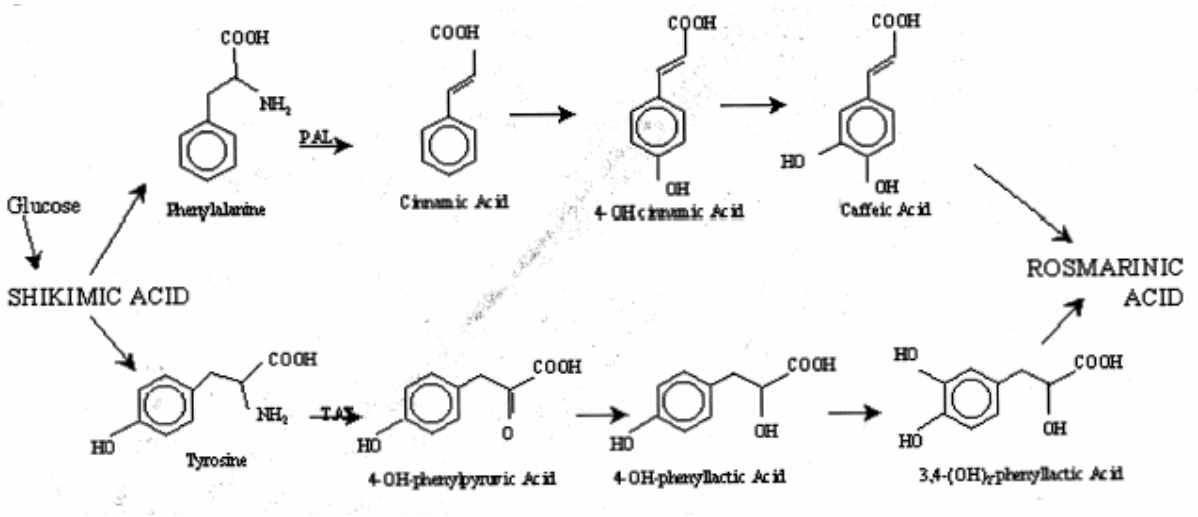

Figure 1.2. Biosynthetic pathway of rosmarinic acid (RA) in Lamiaceae. Both Phe and Tyr are required to synthesise RA, and caffeic acid (CA) is one of the immediate precursor of RA [adapted from Ellis and Towers (1970) and Petersen and Simmonds (2003)].

1.5.2. Basil (*Ocimum basilicum* L.)

The genus *Ocimum* contains up to 150 species of herbs and shrubs that are either annual or perennial and found in tropical regions (Javanmardi *et al.* 2002). Basil is thought to be native to Asia, Africa, South America and the Mediterranean although now widely grown all over the world (Keita *et al.* 2000, Jayasinghe *et al.* 2003). It is also a major commercial crop in countries such as Greece, France, Israel and Italy (Swart and van Niekerk 2003, Biris *et al.* 2004). Basil is universally known for its extracts that are greatly appreciated as spices and for food flavouring (Hirasa and Takemasa 1998, Jirovetz *et al.* 2003), but it has also been traditionally used for the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and also kidney malfunctions (Simon 1998, Javanmardi *et al.* 2002). It is also believed that basil can be used as an insecticide or ointment for insect bites as well as to treat acne (Waltz 1996, Umerie *et al.* 1998, Keita *et al.* 2000).

Basil is generally recognised as safe (GRAS) and is a rich source of phenolic compounds and flavonoids (Juliani and Simon 2002, Jayasinghe *et al.* 2003). A major compound found in basil is RA, which is one of the most abundant caffeic acid esters in *Ocimum* spp. (Javanmardi *et al.* 2002). RA is produced in leaves and shoots of basil, but it was recently suggested that most accumulation can be found in roots (Bais *et al.* 2002). The authors suggested that RA may be released in root exudates only under microbial challenge, which suggests that mycorrhizal colonisation might induce a similar response. RA is also considered to be the major component that confers antioxidant activity in basil (Juliani and Simon 2002, Jayasinghe *et al.* 2003). Besides RA, basil is also a rich source of anthocyanins (Phippen and Simon 1998, Jayasinghe *et al.* 2003). Basil is also valued for its essential oils, which have high concentrations of the aromatic compounds eugenol and linalool (Simon 1998, Jirovetz *et al.* 2003). Essential oils of basil have been shown to have great antimicrobial activity against bacteria such as *Escherichia coli* and *Lactobacillus plantarum* (Bouzouita *et al.* 2003).

The active constituents of basil are influenced by environmental conditions (Javanmardi *et al.* 2002) and it was demonstrated that UV-B light can affect the essential oil content of this plant (Johnson *et al.* 1999). It was also reported that the variability of essential oil content might be dependant of the harvest season (Keita *et al.* 2000), emphasizing the need to consider harvest time as a potential factor influencing optimum concentrations of phytochemicals in basil. In addition, there is little information in the literature about responsiveness of basil to P and AMF (Giovannetti 1997, Dickson 2004, Copetta *et al.* 2006). It is then essential to address this issue to determine whether or not AMF could increase phytochemical

concentrations such as RA and CA in basil, other than through improved P concentrations or content. On the other hand, there have been some reports indicating that both the form and amount of N can affect the concentration of essential oils in basil (Adler *et al.* 1989, Sifola and Barbieri 2006). This information, together with the numerous reports on N transfer to host plants through AMF (Johansen *et al.* 1994, Subramanian and Charest 1999, Toussaint *et al.* 2004, Govindarajulu *et al.* 2005), emphasize the need to investigate the influence of N supply on the production of RA and CA in basil under the AM symbiosis.

Finally, there have been several reports of the increasing occurrence of Fusarium wilt, which causes serious damage to basil production (Gamliel *et al.* 1996, Fravel and Larkin 2002, Swart and van Niekerk 2003, Biris *et al.* 2004, Moya *et al.* 2004). *Fusarium oxysporum* f.sp. *basilici* (*Fob*) has been reported to be the major disease limiting the production of this crop worldwide (Gamliel *et al.* 1996). The symptoms associated with *Fob* include chlorosis, necrosis, wilt of the stems and leaves, root crown and rot, dark lesions as well as vascular discoloration, all of which often lead to plant death. As basil is an economically important crop, efforts have been made to find ways of reducing the damage done by *Fob*. However, better and more efficient methods of controlling the disease and increasing basil resistance to this pathogen need development. As AMF can potentially act as biocontrol agents by inducing systemic resistance to pathogens through increases of defence compounds (*e.g.* RA), this issue was also taken into consideration in the present work.

1.6. Specific aims

On the basis of the information presented in this literature review, the aim of this project was twofold: 1) to evaluate if the AM symbiosis can increase the concentration of phytochemicals (*e.g.* RA and its precursor, CA) in basil, and, if this is the case, 2) to elucidate the mechanisms by which this operates. To address these questions, the project was designed to:

- first, investigate the growing requirements of basil as well as its responsiveness to P and AMF (Chapter 3);
- evaluate the influence of different AMF and their developmental stage on the production of those RA and CA (Chapters 4 and 5);
- determine the influence of N supply on the production of RA and CA under the AM symbiosis (Chapter 6), and;
- investigate the interaction between *G. mosseae* and *Fob* on the production of RA, CA and essential oils, as a potential way to reduce the pathogenic infection while increasing the concentrations of phytochemicals (Chapter 7).

2. General materials and methods

This section describes the general materials and methods that were used in the experiments carried out for this project. Any specific details and modifications in particular experiments are described in the appropriate chapters. Firstly, I describe the steps that led me to choose an appropriate growth medium for basil under the AM symbiosis.

2.1. Plant material and seed sterilisation

Basil (*Ocimum basilicum* L. cv. Genoa, Yates Seed Ltd) was the main basil variety investigated in this work. Leek (*Allium porrum* L. cv. Vertina) was also used as a trap-plant for inoculum production (Section 2.3). Prior to planting, all seeds were surface-sterilised by soaking in a 30% hypochlorite solution (commercial bleach 12.5%) for 10 min and then rinsed for 5 min with reverse osmosis (RO) water. Three germination methods were tested: 1) in Petri dishes on moist Whatman #2 filter papers (Chapters 3); 2) in a tray filled with sterilised fine sand until seedlings were a few days old (Chapter 3, Sections 3.3 – 3.4); or 3) sown directly into pots (Chapters 4 – 6).

2.2. Finding a suitable growth medium

Initially, this project was undertaken with a commercial scope. Therefore, different growth media were tested as a way to be representative of what is done commercially. The search for commercially relevant methods included testing a commercial mycorrhizal inoculum as the sole source of AM fungi.

A small experiment was designed to investigate how well leek could grow on a mixture of Perlite and sand compared to a soil/sand mixture. Perlite is a siliceous rock (granules) that is widely used in horticulture as part of a growth medium to provide aeration and good moisture retention for plant growth (http://www.perlite.net). Mr Ray Prior (McClaren Vale Garden Centre, South Australia) had suggested trying Perlite as a potential medium, as it is commonly used in nurseries to grow many herb varieties. In addition, a commercial AM inoculum was tested, Myco-Gro, which was provided by Mr Sandy Montague (Myco-Gro, New-Edge Microbials Pty. Ltd; further described in Appendix 2). Briefly, leeks were grown for 6 weeks in a glasshouse (Section 2.4) in two different media, both containing Myco-Gro (Table 2.1). After 6 weeks the plants were harvested and root and shoot fresh/dry weights were determined, as well as % colonisation (Sections 2.6 and 2.7).

Table 2.1. Characteristics of two media tested in order to find suitable growth conditions for future experiments. The plants tested were leeks, which were grown for 6 weeks in 400 g pots. The plants were watered three times per week and received 5 ml of a modified Long Ashton solution once a week (Table 2.3).

	Medium 1	**Medium 2**
Perlite/Sand (w/w)	1:1	-
Soil/Sand (w/w)	-	9:1
pH of mixture	7.6	7.5
Colwell P (mg/kg)	6.5	8.0
Myco-Gro inoculum (g/pot)*	0.12	0.12

*recommended application rate

2.2.1. Outcomes

Perlite turned out to be very hard to mix with sand and to use as a growth medium. The mixture was also hard to weigh consistently in order to standardise the amount to put in each pot. Furthermore, the plants growing in the Perlite/sand mix

had quite variable dry weights compared to those grown in the soil/sand medium. In the light of these results it was decided to use a mixture of soil/sand (Sections 2.3 and 2.4) instead of Perlite/sand. In addition, no AM colonisation was detected in any of the plants harvested when using the recommended application rate of Myco-Gro. Another experiment was carried out to further test this commercial inoculum. The main results from this experiment are presented in Appendix 2. As that experiment turned out to be unsuccessful, I decided not to use Myco-Gro for this project. Instead, AM inocula grown from pot cultures were used as described in the next section.

2.3. Inoculum preparation and application

Different AMF obtained from the collection of Soil and Land Systems, School of Earth and Environmental Sciences, University of Adelaide, were used in the experiments that were conducted (Table 2.2). The AMF inocula were prepared as pot cultures with leeks grown in a mixture of soil/sand (1:9, w/w; see Section 2.4). The soil came from Mallala, South Australia (pH ~7.5) and the soil/sand mix contained ~8 mg/kg available P (Colwell 1963). The pot cultures were allowed to grow for eight weeks in Sun-bags (Sigma Aldrich, Australia) and were watered once a week to 10% (w/w) of soil dry weight. In addition, the plants received 10 ml of a modified Long Ashton solution minus phosphorus (P) once a week (Cavagnaro *et al*. 2001) (Table 2.3). Non-mycorrhizal (NM) pot cultures were also prepared in which leeks were grown without AMF under the same conditions. With the exception of experiments described in Chapters 3 and 7, all pots received 25% (w/w) of one of the dry pot cultures, consisting of the soil/sand mix, colonised root pieces of leeks, spores and hyphae. Control pots received 25%

(w/w) of the NM pot cultures (soil/sand mix and non-colonised root pieces of leek).

Table 2.2. Name, authority and accession number of the different AM fungi species used during the course of this work.

Fungus	Authority	Accession
Glomus caledonium	(Nicolson & Gerdemann) Trappe & Gerdemann	BEG 162
Glomus intraradices	Schenck & Smith	BEG 159
Glomus mosseae	(Nicolson & Gerdemann) Trappe & Gerdemann	NBR 1-2

Table 2.3. Modified Long Ashton solution (Cavagnaro et al. 2001).

NOTE: This table is included on page 26 of the print copy of the thesis held in the University of Adelaide Library.

2.4. Plant growth

Plants were grown in a mixture of sand and Mallala soil. This mixture originally consisted of sterilised sand (3 parts coarse sand and 1 part fine sand) and soil, with a final ratio of 1:9 (w/w) soil/sand. This ratio was modified during the course of this work to a final ratio of 1:3 (w/w) soil/sand (Chapter 3, Section 3.4 and Chapters 4-6). Unless otherwise specified, nutrients were mixed with the soil at the following rates (mg/kg dry soil, based on Jakobsen *et al*. (1992): K_2SO_4, 75; $CaCl_2$ x $2H_2O$, 75; $CuSO_4$ x $5H_2O$, 2.1; $ZnSO_4$ x $7H_2O$, 5.4; $MnSO_4$ x H_2O, 10.5; $CoSO_4$ x $7H_2O$, 0.39; $MgSO_4$ x $7H_2O$, 45.0; Na_2MoO_4 x $2H_2O$, 0.18; and

NH_4NO_3 85.7. All plants were grown in a glasshouse on the Waite Campus, University of Adelaide, South Australia, during summer unless otherwise specified (Chapter 3). The mean temperature range during summer was 25 - 30^oC and the light intensity range was 400 - 1100 μmol/m^2/sec, depending on the position in the glasshouse and weather conditions. In winter, the temperature range was 20 - 25^oC and the light intensity range was 250 – 550 μmol/m^2/sec. During that time, supplementary light was provided, giving an additional 100 μmol/m^2/sec and extending day length to ~16 hours. The plants were watered once a day at first until they were well established, then three times a week to 10% (w/w) of soil dry weight for the remainder of the experiment.

2.5. Phosphorus supply

Phosphorus (P) was added separately to the soil/sand mix as calcium hydrogen orthophosphate ($CaHPO_4$) powder, a slow release form of P, which allows the plants and AMF to grow well together (Gao 2002). As different P supplies were used throughout the various experiments, the rates at which they were applied will be described in the specific chapters.

2.6. Harvesting

At harvest, the following main parameters were recorded, unless otherwise specified in the appropriate section: number of leaves, shoot and root fresh and dry weights, plant P concentration (Section 2.7), RA and CA concentrations (Section 2.9) and percent colonisation (Section 2.7). Fresh weights were recorded on the harvest day and a weighed sub-sample was kept for determination of mycorrhizal colonisation. The remainder of the plant material was oven-dried at

45°C for 3 days before dry weights were recorded. This temperature prevented any breakdown of RA and CA. The total root dry weight was determined from the total fresh weight and the fresh/dry weight ratio of the remainder of the sample. As the trends of fresh and dry weights were the same within each experiment, only dry weights data were reported in the results sections of each chapter. Similarly, the trends in root and shoot dry weights followed similar trends, therefore only total plant dry weights were presented (except in Section 3.1 and Chapter 7). Any additional parameters recorded will be described in the appropriate sections (Chapters 6 and 7).

2.7. Mycorrhizal colonisation

At harvest, root samples (~100 mg) were prepared for determination of percent colonisation using a modified method of Vierheilig *et al.* (1998). Roots were cut into ~1 cm segments and cleared in 10% KOH for two days at room temperature. After rinsing several times in RO water, roots were stained in a 5% ink-vinegar solution (black ink, Sheaffer) for 30 min at 80°C and subsequently destained overnight in acidified water (using a few drops of vinegar). Colonisation was recorded according to the magnified intersections method of McGonigle *et al.* (1990) as modified by Cavagnaro *et al.* (2001). Root pieces were mounted on slides and examined at X 100 magnification, using a bright-field microscope containing an ocular cross-hair eyepiece. One hundred intersections per sample were observed and the incidence of AM structures was scored for internal and external hyphae, arbuscules and vesicles. The percent incidence of each structure over total intersections was calculated and the total percent colonisation was determined.

2.8. Plant P determination

Plant P concentration was determined using the method of Hanson (1950). Dried material (shoot or root, ~50 mg) was ground and put in a nitric-perchloric acid mixture (6:1, v/v). The samples were digested on a programmed Tecator digestion block (Table 2.4). The digests where diluted to 25 ml with RO water and an 8 ml aliquot was made up to a final volume of 25 ml with 2 ml of colour reagent (nitric acid, 0.25% ammonium vanadate and 5% ammonium molybdate, 1:1:1, v/v/v) and RO water. After 30 min, absorbances were read at 390 nm on a Shimadzu UV-601 spectrophotometer. In order to calculate the plant tissue P concentration, a standard curve was prepared using a range of P concentrations from 0 to 10 μg/ml.

Table 2.4. Digestion steps for plant P determination.

Step	Temperature (^{o}C)	Ramp (min)	Time (h)
1	70	10	0.5
2	150	10	5
3	180	10	5

2.9. High Performance Liquid Chromatography (HPLC) analysis for determination of rosmarinic and caffeic acid

The ethanol used for sample preparation was of analytical grade, the methanol was of HPLC grade, and the water purified with a MilliQ apparatus. RA and CA standards were purchased from Adelab Scientific, Australia. The extraction and analytical methods were adapted from Wang *et al.* (2004). Briefly, 50 mg of dried material (shoot or root) was ground and extracted with 25 ml ethanol/water (30:70, v/v) with 10 min of sonication. The mixture was then centrifuged for 5 min at 4500 rpm in a Sorvall Legend RT (Kendro Laboratory Products, Germany) centrifuge, and the supernatant transferred to a 50 ml tube. The residual material was further extracted with 20 ml ethanol/water followed by 5 min of sonication

and centrifuged again. The supernatants were combined and further centrifuged at 4500 rpm for an additional 20 min, then filtered through a 0.45 μm filter and transferred to 2 ml vials prior to injection to HPLC.

2.9.1. Instrumentation

The HPLC apparatus used was an Agilent 1100 Series liquid chromatograph system comprising a quaternary pump, thermostatted column compartment, vacuum degasser, autosampler and diode-array detector. An Apollo C_{18} column from Alltech (Model 36511) 5 μm, 250 x 4.6 mm was used for the analyses and maintained at 30^{o}C. The solvents used for separation were 0.1% orthophosphoric acid in water (v/v; eluent A) and 0.1% orthophosphoric acid in methanol (v/v; eluent B). After trying various gradient steps to isolate RA and CA, it was found that the best way to separate the compounds was to use an isocratic step (*i.e.* using an unchanging mobile phase). Hence, eluent A and B were maintained at 50% for 12 min with a flow rate of 1.0 ml/min with an injection volume of 50 μl. The detection wavelength was 330 nm and the chromatographic peaks were confirmed and quantified by comparing their retention times and integrating the area of their peaks with that of the standards after obtaining a calibration curve.

2.10. Statistical analyses

Treatment effects were determined by analyses of variance (ANOVAs) and any difference between treatments were determined using Tukey's pairwise comparison test at a significance level of 95% (SYSTAT 11®), except where otherwise indicated in the text. The data were treated in order to meet the assumptions of ANOVA (*i.e.* normality of data and evenness of variance) and

transformations – natural log, arcsin (for % colonisation) – were done when required.

3. Determining the growing requirements for basil and obtaining matched tissue P concentrations in AM and NM plants

This chapter describes the experiments that were carried out in order to attempt to optimise the growth conditions of basil. This led to the investigation and selection of an adequate P supply that would result in NM and AM plants with matched tissue P concentrations in shoots.

3.1. Preliminary experiment

Aims

This experiment was designed to investigate the growth rate of basil and timing of harvest, as the developmental stage of the plant is known to influence the production of phytochemicals such as RA and CA. As a means to estimate the growth of basil, the number of leaves was counted throughout the experiment and related to plant biomass. I also wanted to test the suitability of the soil/sand mixture (Chapter 2) to grow basil under my experimental conditions. Finally, as very little work has been done on basil and AMF (Giovannetti 1997, Dickson 2004, Copetta *et al.* 2006), the experiment also aimed at evaluating the responsiveness of basil to an AM fungus.

Materials and methods

The experiment was set up as follows: there were two P supplies, two mycorrhizal treatments (NM controls and AM) and three harvests (at 3, 6 and 10 wks). This factorial design was completely randomised and there were four replicates per treatment at each harvest.

The seeds were sterilised and germinated in a Petri dish as described in Section 2.1. Three day-old seedlings were transferred into 700 g pots (1 seedling per pot) containing the soil/sand mixture (1:9, w/w) as described in Section 2.4. Nutrients were mixed with the soil and P was added at two different supplies: 0.025 (P1) or 0.05 (P2) g/kg $CaHPO_4$, as described in Sections 2.4 and 2.5. These nutrient supplies were adequate to sustain plant growth for the first six weeks of the experiment. From week 7 all the remaining plants (NM and AM) received additional nitrogen (N) as NH_4NO_3 solution (0.28 g total extra N per pot) once a week as some AM plants showed signs of N deficiency (*i.e.* chlorosis). The mycorrhizal treatments consisted of the soil/sand mix with 10% inoculum (w/w, *i.e.* 70 g per pot, dry soil) of the AM fungus *G. intraradices*, whereas NM treatments received 10% (w/w, dry soil) of NM pot culture (Section 2.3). All plants were grown in a glasshouse during summer as described in Section 2.4.

At each harvest the following parameters were recorded: number of leaves, fresh and dry weights, plant P concentration (at 6 and 10 wks only), RA and CA concentrations, and percent colonisation, according to the methods described in Chapter 2. As the plants were quite small at 3 wks, it was not possible to conduct plant P analyses for this harvest. Hence, P concentrations and content were determined for plants harvested at 6 and 10 wks. NM root samples at P1 were also pooled due to the lack of material to conduct analyses.

Results

NM plants were not colonised at all. After 3 wks growth (first harvest) AM colonisation was already fully established in inoculated plants, with the main

structures found in the root cortex (arbuscules and vesicles). Although percent colonisation appeared higher in plants growing at P1 than P2, the difference was not statistically significant (Table 3.1). At 6 and 10 wks, colonisation in plants growing at P2 was as high as that of those growing at P1. The number of leaves increased throughout the experiment as the plants grew bigger, and at each harvest there were no significant differences between treatments (Table 3.1).

Table 3.1. Percent colonisation and number of leaves of *O. basilicum* grown under two P supplies with (AM) or without (NM) *G. intraradices*. There were three harvests at 3, 6 and 10 wks after planting. Means (n=4) ± SE are presented. There were no significant differences at each harvest for each parameter.

Harvest	Treatment	% colonisation	# leaves
3 wks	NM P1	0	3 ± 0 a
	AM P1	51 ± 7 a	3 ± 0 a
	NM P2	0	2 ± 0 a
	AM P2	31 ± 10 a	4 ± 0 a
6 wks	NM P1	0	6 ± 0 a
	AM P1	59 ± 9 a	9 ± 1 a
	NM P2	0	7 ± 1 a
	AM P2	52 ± 5 a	13 ± 1 a
10 wks	NM P1	0	30 ± 2 a
	AM P1	75 ± 7 a	33 ± 4 a
	NM P2	0	41 ± 3 a
	AM P2	73 ± 4 a	34 ± 2 a

The plant biomass for roots and shoots followed similar trends (Fig. 3.1). At 3 wks, there were no significant differences between AM and NM plants at any P supply. At 6 wks, AM plants had significantly higher shoot and root dry weight than NM plants, at both P supplies. At 10 wks, there were no differences in shoot dry weight between AM and NM plants at P1, but higher P supply resulted in a significant increase of shoot dry weight in NM plants. At 10 wks, root dry weights were not significantly different between most treatments, except for AM plants at

P2, which had higher root biomass than NM plants at P1. There was also a strong correlation between the total plant biomass and the number of leaves (Fig. 3.2).

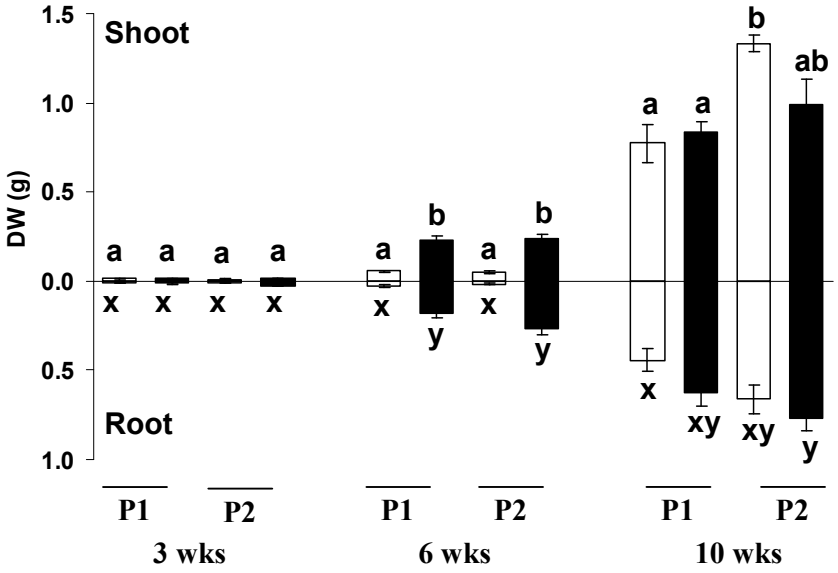

Figure 3.1. Shoot and root dry weight (DW, g) of *O. basilicum* grown under two P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There were three harvests at 3, 6 and 10 wks after planting. Means (n=4) ± SE are presented. For each harvest, different letters indicate significant differences according to Tukey's test (p=0.05).

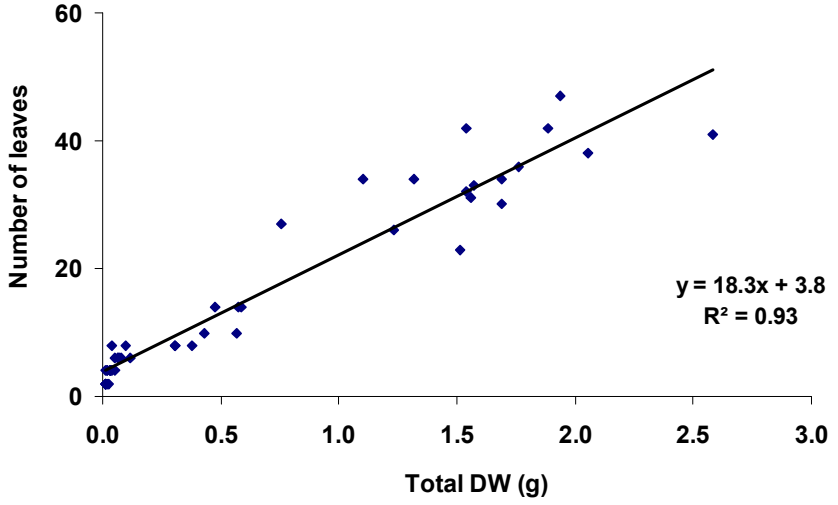

Figure 3.2. Correlation between the number of leaves per plant of *O. basilicum* and total plant dry weight (DW, g) for all AM and NM plants. Each dot represents one replicate.

Table 3.2. Shoot and root P concentrations (mg/g DW), and total plant P content (mg/plant), in *O. basilicum* grown under two P supplies with (AM) or without (NM) *G. intraradices*. The analyses were done for plants harvested at 6 and 10 wks after planting. Means (n=2-4; pooled samples for NM plants) ± SE are presented. For each harvest and each parameter, different letters indicate significant differences according to Tukey's test (p=0.05); n/a: data not available.

Harvest	Treatment	Shoot P conc. (mg/g DW)	Root P conc. (mg/g DW)	Total plant P (mg)
6 wks	NM P1	1.85 ± 0.23 a	1.71 (n=1)	0.15 ± 0.00 a
	AM P1	1.46 ± 0.23 a	2.57 ± 0.43	0.81 ± 0.19 b
	NM P2	1.78 ± 0.34 a	n/a	0.08 ± 0.00 a
	AM P2	2.03 ± 0.26 a	3.02 ± 0.31	1.28 ± 0.15 b
10 wks	NM P1	0.91 ± 0.12 a	1.47 ± 0.40 a	1.30 ± 0.24 a
	AM P1	1.11 ± 0.09 a	1.88 ± 0.14 a	2.08 ± 0.20 b
	NM P2	1.24 ± 0.34 a	1.61 ± 0.17 a	2.25 ± 0.35 b
	AM P2	1.69 ± 0.18 a	1.80 ± 0.25 a	2.44 ± 0.20 b

Shoot and root P concentrations did not vary significantly between treatments at either harvest (Table 3.2). At 6 wks, AM plants had taken up significantly (p=0.035) more P than NM plants in all P treatments. At 10 wks, NM plants growing at P1 took up significantly (p=0.031) less P than all other treatments.

Shoot RA concentrations ranged from 1.5 to 10.5 mg/g DW (Fig. 3.3). At 3 wks NM plants growing at P2 had significantly higher shoot RA concentrations than all other treatments. At 6 wks, AM plants had significantly higher shoot RA concentrations than NM plants, at all P supplies. However, at 10 wks, NM plants at P2 had significantly higher shoot RA concentrations compared to most other treatments. Shoot RA concentrations in AM and NM plants followed a similar trend to that observed for total biomass and total P content. Regression analyses performed on the data indicated that shoot dry weight and shoot P content were positively correlated to shoot RA concentrations in NM plants (R^2=0.44;

y=4.6x+3.17; p=0.000 and R^2=0.53; y=4.1x+2.8; p=0.017, respectively). There were no such correlations for AM plants.

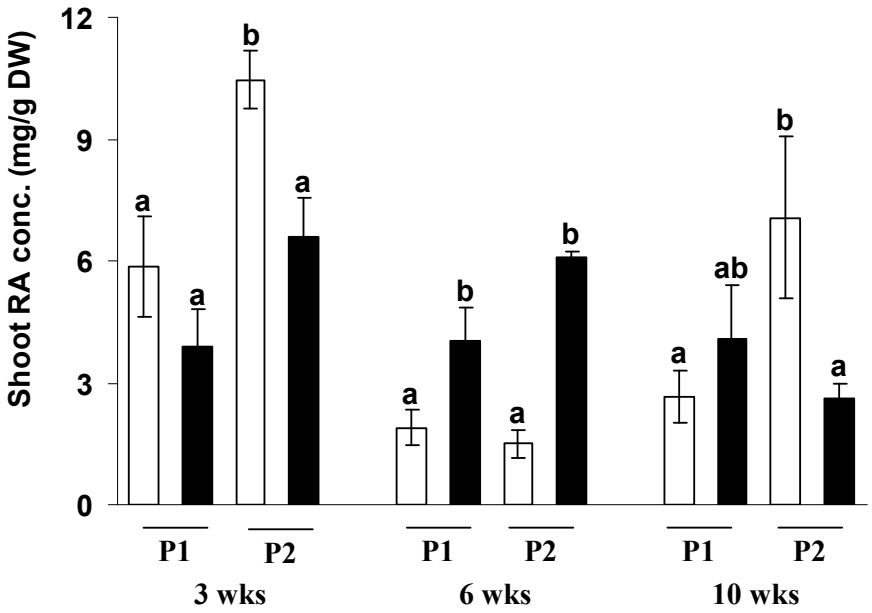

Figure 3.3. Shoot RA concentrations in *O. basilicum* grown under two P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There were three harvests at 3, 6 and 10 wks after planting. Means (n=4) and SE are presented. For each harvest different letters indicate significant differences according to Tukey's test (p=0.05).

Root RA concentrations were generally higher than shoot concentrations, ranging from 7.0 to 44.0 mg/g DW (Fig. 3.4). Root RA concentrations were quite low at 3 wks for all treatments, but increased by 6 wks and remained similar at 10 wks for most treatments. There was also a positive correlation between root RA concentrations and root dry weight in AM (R^2=0.48; y=35.9x+18.4; p= 0.000) and NM plants (R^2=0.80; y=43.2x+11.4; p=0.000).

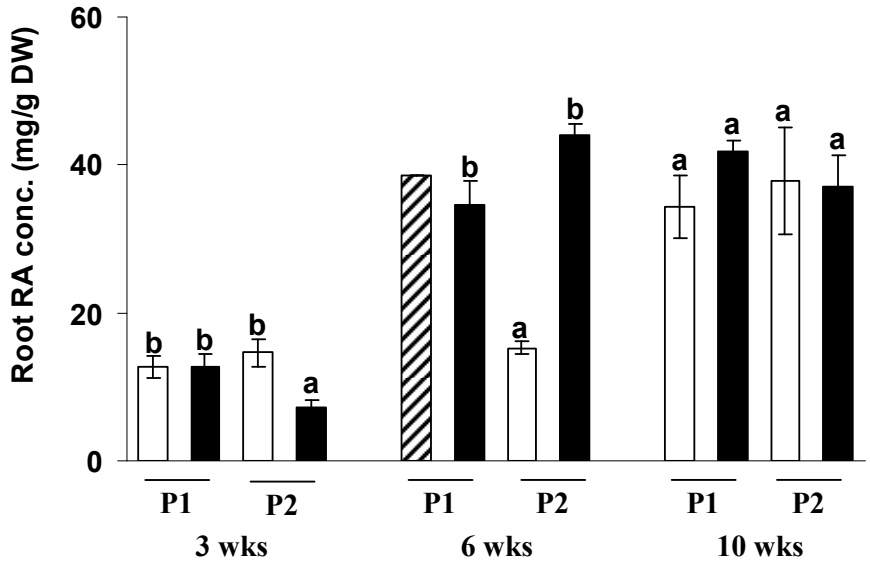

Figure 3.4. Root RA concentrations in *O. basilicum* grown under two P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There were three harvests at 3, 6 and 10 wks after planting. The hatched bar represents a single pooled sample of the NM treatment. Means (n=4) and SE are presented. For each harvest different letters indicate significant differences according to Tukey's test (p=0.05).

Shoot CA concentrations ranged from 1.5 to 9.5 mg/g DW (Fig. 3.5). These concentrations decreased over time for all treatments as the plants grew bigger. At 3 wks, NM plants grown at P2 had significantly higher (p=0.05) CA concentrations than those grown at P1. At 6 wks, all AM plants had significantly higher CA concentrations than NM plants at P2, but at 10 wks all treatments had similar shoot CA concentrations. Shoot CA concentrations and shoot dry weight were negatively correlated in NM (R^2=0.24; y=-2.8x+4.9; p=0.015) and AM plants (R^2=0.62; y=-5.3x+6.4; p=0.000), although this correlation was weak in NM plants.

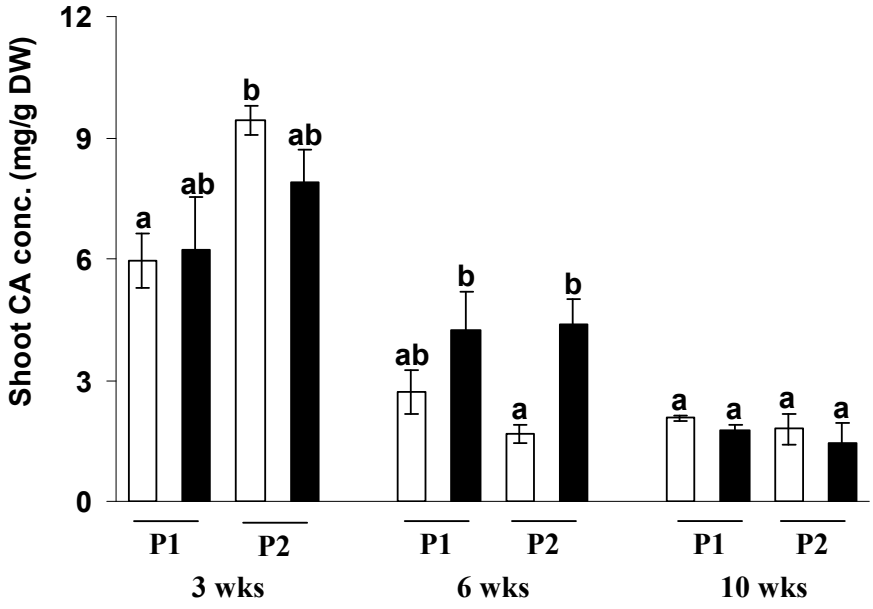

Figure 3.5. Shoot CA concentrations in *O. basilicum* grown under two P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There were three harvests at 3, 6 and 10 wks after planting. Means (n=4) and SE are presented. For each harvest different letters indicate significant differences according to Tukey's test (p=0.05).

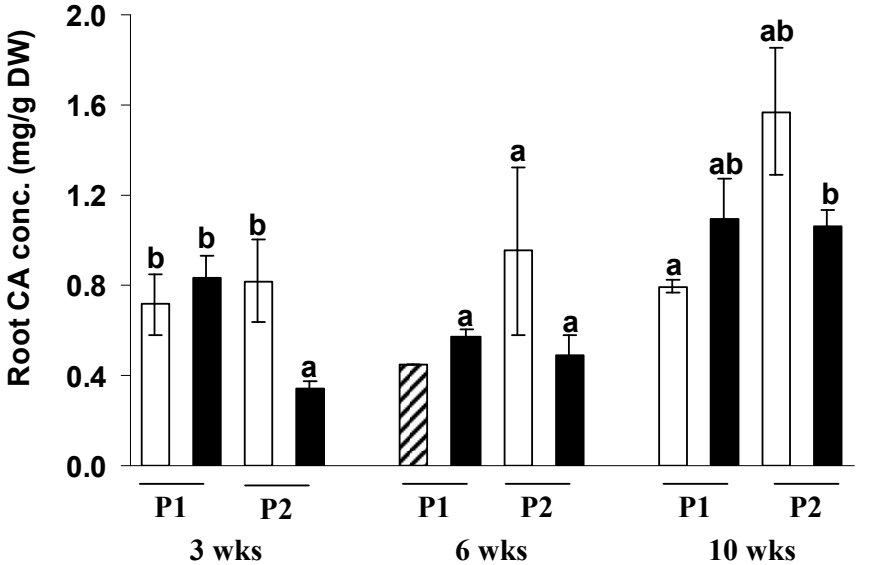

Figure 3.6. Root CA concentrations in *O. basilicum* grown under two P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There were three harvests at 3, 6 and 10 wks after planting. The hatched bar represents a single pooled sample of the NM treatment. Means (n=4) and SE are presented. For each harvest different letters indicate significant differences according to Tukey's test (p=0.05).

Root CA concentrations were lower than shoot concentrations, with values ranging from 0.3 to 1.5 mg/g DW (Fig. 3.6). At 3 wks, AM plants grown under P2 had significantly lower root CA concentrations than other treatments. There were no differences between treatments at 6 wks. At 10 wks, AM plants grown under P2 had significantly higher root CA concentrations than NM plants at P1. Root CA concentrations were also positively correlated to root dry weight in AM plants (R^2=0.42; y=1.1x+0.5). Finally, at 6 and 10 wks, it was observed that the roots of both AM and NM plants were pot-bound, and that some of the plants had flower buds (10 wks only), factors that might have influenced the concentrations of RA and CA.

Discussion

Responsiveness of basil to P supply and AMF has been poorly documented in the literature. In the scope of this project, it was important to investigate such issues, which have been partly addressed in this preliminary experiment. Basil grew well in the soil/sand mix, which therefore proved to be a suitable medium to conduct experiments. The rate of growth of basil was relatively slow in the first 3 wks, but was followed by a sharp increase in growth rate in the last 4 wks of the experiment. AM plants had a higher growth rate than NM plants in the first 6 wks, at all P supplies. Hence, 3 wks is too short a growing time to provide enough material for the full suite of analyses (*i.e.* P determination, RA and CA extractions, mycorrhizal colonisation). Growing the plants for only 3 wks is also not relevant for commercial practices. The number of leaves was shown to be a good indicator of the total plant biomass. This parameter was used in subsequent experiments to monitor the growth rate of basil and decide on harvest times. As a

general rule, when most plants reached an average of 40 leaves, it was decided that there would be enough biomass to harvest them (generally after ~7 wks growth). Basil proved to be very responsive to the AM fungus *G. intraradices*, as mycorrhizal colonisation was well established at 3 wks and AM plants were much bigger than NM plants by 6 wks. The higher growth rate of AM plants compared to NM plants was most probably due to improved P uptake as shown by the results at 6 wks (see Table 3.2). However, despite higher P uptake and better growth in the first 6 wks of the experiment, AM plants had similar shoot and root P concentrations to NM plants. NM plants only had similar growth rates and P uptake to AM plants at 10 wks, at which point both NM and AM plants showed a clear response to P supply. Thus, the main objectives set for this experiment were fulfilled.

The fact that AM plants had higher shoot RA and CA concentrations than NM plants under similar shoot P concentrations at 6 wks suggests that the AM fungus contributed to higher phytochemical concentrations other than through improved P content. The regression analyses performed between shoot biomass, P content and shoot RA concentrations further indicated that P supply affects the production of RA in the shoots of NM plants, but not of AM plants. In addition, the decrease in shoot CA concentrations in NM and AM plants over time may explain why RA concentrations reached a "plateau" by 6 wks, as CA is the direct precursor of RA (see Fig. 1.2, Chapter 1). Overall, the range of shoot RA concentrations observed in this experiment was consistent with what has been reported in the literature for NM plants belonging to the Lamiaceae (Beier and Nigg 1992, Zgorka and Glowniak 2001, Javanmardi *et al.* 2002).

The small pot size used in this experiment might have hindered plant growth, as the roots were pot-bound at 6 and 10 wks. In light of these observations it was decided to use bigger pots (1.4 kg pots) for future experiments. Finally, anthesis (at 10 wks in this experiment) is the developmental stage at which anthocyanins (phenolic compounds) have been shown to be at their highest concentrations in basil (Phippen and Simon 1998). However, in the current experiment shoot RA and CA concentrations peaked at 3 wks, regardless of the treatment. The discrepancy between the results reported in this experiment and those of Phippen and Simon (1998) is likely to be due to the difference in phenolic compounds analysed in both studies.

Outcome

From these results, it was shown that *G. intraradices* can be used as a satisfactory inoculum. It was also decided to grow basil in 1.4 kg pots for at least six weeks, allowing the plants to grow large enough to conduct all analyses required. More specifically, the harvest time would be decided by the number of leaves as an indicator of growth rate. Despite similar shoot P concentrations in AM and NM plants in this experiment, the effect of different P supplies on the growth of basil was further investigated (Sections 3.2 – 3.4). As discussed in Section 1.3.2, to distinguish any physiological response due to AMF that is unrelated to nutritional benefits from the symbiosis, it is essential to obtain AM and NM plants with similar growth rate and tissue P concentrations (Smith and Read 1997).

3.2. First P experiment

Aims

This section and the next further explore the responsiveness of basil to different P supplies and aim at obtaining NM and AM plants with matched tissue P concentrations. The responsiveness of basil to P was tested using six different P supplies for NM plants, the highest supply corresponding to the maximum amount of P applied when growing this plant in the field. AM plants were tested on three of those six P supplies only.

Materials and methods

The experiment consisted of the following treatments: two mycorrhizal treatments (NM or AM) and three or six P supplies (AM and NM plants, respectively). The experiment was set up in a complete randomised design and there were six replicates per treatment.

Basil seeds were sterilised and germinated in a Petri dish as described in Section 2.1. The germinated seedlings were transplanted into 1400 g pots (1 seedling per pot) containing the soil/sand mixture (1:9, w/w) in which nutrients were mixed as described in Section 2.4. As AM plants showed N deficiency in the preliminary experiment (Section 3.1), twice the amount was added this time, which corresponded to 171.4 mg/kg NH_4NO_3. Phosphorus was added separately in the soil/sand mix at six different supplies for NM plants: no added P (P0), 0.05 (P1); 0.1 (P2); 0.25 (P3); 0.4 (P4) and 0.5 (P5) g/kg $CaHPO_4$. For AM plants, the three P supplies used were P0, P3 and P5. The AM treatments consisted of the soil/sand mix with 10% inoculum (w/w, 140 g per pot, dry soil) of *G. intraradices* (Section

2.3), whereas NM treatments received 10% (w/w, dry soil) of the NM pot culture (Section 2.3). All plants were grown in a glasshouse during winter as described in Section 2.4. The number of leaves was monitored during the experiment in order to estimate the biomass of the plants and select an appropriate harvest time (see Section 3.1). At 8 wks, when the number of leaves per plant averaged ~40, all plants were harvested and the following parameters were recorded: shoot and root fresh and dry weights, plant P concentration and percent colonisation, as described in Chapter 2.

Results

Many plants died during the course of this experiment and some AM plants were infected with binucleate *Rhizoctonia* (BNR; "chain fungus"), which made data analyses and interpretation difficult. As a consequence, P analyses were only performed for a few treatments and statistical comparisons were not conducted due to the high variability of the data. No NM plants were colonised by *G. intraradices* in the experiment. In AM plants, colonisation levels decreased as P supply increased, except at P5 where a high level of colonisation was observed (Table 3.3). NM and AM plants had higher biomass under high P supplies, despite large variation within each treatment (Fig. 3.7). There was a growth response of basil to *G. intraradices* at P0, corresponding well to the high colonisation level at this P supply. Shoot and root P concentrations were similar in both NM and AM plants at all P treatments, whereas P uptake increased with increasing P supply in all plants (Table 3.3).

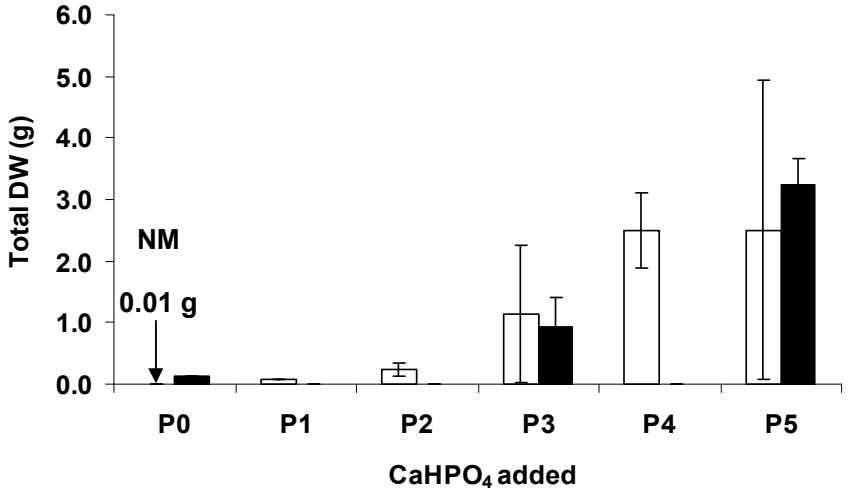

Figure 3.7. Total plant dry weight (DW, g) of *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. Means (n=2-5) and SE are shown. No statistical analyses were performed due to the high variability of data and because of the loss of many replicates.

Table 3.3. Percent colonisation, shoot and root P concentrations (mg/g DW) and total plant P content (mg) in *O. basilicum* grown under different P supplies with (AM) or without (NM) *G. intraradices*. Means (n=2-5) and SE are shown. No statistical analyses were performed due to the high variability of data and because of the loss of many replicates; n/a: data not available.

Treatment	% colonisation	Shoot P conc. (mg/g DW)	Root P conc. (mg/g DW)	Total P (mg)
NM P0	0	n/a	n/a	n/a
AM P0	94 (n=1)	1.69 (n=1)	1.46 (n=1)	0.210 (n=1)
NM P2	0	2.28 ± 0.35	2.60 ± 0.09	0.85 ± 0.09
AM P2	n/a	n/a	n/a	n/a
NM P3	0	n/a	n/a	n/a
AM P3	20 ± 8	n/a	n/a	n/a
NM P4	0	2.94 ± 0.29	2.80 ± 0.40	6.94 ± 1.54
AM P4	n/a	n/a	n/a	n/a
NM P5	0	2.24 (n=1)	2.34 (n=1)	11.25 (n=1)
AM P5	48 ± 8	1.35 ± 0.25	1.65 ± 0.10	4.75 ± 0.36

Discussion

Many plants were lost in this experiment and on the basis of the data available it was not possible to draw strong conclusions or to select an appropriate P supply at which NM and AM plants have similar growth rates and shoot P concentrations.

Two main reasons could explain the loss of plants: 1) some AM plants were infected with BNR, which might have affected plant growth and led to plant death in some cases; 2) the experiment was carried out in winter, which probably made it harder for basil to grow well, even in a semi-controlled environment. The first reason is unlikely to have significantly contributed to plant death as it has been shown that BNR does not affect plant growth or mycorrhizal colonisation in mung bean (*Vignia radiata* L.) (Kasiamdari *et al.* 2002). Moreover, young seedlings that were germinated in Petri dishes then transferred into pots died within the first few days of transfer. As the root system was not well developed in those young seedlings, this could also have contributed to the loss of several plants. Despite high variability of the data, AM plants grew better than NM plants when no P was supplied, indicating that basil is quite responsive to *G. intraradices*. It also appeared as though all plants were P limited during the experiment, unless supplied with more than 0.25 (P3) g/kg $CaHPO_4$. However, under the highest P supply, the plants did not appear to be P limited. This high P supply (P5) did not contribute to significant increases in plant biomass compared to plants at P4. Furthermore, as AM colonisation was reduced with increasing P supplies, it was decided to eliminate the P5 treatment (0.5 g/kg $CaHPO_4$) in the next experiment.

3.3. Second P experiment

Aims

The aims were essentially the same as stated in Section 3.2. Some modifications were made in the experimental set-up in order to avoid the loss of replicates. The seed germination method was modified and more AM treatments as well as replicates were added (see below).

Materials and methods

The experiment consisted of the following treatments: two mycorrhizal treatments (NM or AM), five P supplies with six replicates per treatment, in a complete randomised design.

Basil seeds were sterilised and germinated in a tray filled with sterilised fine sand until seedlings were 7 day-old as described in Section 2.1. The seedlings were then transferred into 1400 g pots (one seedling per pot) containing the same soil/sand mixture (1:9, w/w) as described in Section 2.4. Nutrients were mixed with the soil as described in Section 3.2. There were five P supplies: no added P (P0), 0.1 (P1); 0.2 (P2); 0.3 (P3) and 0.4 (P4) g/kg $CaHPO_4$. AM and NM treatments were prepared as described before (Section 2.3). The plants were grown in a glasshouse during winter as described in Section 2.4. Once again, the number of leaves was monitored during the experiment to help select an appropriate harvest time (see Section 3.1). After 7 wks (~40 leaves per plant), the plants were harvested and the following parameters were recorded: shoot and root fresh and dry weights, plant P concentration, RA and CA concentrations and percent colonisation as described in Chapter 2.

Results

NM plants did not show any sign of root colonisation or infection by BNR, but several replicates of this treatment were still lost (P0, n=0; P1, n=1; P2, n=4, P3, n=3 and P4, n=4). Many AM plants were again infected by BNR. AM colonisation was highest at P0 and significantly lower at other P supplies (Table 3.4).

Table 3.4. Percent colonisation in *O. basilicum* grown with *G. intraradices* under different P supplies Means (n=number of surviving plants, *i.e.* replicates) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Treatment	% colonisation
AM P0	83 ± 7 b (n=3)
AM P1	40 ± 8 a (n=4)
AM P2	12 ± 6 a (n=2)
AM P3	17 ± 3 a (n=4)
AM P5	22 ± 7 a (n=4)

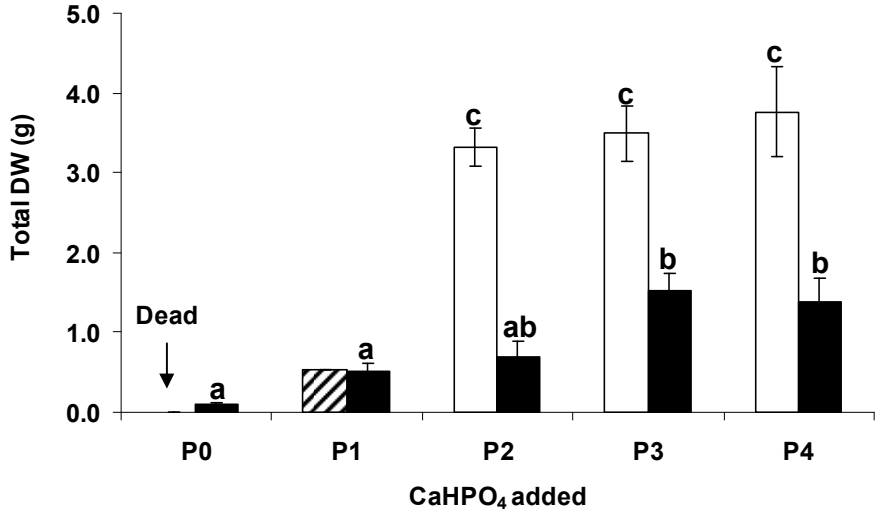

Figure 3.8. Total plant dry weight (DW, g) of *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. The hatched bar represents one replicate of the NM treatment. Means (n=2-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

NM plants did not grow well under no P or low P supplies (P0 and P1) as most of them died, whereas AM plants grew under these conditions (Fig. 3.8). At P2 and higher P supplies, there was a large increase of dry weight of NM plants compared to AM plants which also grew better under high P supplies (P3 and P4). There were no significant differences in root/shoot ratio between NM and AM plants at any P supply (results not shown). Shoot P concentrations in NM plants increased with increasing P supply and reached a maximum at P3 (Fig. 3.9). Shoot P concentrations did not increase significantly when AM plants were supplied with more than 0.2 g/kg (P2) $CaHPO_4$. NM and AM plants had similar shoot P concentrations at each P supply.

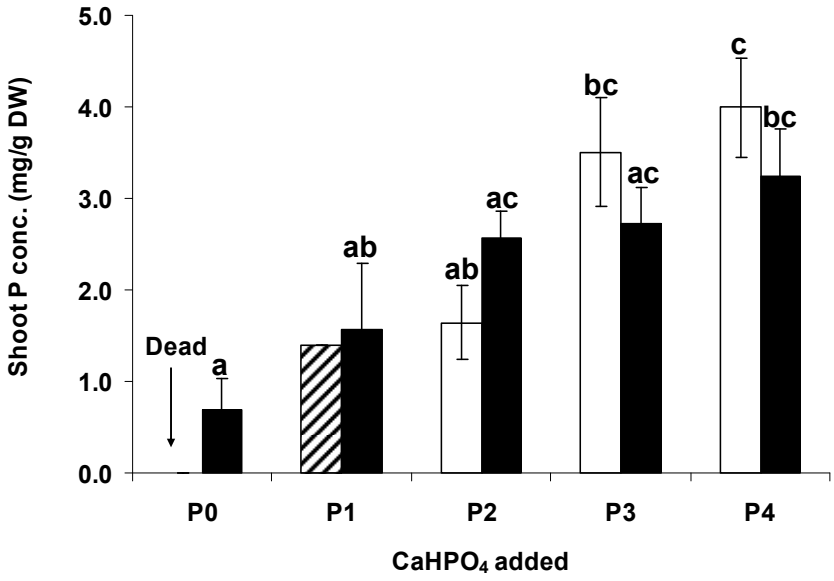

Figure 3.9. Shoot P concentration (mg/g DW) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. The hatched bar represents one replicate of the NM treatment. Means (n=2-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

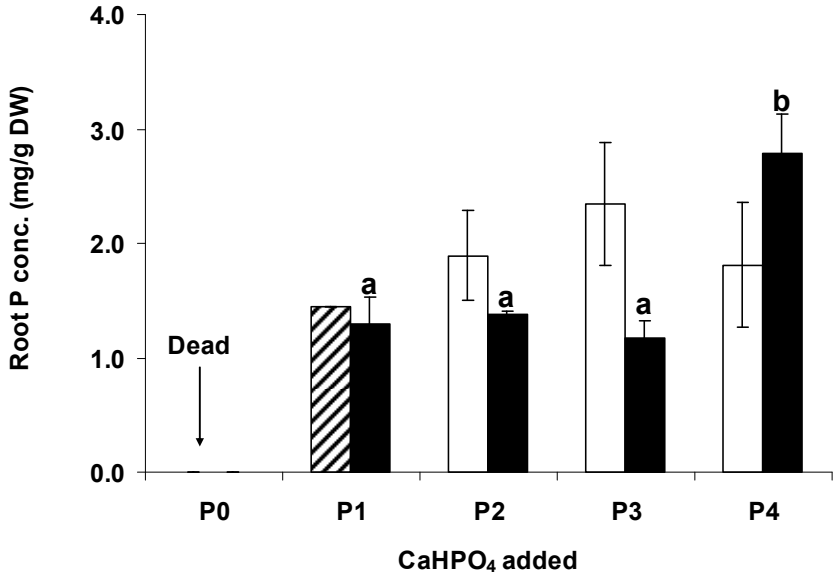

Figure 3.10. Root P concentration (mg/g DW) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. The hatched bar represents one replicate of the NM treatment. Means (n=2-6) and SE are shown. For AM plants, different letters indicate significant differences according to Tukey's test (p=0.05). There were no differences between NM and AM plants at any P supply.

Root P concentrations did not differ significantly between P supplies in NM plants (Fig. 3.10). The same was observed for AM plants, with the exception of plants grown at P4, which had significantly higher root P concentrations than other AM plants. There were no significant differences in root P concentrations between NM and AM plants at any P supply. Plant P content was quite low at P1 in NM plants, but increased significantly with higher P supplies for both NM and AM plants (Fig. 3.11). Overall, the high P contents observed at P2, P3 and P4 corresponded to higher dry weight under those P supplies for NM and AM plants. NM plants always had higher P content compared to AM plants at any P supply.

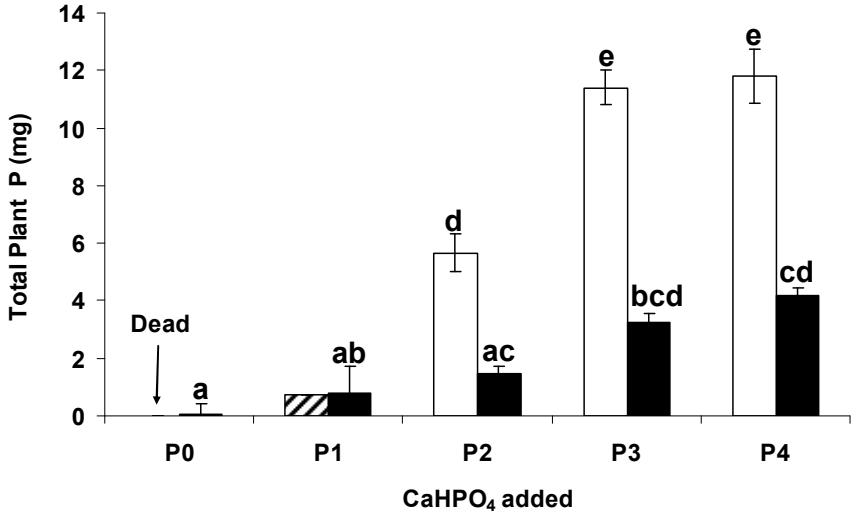

Figure 3.11. Total plant P content (mg) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. The hatched bar represents only one replicate of the NM treatment. Means (n=s-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Shoot RA concentrations in NM plants were highest at P2, P3 and P4 but not significantly different from each other, although significantly higher than all AM plants (Fig. 3.12). In AM plants, shoot RA concentrations remained relatively low but increased significantly with increasing P supply to reach a maximum at P4. Root RA concentrations of NM plants were also highest at P2, P3 and P4, but not significantly different from each other (Fig. 3.13). Most NM plants had significantly higher root RA concentrations than AM plants, except at P4. Root RA concentrations of AM plants were highest at P4 and significantly different from most other AM plants, except those grown without P (P0). Root RA concentrations were twice as high as those in shoots of both AM and NM plants.

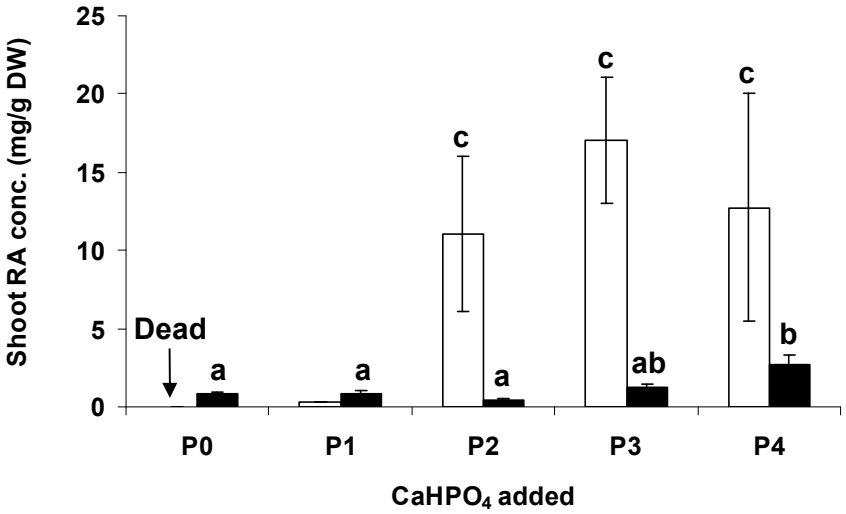

Figure 3.12. Shoot RA concentration (mg/g DW) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There was only one replicate of the NM treatment at P1. Means (n=2-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

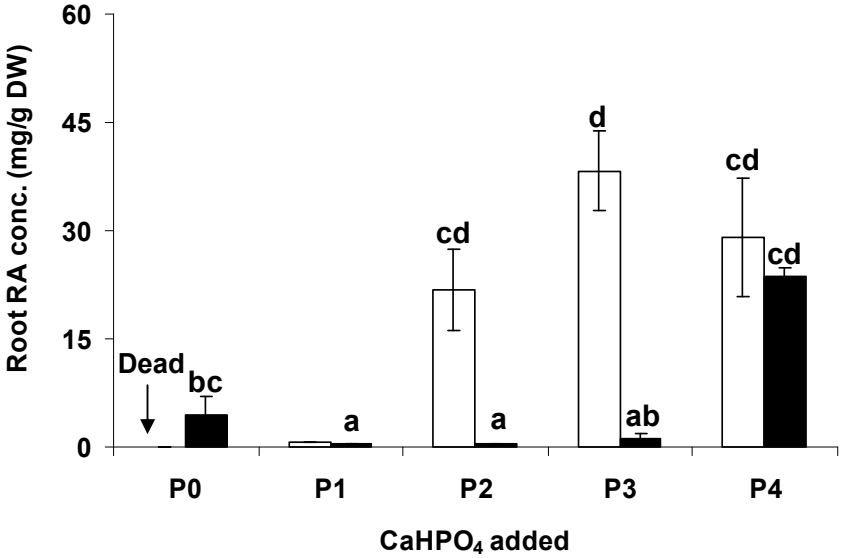

Figure 3.13. Root RA concentration (mg/g DW) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There was only one replicate of the NM treatment at P1. Means (n=2-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Shoot CA concentrations in NM plants were highest at P2, P3 and P4, but not significantly different from each other (Fig. 3.14). In AM plants, shoot CA concentrations did not vary significantly between P treatments, except at P4 (Fig. 3.14). Generally, NM plants had significantly higher shoot CA concentrations than AM plants, except at P4. Although root CA concentrations appeared to increase with increasing P supply in NM plants, there were no significant differences between treatments, which is probably the result of the high variability of the data (Fig. 3.15).

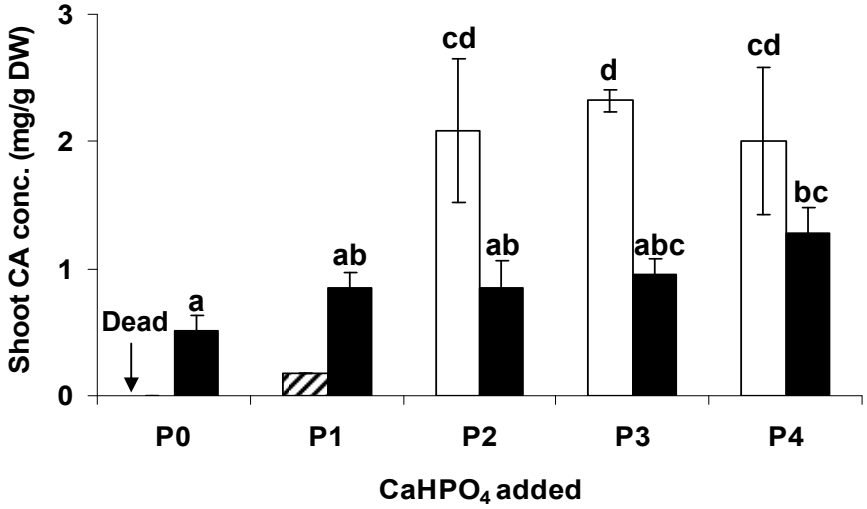

Figure 3.14. Shoot CA concentration (mg/g DW) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There was only one replicate of the NM treatment at P1. Means (n=2-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Root CA concentrations were significantly higher in AM plants at P0, P3 and P4, compared to the other two P supplies. At P2, NM plants had significantly higher root CA concentration than AM plants. Root CA concentrations were lower in both NM and AM plants compared to shoot CA concentrations, which is opposite to what was observed in the preliminary experiment. Finally, there were no relationships between shoot and root RA and CA concentrations and P concentrations in either AM and NM plants.

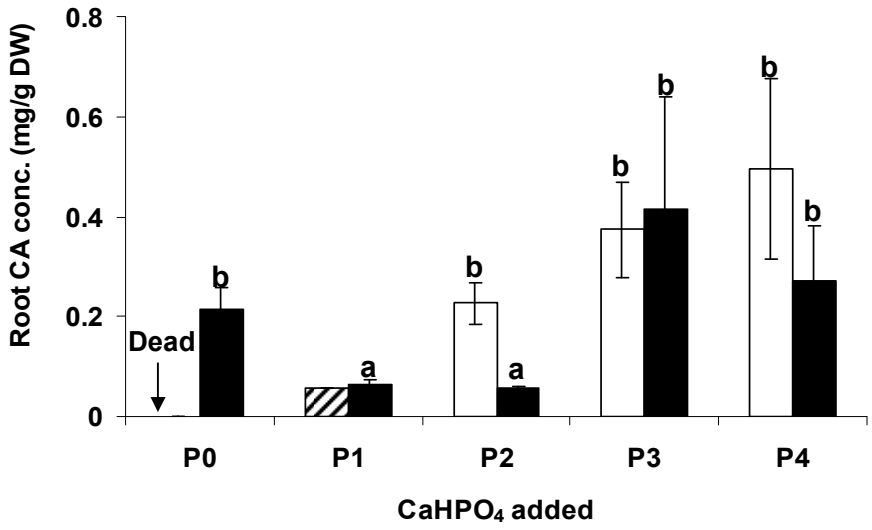

Figure 3.15. Root CA concentration (mg/g DW) in *O. basilicum* grown under different P supplies. White bars NM plants, black bars AM plants colonised by *G. intraradices*. There was only one replicate of the NM treatment at P1. Means (n=2-6) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Discussion

The results obtained here clearly indicate that basil is very responsive to P, as the plants grew bigger and took up more P under high P supplies, especially when NM. The high requirement for P, and hence responsiveness to P supply, could explain why many NM plants died within a few weeks when no or low P was supplied. Only AM plants survived at P0, which is consistent with the results

described in Section 3.2., and indicates that basil might have benefited from being colonised by *G. intraradices*. NM and AM plants also had similar shoot P concentrations at all P supplies, indicating that no "luxury" P was accumulated in NM or AM plants. Despite high variability of the data and loss of replicates, shoot P concentrations were the same in NM and AM plants at the same P supply. This confirmed the results of earlier experiments and showed that it is relatively easy to obtain plants matched for P concentrations. Despite matched P concentrations, growth rates were not similar between NM and AM plants, the latter showing growth depressions. Furthermore, NM plants did not grow any better when supplied with more than 0.2 g/kg $CaHPO_4$ (P2), in agreement with previous results (Section 3.2). This finding suggests that this P supply is potentially suitable for subsequent experiments. Mycorrhizal colonisation also decreased significantly in AM plants under high P supplies, further emphasizing that P2 is a good P supply to grow AM and NM plants. The relatively low colonisation levels obtained in this experiment might have been due to the presence of BNR in most AM roots, although this hypothesis remains to be proven (see discussion Section 3.2). The presence of BNR could have also influenced the production of RA and CA in AM basil, although there is no specific evidence to support this hypothesis.

Shoot and root RA concentrations were much higher in NM plants than AM plants, regardless of P supply, although the data were quite variable. Due to this high variability and the presence of BNR in AM plants, the results obtained for RA and CA concentrations cannot be used with confidence. The results for shoot and root RA and CA concentrations were also quite different from those obtained in the preliminary experiment, but the higher RA concentrations found in roots

compared to that of shoots were consistent with what was previously observed (Section 3.1). The differences in growing seasons and P supplies added to the soil in the preliminary experiment compared to the second P experiment might explain such disparate results. The low light intensity in winter might have affected plant growth (basil being a summer plant), which in turn might have affected the production of RA and CA in plant tissues. Clearly, AM plants suffered from growth depression compared to their NM counterparts in this experiment, which is likely to have been the result of AMF being a relatively large sink of carbon under low light conditions (Smith and Read 1997). Thus, the results obtained for RA and CA concentrations in this experiment must be interpreted with caution because of the growth conditions that were probably not optimal.

Only AM plants were infected with BNR, which led me to think that the chain fungus might have been associated with the mycorrhizal inoculum (pot cultures). Hence, new pot cultures were prepared at the end of this experiment in order to obtain fresh AM inoculum free of any other fungi. These pot cultures were established from standard AM inoculum that had been thoroughly checked for BNR. Finally, another hypothesis for the loss of several replicates in this experiment (other than growth conditions) is that some plants might have suffered from N toxicity as N was applied as NH_4NO_3 at a higher concentration than previously (Section 3.1). As the nutrients used in my experiment were originally used in experiments conducted in 50% soil (Jakobsen *et al.* 1992), a toxicity effect could have been the result of a low buffering capacity of the soil/sand mix used here (1:9, w/w dry soil).

3.4. Third P experiment

Aims

This experiment was conducted to verify that the P supply selected in Section 3.3 would be suitable to obtain plants with similar P concentrations and growth rates, as well as further adjusting the growth conditions of basil in a soil/sand mix that provides a better buffering capacity against potential nutrient toxicity. Two percentages of soil were tested in the soil/sand mix as well as a low and high percentage of AM inoculum. The higher amount of inoculum application was to assure good colonisation of basil despite a relatively high soil P supply (0.2 g/kg $CaHPO_4$).

Materials and methods

The experiment consisted of the following treatments: 1) three mycorrhizal treatments, NM plants as well as AM plants with either 10 or 25% inoculum (w/w, dry soil); and 2) two P supplies with six replicates per treatment, in a complete randomised design.

Basil seeds were sterilised and germinated directly in the growth pots (1400 g), as described in Section 2.1. Once well established, the seedlings were thinned to one plant per pot, which contained a soil/sand mixture (1:3, w/w), as described in Section 2.4. Nutrients were mixed with the soil as described in Section 3.2 and there were two different P supplies: 0.1 (P1) and 0.2 g/kg (P2) $CaHPO_4$. NM and AM treatments were prepared as described earlier (Section 2.3), with two amounts of inoculum application: 10 and 25% (w/w, 140 and 350 g per pot, dry soil, respectively). The plants were grown in a glasshouse towards the end of winter as

described in Section 2.4. The number of leaves was monitored once again as a way to estimate plant biomass (see Section 3.1). When the plants had ~40 leaves, after 7 wks, they were harvested and the following parameters were recorded: shoot and root fresh and dry weights, plant P concentration and percent AM colonisation as described in Chapter 2.

Results

No plants were lost during the experiment and no BNR was detected in any plants. NM plants did not show any sign of AM colonisation. AM colonisation was quite negligible when 10% inoculum was applied and only some external hyphae (surface colonisation) were observed. Colonisation was consistently well established with 25% inoculum at both P supplies (Table 3.5). NM plants grew equally well under both P supplies. AM plants did not grow well at P1, but significantly better at P2. At P2, NM and AM plants grown with 25% inoculum grew equally well and significantly better than AM plants grown with 10% inoculum.

At P1, all plants had similar shoot P concentrations. At P2, AM plants grown with 25% inoculum had similar shoot P concentrations to NM plants. AM plants that received 10% inoculum had much higher shoot P concentrations than NM plants at P2. Root P concentrations were significantly higher in NM and AM plants grown with 25% inoculum than those grown with 10% inoculum at P1. At P2, there were no significant differences in root P concentrations between plants. NM plants took up more P than AM plants at P1, regardless of how much inoculum

was applied to AM plants (Table 3.6). However, at P2, AM plants grown with 25% inoculum had similar P content to NM plants.

Table 3.5. Percent colonisation and total plant dry weight (DW, g) in *O. basilicum* grown under two P supplies with (AM) or without (NM) *G. intraradices*. There were two inoculum applications. Means (n=3-6) and SE are shown. Within each P supply, different letters indicate significant differences according to Tukey's test (p=0.05). Note the high variability in % colonisation at P1; n/a: data not available.

Treatment	% inoculum (AM only)	% colonisation	Total plant DW (g)
NM P1	n/a	0	0.53 ± 0.25 bc
AM P1	10	15 ± 14 a	0.08 ± 0.05 a
AM P1	25	32 ± 8 a	0.16 ± 0.09 ab
NM P2	n/a	0	0.76 ± 0.24 c
AM P2	10	0*	0.13 ± 0.04 a
AM P2	25	40 ± 10 a	0.74 ± 0.22 c

*Some external hyphae were detected

Table 3.6. Shoot and root P concentrations (mg/g DW), and total plant P content (mg) in *O. basilicum* grown under two P supplies with (AM) or without (NM) *G. intraradices*. There were two inoculum applications. Means (n=3-6) and SE are shown. Within each P supply, different letters indicate significant differences according to Tukey's test (p=0.05); n/a: data not available.

Treatment	% inoculum (AM only)	Shoot P conc. (mg/g DW)	Root P conc. (mg/g DW)	Total plant P (mg)
NM P1	n/a	1.94 ± 0.51 ab	1.64 ± 0.40 b	1.27 ± 0.81 b
AM P1	10	1.05 ± 0.33 a	0.69 ± 0.29 a	0.04 ± 0.01 a
AM P1	25	1.29 ± 0.54 a	1.56 ± 0.22 b	0.21 ± 0.11 a
NM P2	n/a	1.59 ± 0.24 a	0.92 ± 0.22 a	1.09 ± 0.46 ab
AM P2	10	2.46 ± 0.29 b	1.44 ± 0.12 a	0.29 ± 0.12 b
AM P2	25	1.67 ± 0.09 ab	1.15 ± 0.18 a	1.03 ± 0.27 b

Discussion

The absence of BNR indicated that the new pot cultures were not contaminated with fungi other than AMF (see discussion Section 3.3). NM plants grew as well as those from the second P experiment at P1, whereas NM plants at P2 grew worse than seen previously (see Fig. 3.8, Section 3.3). When treated with only

10% inoculum AM plants did not grow well at all, but increasing the amount of inoculum appeared to alleviate the growth depressions. Indeed, at P2, AM plants treated with 25% inoculum, and NM plants grown in the new soil/sand mixture (1:3, w/w) had similar growth rates as well as being matched for shoot P concentrations. The higher amount of inoculum application also resulted in better and more consistent colonisation levels in AM plants than those obtained in Section 3.3.

The mycorrhizal growth depressions observed here (AM plants at P1) and in Section 3.3 appeared to be associated with low percent colonisation in AM plants. Similar effects have been observed by other authors (Li *et al.* unpublished results, F.A. Smith personal communication). It is therefore possible that AMF might be "switching off" the direct root P uptake, even under low colonisation levels, but are not contributing much to P uptake via the AM pathway (acting as "cheaters"). If such is the case, the AM symbiont can therefore become a high cost (large carbon sink) for the host plant, rather than benefiting it through improved P uptake, resulting in mycorrhizal growth depressions.

General outcomes

From the experiments described in this chapter, the following conclusions were made: 1) mycorrhizal responsiveness varied from a positive response with low P supplied (Section 3.1) to a negative response with higher P supplies (Section 3.3); 2) in Section 3.4, higher inoculum application resulted in higher percentage colonisation (see Table 3.5), which in turn resulted in AM plants with higher biomass (see Table 3.6), suggesting that the AM P uptake pathway might have

been operating under such circumstances; and 3) in general, shoot RA and CA concentrations were high in plants with higher biomass, regardless of the mycorrhizal status, and thus the current results do not provide an indication that AMF significantly improve shoot RA or CA concentrations.

In the light of the results presented here, the objectives set out at the beginning of this chapter were thus attained: satisfactory growth conditions for basil were identified; basil was shown to be highly responsive to P supply and to *G. intraradices* (under low P supply), which had not been well documented before; and a specific P supply was selected to grow NM and AM plants in future experiments. Hence, it was decided to use the new soil/sand mixture (1:3, w/w) along with 25% inoculum (w/w, dry soil; AM plants) and 0.2 g/kg (P2) $CaHPO_4$ added to the soil for the rest of the experiments carried out in this project. The combination of the latter amendments resulted in AM and NM plants with matched growth rate and shoot P concentrations, a prerequisite for determining the direct contribution of AMF in the production of RA and CA in basil.

4. Different AMF can have different effects on the production of RA and CA in basil

The results from this chapter were recently published by J.-P. Toussaint, F.A. Smith and S.E. Smith in the journal Mycorrhiza, 17 (4): 291-297 (2007).

It was recently reported that *G. mosseae* can directly increase essential oil concentrations in *Origanum* sp. (Khaosaad *et al.* 2006) as well as in basil (Copetta *et al.* 2006). However, similar effects have not been reported with respect to the production of RA and CA, the main antioxidants found in basil. After finding that a combination of 0.2 g/kg $CaHPO_4$ and 25% AM inoculum yielded similar growth rates and shoot P concentrations in AM and NM plants (Chapter 3), the next step was to investigate if any changes in the production of RA and CA in basil could be attributed to AMF and/or P.

Aims

This experiment was designed to answer the following questions: can the increases in RA and CA concentrations be the direct result of AM colonisation (depending on the fungal species)? Or, are such increases (if any) the result of an indirect effect of improved P uptake via the fungi? As different species of AMF can have different effects on the same host plant, another objective was to investigate the effects of different species of AMF on the production of RA and CA in basil.

Materials and methods

The experimental design involved different P supplies (NM plants grown at three P supplies and AM plants at one), four inoculation treatments (three AM species and one NM) with six replicates (one plant/pot) per treatment, in a complete randomised design.

Basil seeds were sterilised and germinated directly into pots (1 seedling per pot) as described in Section 2.1. The pots contained 1400 g of the soil/sand mixture (1:3, w/w) as modified following the results described in Section 3.4. Nutrients were mixed with the soil as described in Section 3.2 and P was added to the soil/sand mix at three different supplies for NM plants: 0.1 (P1), 0.2 (P2) or 0.3 (P3) g/kg $CaHPO_4$. There was one P supply for AM plants (P2). As described in Section 3.4, when grown at P2 and when plants were treated with 25% AM inoculum, both NM and plants colonised by *G. intraradices* had similar growth rates and shoot P concentrations. Under these conditions mycorrhizal colonisation was also high. Different P supplies were used for NM plants in order to obtain matched tissue P concentrations between the NM and AM plants colonised by *G. mosseae* and *G. caledonium*, as these plant/fungus combinations were not tested previously. NM and AM treatments were prepared as described in Section 2.3, with 25% inoculum (w/w, 350 g per pot, dry soil) of one of the following AMF: *G. caledonium*, *G. intraradices*, *G. mosseae* or NM inoculum. The inocula came from new pot cultures free of BNR as discussed in Sections 3.3 and 3.4 (Chapter 3). These pot cultures averaged 45, 75 and 60% colonisation (in leek roots) for each fungal species (*G. caledonium*, *G. intraradices*, *G. mosseae*, respectively). The plants were grown in a glasshouse during summer as described in Section 2.4

and the number of leaves was monitored in the course of the experiment to help choose a harvest time, according to the estimated biomass (Fig. 4.1; see Section 3.1). When the plants reached the targeted number of leaves (~40) after 7 wks growth, they were harvested and the following parameters were recorded: shoot and root fresh and dry weights, plant P concentration, RA and CA concentrations and percent AM colonisation, as described in Chapter 2.

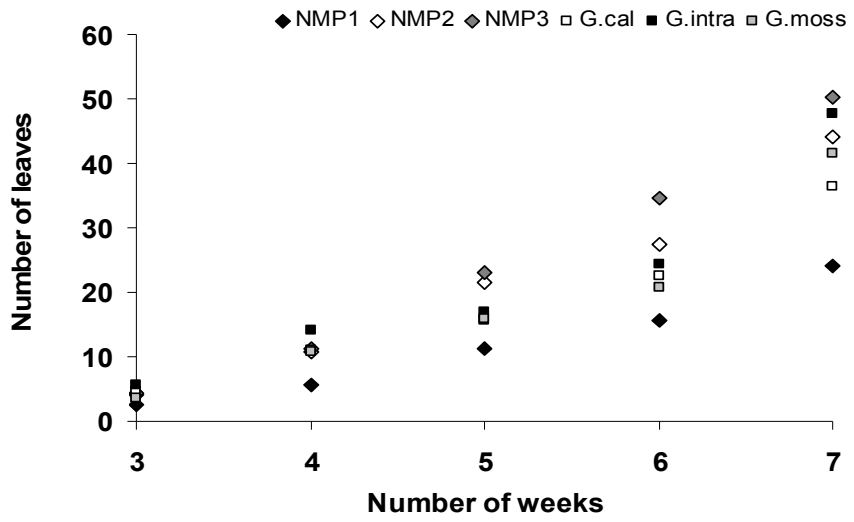

Figure 4.1. Number of leaves per plant of *O. basilicum* counted at weekly intervals in order to decide on harvest time (7 wks). *O. basilicum* was grown under three different P supplies, with or without AMF. Each dot represents the average number of leaves for each mycorrhizal treatment (n=4-6). SE were omitted to make the figure easier to read.

Results

As indicated by the number of leaves (Fig. 4.1), the plants were harvested after 7 wks. Only two replicates were lost during the course of the experiment in plants colonised by *G. caledonium*, indicating that the growth conditions had been improved compared to those described previously (see Sections 3.2 – 3.4). No NM plants were colonised by AMF. The percent AM colonisation was relatively low for plants inoculated with *G. caledonium*, whereas plants inoculated with *G.*

mosseae and *G. intraradices* had a significantly higher percent colonisation (Table 4.1). The percent arbuscules was lowest in *G. caledonium*, followed by *G. mosseae* and *G. intraradices*. The dry weight of NM plants increased with increasing P supply. At P1, NM plants had a significantly lower dry weight than other treatments and, at P2 NM and AM plants had similar dry weights (Fig. 4.2).

Table 4.1. Percent total colonisation and arbuscules in *O. basilicum* grown with *G. caledonium*, *G. intraradices* or *G. mosseae* at P2. Means (n=6, except for *G. caledonium* where n=4) and SE are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Fungus	% colonisation	% arbuscules
G. caledonium	15 ± 4 a	2 ± 2 a
G. intraradices	76 ± 6 c	31 ± 5 c
G. mosseae	39 ± 5 b	8 ± 2 b

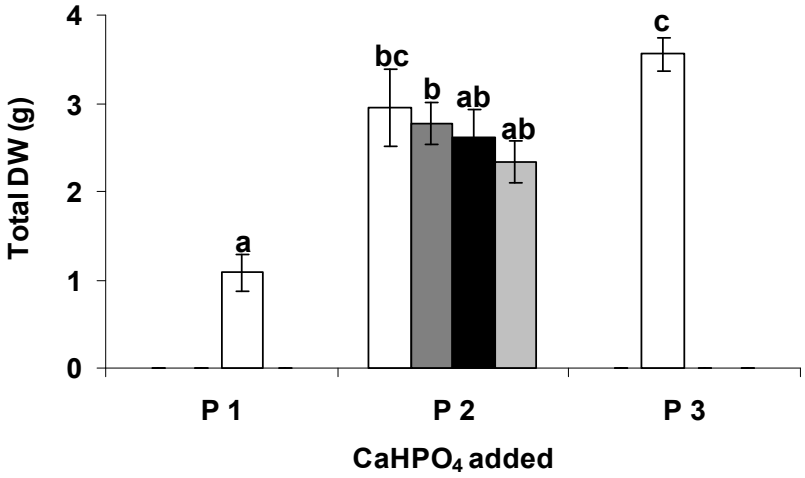

Figure 4.2. Total plant dry weight (DW, g) of *O. basilicum* grown under three different P supplies. The plants were NM (white bars) or colonised by one of the following AMF: *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=6, except for *G. caledonium* where n=4) and SE bars are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

NM plants had similar shoot P concentrations regardless of treatment. At P1 and P2, NM plants also had similar shoot P concentrations to plants colonised by *G. caledonium* (Fig. 4.3a). Plants colonised by *G. intraradices* and *G. mosseae* had

significantly higher shoot P concentrations than those colonised by *G. caledonium* and NM plants at P1. Thus, plants colonised by *G. caledonium* matched NM plants at P1 and P2, and those colonised by *G. intraradices* and *G. mosseae* matched NM plants at P3.

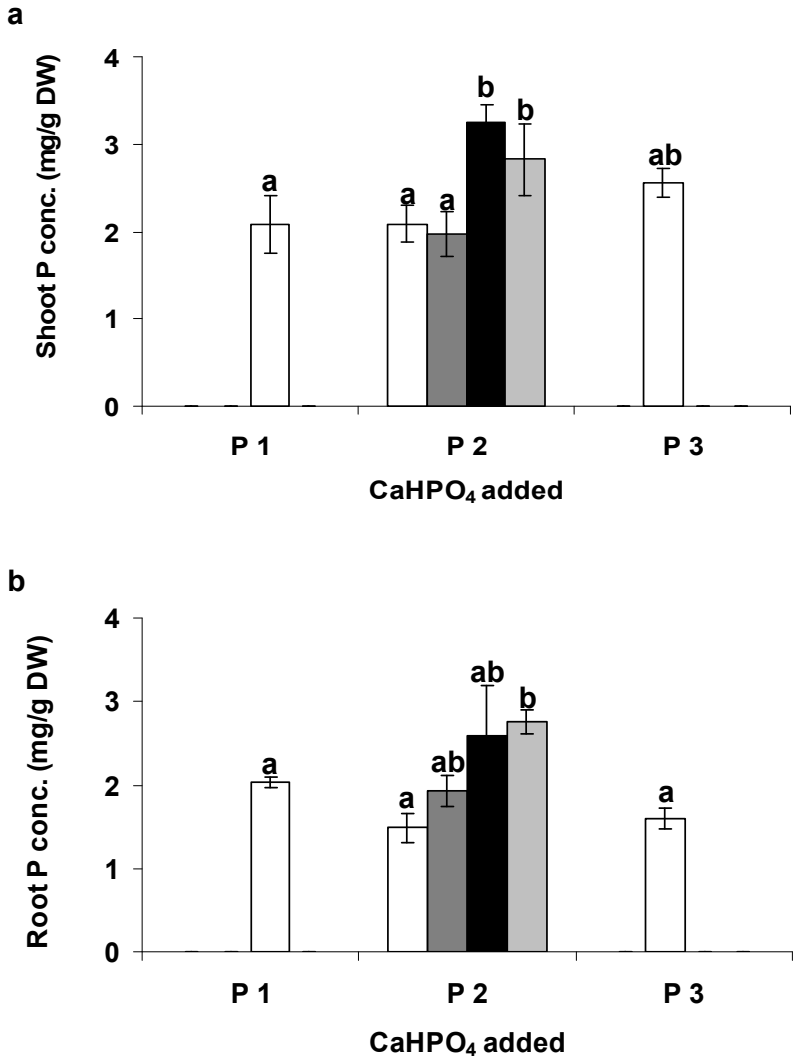

Figure 4.3. Shoot (a) and root (b) P concentrations (mg/g DW) in *O. basilicum* grown under three different P supplies. The plants were NM (white bars) or colonised by one of the following AMF: *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=6, except for *G. caledonium* where n=4) and SE bars are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Root P concentrations of NM and AM plants were similar between treatments, except for plants colonised by *G. mosseae*, which had higher root P concentrations than NM plants at all P supplies (Fig. 4.3b). Total plant P content of NM plants increased as P supply increased. All AM plants had similar P contents to NM plants grown at P2 and P3 (Fig. 4.4).

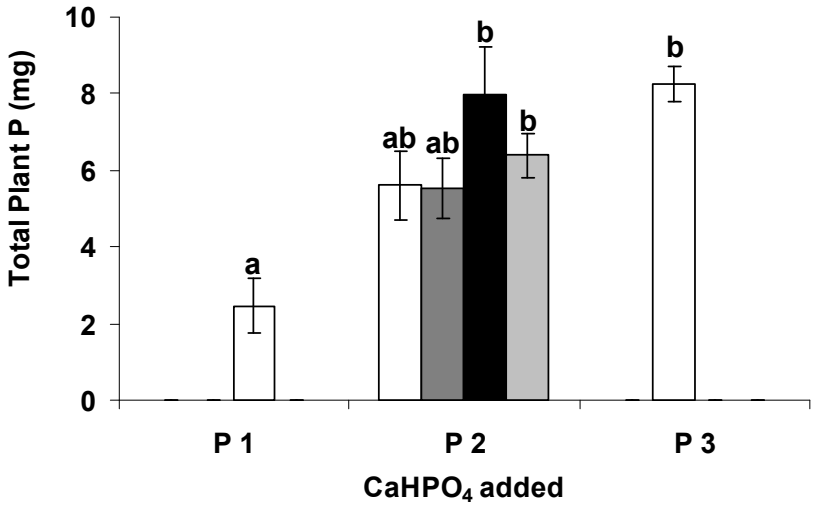

Figure 4.4. Total plant P content (mg) in *O. basilicum* grown under three different P supplies. The plants were NM (white bars) or colonised by one of the following AMF: *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=6, except for *G. caledonium* where n=4) and SE bars are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

At P1, shoot RA concentrations of NM plants were significantly lower than other NM treatments (Fig. 4.5a). Shoot RA concentrations of plants colonised by *G. caledonium* and *G. mosseae* were similar to or greater than those of NM plants at P2 and P3, with values for *G. caledonium* higher (marginally significant at p=0.08) than all other treatments. Shoot RA concentrations in plants colonised by *G. intraradices* were low and similar to NM plants at P1. Root RA concentrations were generally much lower than shoot concentrations (compare Figs. 4.5a and b; note the different scales). Root RA concentrations of NM plants increased

significantly as P supply increased. All AM plants had similar root RA concentrations, which were not significantly different from NM plants at P2 (Fig. 4.5b).

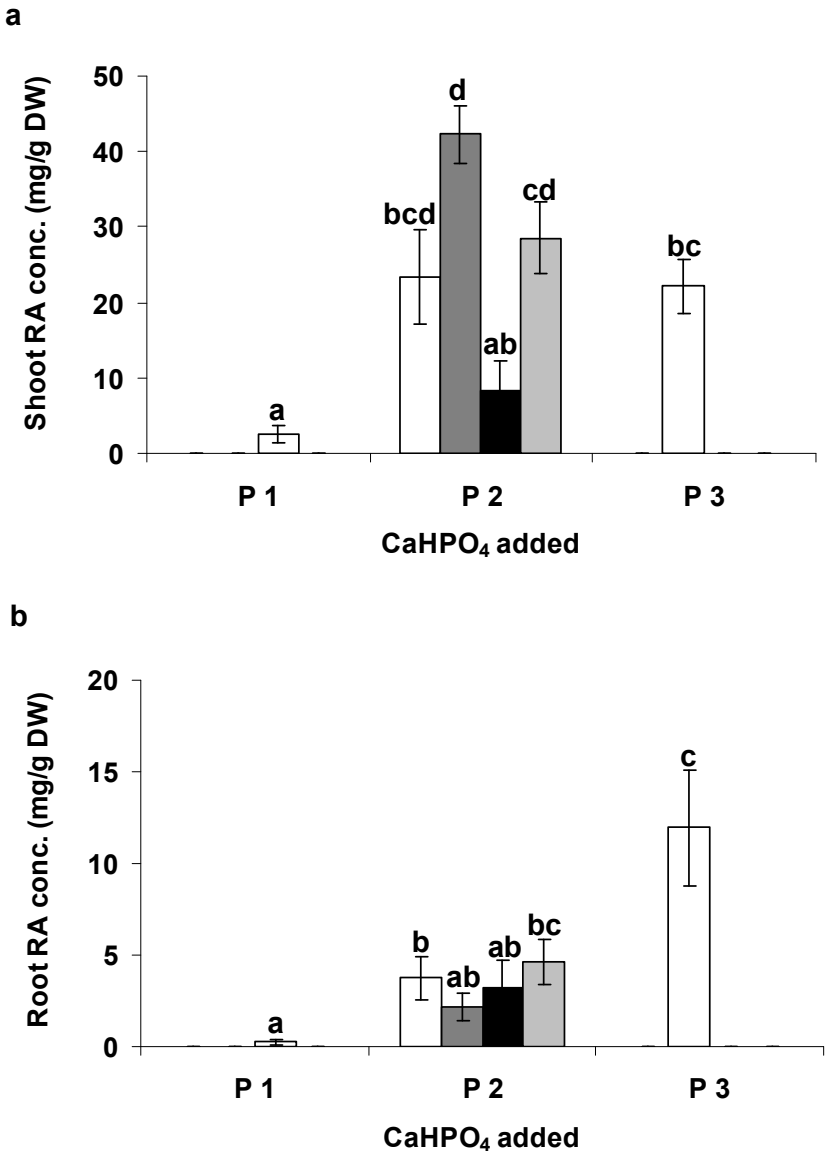

Figure 4.5. Shoot (a) and root (b) RA concentrations (mg/g DW) in *O. basilicum* grown under three different P supplies. The plants were NM (white bars) or colonised by one of the following AMF: *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=6, except for *G. caledonium* where n=4) and SE bars are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

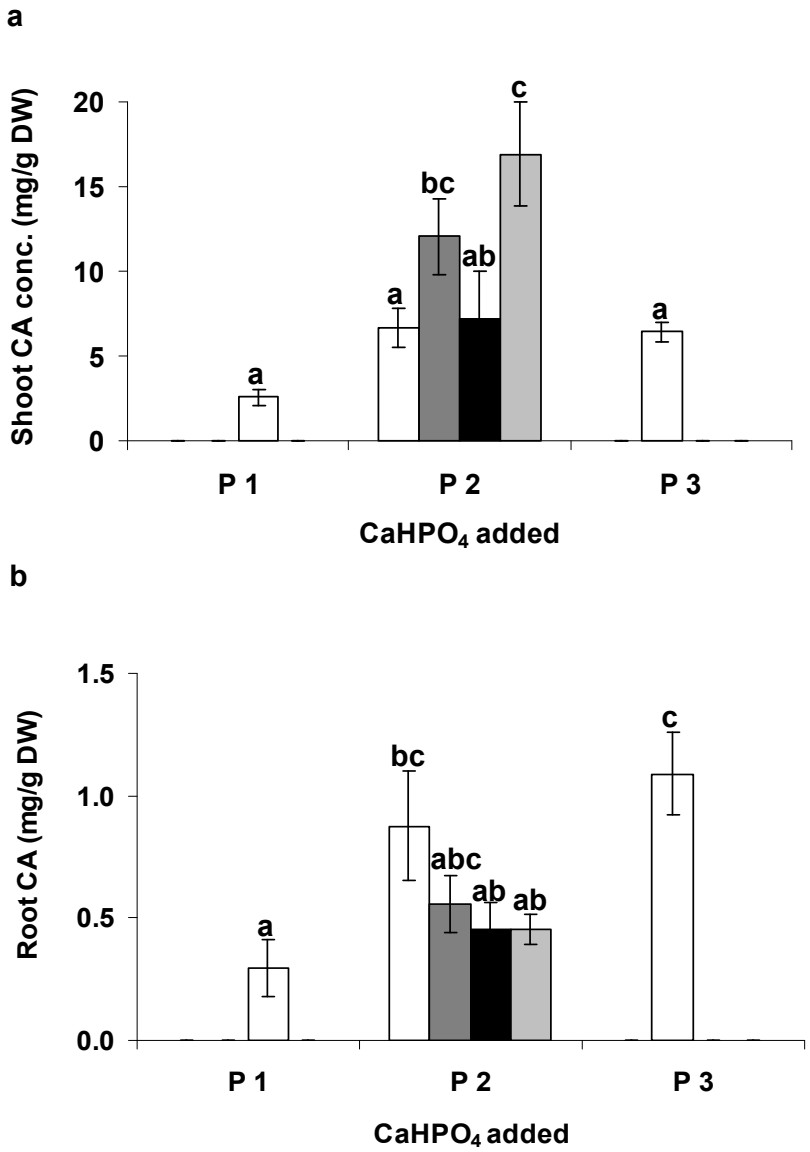

Figure 4.6. Shoot (a) and root (b) CA concentrations (mg/g DW) in *O. basilicum* grown under three different P supplies. The plants were NM (white bars) or colonised by one of the following AMF: *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=6, except for *G. caledonium* where n=4) and SE bars are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Shoot CA concentrations showed a similar pattern to shoot RA concentrations. Those of NM plants were similar at all P supplies and were significantly lower than shoot CA concentrations of plants colonised by *G. caledonium* or *G. mosseae*

(Fig. 4.6a). Shoot CA concentrations in plants colonised by *G. intraradices* were not significantly different from those of NM plants or plants colonised by *G. caledonium*, but were significantly lower than in plants colonised by *G. mosseae*. Root CA concentrations were much lower than shoot concentrations (compare Figs. 4.6a and b; note the different scales). Root CA concentrations of NM plants increased as P supply increased, whereas those of AM plants did not differ between fungal treatments (Fig. 4.6b) and were not significantly different from NM plants at P2.

Finally, there was a negative relationship (R^2=0.34; y=-12.3x + 58.5 p=0.01) between shoot P and RA concentrations in AM plants, whereas in NM plants shoot dry weight and shoot P content were positively correlated to shoot RA concentrations (R^2=0.73; y=12.4x – 7.4; p=0.000 and R^2=0.43; y=3.5x + 0.5; p=0.03).

Discussion

AM and NM plants were successfully matched for tissue P concentrations and under these conditions it was demonstrated that 1) AM plants grow as well as NM plants (as seen in Section 3.4, Chapter 3), that 2) basil is quite responsive to P supply when NM, as seen in Chapter 3, and that 3) plants colonised by *G. caledonium* had higher shoot RA and CA concentrations compared to NM plants with the same shoot P concentrations. *G. mosseae* also increased shoot CA concentrations compared to NM plants. These findings show that the increase in shoot RA and CA concentrations is influenced both by improved P uptake (NM plants) and also by a direct effect of AM colonisation (AM plants). The negative

relationship between shoot P and RA concentrations in AM plants, as well as the effect of P supply on the production of RA and CA in NM plants further support these conclusions. The fact that plants colonised by *G. intraradices* did not show increased shoot or root RA and CA concentrations, contrary to what was observed in Chapter 3 (Section 3.1), might be explained by differences in growth medium (25 vs. 10% soil and different P supplies). The differences observed in shoot and root RA concentrations in this experiment compared with those obtained in Chapter 3 remain to be explained. Different growing seasons as well as different P supplies used in the experiments might partly explain such discrepancies. The variability in the data in terms of root RA concentrations could also be linked to the presence of BNR in the experiments described in Chapter 3 (Sections 3.2. and 3.3). As discussed in Chapter 1 (Section 1.5.2), RA can accumulate in the roots of basil under microbial challenge. Hence, the presence of both BNR and *G. intraradices* in the experiments described in Sections 3.2 and 3.3 might have triggered the production of RA in the roots. This hypothesis remains to be tested. On the other hand, in the current experiment, shoot and root CA concentrations of NM plants were in the same range to those observed in Chapter 3. Altogether, the results from the current experiment indicate that it is possible to grow basil equally well using AMF or added P supply to produce more biomass of the harvested product, with high concentrations of phytochemicals, as sought after in the herbal industry.

As discussed in Chapter 1, until recently few investigations had focussed on the production of phytochemicals in the shoots of AM plants. In most studies that have attempted this, AM and NM plants were not matched for tissue P

concentrations. Therefore, increases in phytochemical concentrations in AM plants might have been due to an improved P uptake via the fungus. Nevertheless, recent studies have shown increases of essential oils in shoots of basil colonised by *Gigaspora rosea* (Copetta *et al.* 2006), and in oregano (*O. vulgare*) colonised by *G. mosseae* (Khaosaad *et al.* 2006). In neither case was the effect due to an improved P concentration of AM plants, as both NM and AM plants had similar shoot P concentrations. Similar results were also recently reported in which wormwood (*Artemisia annua* L.) colonised by *G. macrocarpum* or *G. fasciculatum* showed significant increases of shoot artemisinin concentrations compared to NM plants that were matched for P concentrations (Kapoor *et al.* 2007). The findings from the present experiment provide further evidence that AMF can also directly increase the concentration of phenolic compounds, such as RA and CA, in the shoots of basil.

The variability of RA and CA concentrations observed in plants colonised by different AMF highlights the functional diversity that exists between fungal species belonging to the same genus in their effects on host plants. Other reports have also acknowledged the variable effects of different AMF species in altering the production of phytochemicals (Kapoor *et al.* 2002b, Copetta *et al.* 2006). The relatively low colonisation levels in plants colonised by *G. caledonium* (and to some extent *G. mosseae*) indicate that AMF can have considerable effects on the host plant physiology, even when their biomass in roots is low. These variations may have significant ecological implications via effects on the above-ground interactions between herbivores and host plants through the alteration of production of defence compounds (Gehring and Whitham 2002). Interestingly,

plants colonised by *G. caledonium* had the lowest percentage of arbuscules, followed by *G. mosseae* and *G. intraradices* (see Table 1). This low frequency of arbuscules could relate to the high concentrations of RA and CA observed in the shoot of basil. As reviewed by Harrison (2005), the down regulation of defence responses usually correlates well with an increase in arbuscule development. In the current experiment, the high RA concentrations observed in plants colonised by *G. caledonium* might have been the result of the inability (or difficulty) of the fungus to colonise the host plant and to produce a large number of arbuscules, despite good inoculum quality (well colonised pot cultures of leek). This hypothesis will be further addressed in the next chapter.

More work is needed in order to elucidate the mechanisms by which *G. caledonium* specifically altered the production of RA and CA in basil, as the current experimental design did not allow me to answer this question. To address this point, the next chapter will describe work that investigated the impact of developmental stages of AM by the three AMF tested in the current experiment on the production of RA and CA.

5. Relationship between the time course of colonisation and RA and CA production in basil

As discussed in Chapter 1, there have been reports on the induction of defence-related compounds by AMF in the roots of host plants (VanEtten *et al.* 1994, Bonfante and Perotto 1995, Benhamou 1996, Scharff *et al.* 1997), but such effects have been shown to be transient. For instance, Harrison and Dixon (1993) found that the transcript levels of isoflavone reductase slightly increased in AM *M. truncatula* roots 7 days after inoculation and were then significantly reduced 13 days after inoculation. Similarly, Volpin *et al.* (1995) demonstrated that the relative amounts of phenylalanine ammonia-lyase (PAL) mRNA increased in AM *M. sativa* roots between 14 and 18 days after seed germination and were then subsequently significantly reduced to reach a value lower than that of the control roots. As reviewed by Garcia-Garrido and Ocampo (2002), such transient defence responses have been shown to be small in compatible host plant/AMF interactions. Compatibility here refers to the ability of AMF to penetrate the root cortex and form arbuscules (see Fig. 1.1, Section 1.3.1).

It was therefore hypothesised that the production of RA and CA in the shoots of basil might change depending on the developmental stage of the AM symbiosis (*i.e.* appressorium formation, development of intraradical hyphae, presence of arbuscules). Due to the difficulty or slow colonisation rate of *G. caledonium* (see results and discussion Chapter 4), it was suspected that this fungus might trigger a defence response (*i.e.* higher RA concentrations) in the host plant as it "forced" its way through the cortical cells.

Aims

This experiment was designed to understand how the stages of development reached by different fungal partners can influence the production of RA and CA in the shoots of basil. To measure these effects in the short term, nurse pots of various AMF (including NM nurse pots) were established in which uninoculated basil plants were transplanted after a few weeks growth. This nurse pot system was adapted from Rosewarne *et al.* (1997), as the presence of an established fungal mycelium allowed more precise determination of the onset of colonisation and synchronous production of different structures than would have been possible using a mixed inoculum technique as described in Chapter 2. Two time course experiments were conducted to fulfil the objective because not all basil seeds germinated simultaneously to allow conducting the full experiment at one time. The results of both experiments will be discussed together in Section 5.3.

5.1. First time course experiment

Materials and methods

There were two mycorrhizal treatments (NM and *G. caledonium* nurse pots) and four replicates per treatment, including "background control" plants (see below). The experiment was set up as a completely randomised factorial design.

Nurse pots were established in a glasshouse during summer with similar growth conditions as described in Section 2.4. Each nurse pot consisted of a 1400 g pot containing the soil/sand mixture (1:3, w/w). Four nurse pots of each *G. caledonium* and NM inocula were set up, using leeks as the nurse plants (three plants per pot). Pots were kept enclosed in plastic sun-bags, watered once a week

and each received 10 ml of half strength modified Long Ashton solution minus P once a week (Section 2.3). The nurse pots were grown for 16 wks before being used in the experiment to allow extensive production of fungal inoculum (~40% colonisation in leek roots).

Many basil seeds were sterilised and sown directly into a tray containing the soil/sand mixture (1:3, w/w) as described in Section 2.1. The mixture was supplied with 0.2 g/kg $CaHPO_4$ (Section 2.5). After ~5 wks growth, when vigorous enough, these uninoculated seedlings were transplanted into the nurse pots (AM and NM) at different intervals (days) to obtain specific mycorrhizal developmental stages. Four uninoculated seedlings were also used as "background control" plants. These were not transferred into the nurse pots and were 5 wks and 5 day-old when harvested at the same time as the other plants (see below). This procedure was carried out in order to assess if there was any stress-induced response (*e.g.* through root damage) due to transplanting the plants into nurse pots, which might have triggered the production of RA or CA. All plants were harvested on the same day, and therefore were the same age at that time. At the indicated time after transplantation into the nurse pots (1, 3 or 5 days after transplanting, DAP), the plants were harvested and the following parameters were recorded: fresh and dry weight, percent colonisation and fungal structures, plant P concentration and shoot RA and CA concentrations as described in Chapter 2. The percentages of fungal structures (external and internal hyphae, arbuscules) were calculated as a percentage of the total colonisation.

Results

NM and background control plants did not become colonised. Plants harvested 1 DAP from AM nurse pots were not colonised, although some external hyphae were detected (Table 5.1). Total colonisation was similar in plants harvested 3 DAP and 5 DAP. Arbuscules were detected 5 DAP, but were very sparse. Plant biomass was relatively low and not significantly different between any treatments. Growth was, however, somewhat above that of the background control plants (Fig. 5.1).

Table 5.1. Percent (%) total colonisation (Total), internal hyphae (IH), external hyphae (EH) and arbuscules (Arbs) in *O. basilicum* grown with *G. caledonium* and harvested at 1, 3 or 5 DAP. Means (n=4) and SE are shown.

Time course	Total	IH	EH	Arbs
1 DAP	0.8 ± 0.5	0	0.01 ± 0.01	0
3 DAP	6 ± 2	0.1 ± 0.1	0.3 ± 0.2	0
5 DAP	8 ± 4	0.2 ± 0.06	0.5 ± 0.1	0.04 ± 0.02

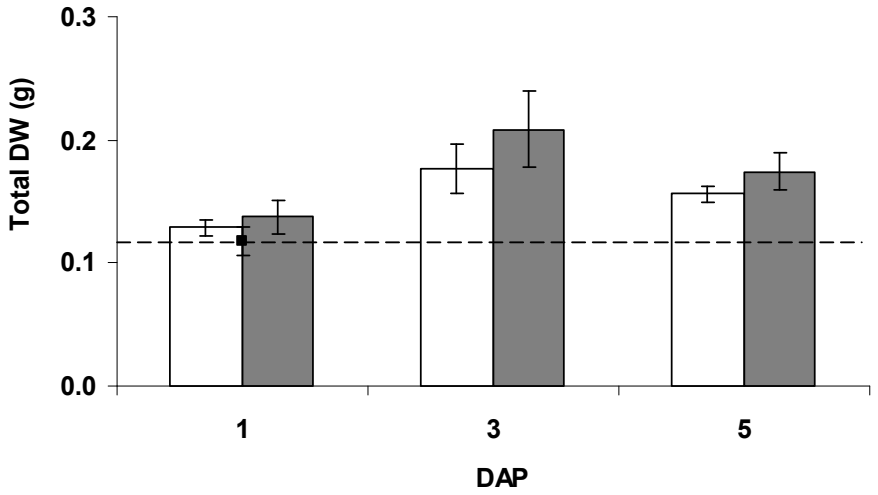

Figure 5.1. Total plant dry weight (DW, g) of *O. basilicum* harvested at 1, 3 or 5 DAP. Plants were grown with (dark grey bars) or without (white bars) *G. caledonium*. The black square and dashed line represent the "background control" plants. Means (n=4) and SE are presented. There were no significant differences between treatments.

Shoot P concentrations were matched between AM and NM plants for each time point and there were no significant differences between any of the treatments (Fig. 5.2). Shoot P concentrations of all treatments were somewhat lower than those of the background control plants.

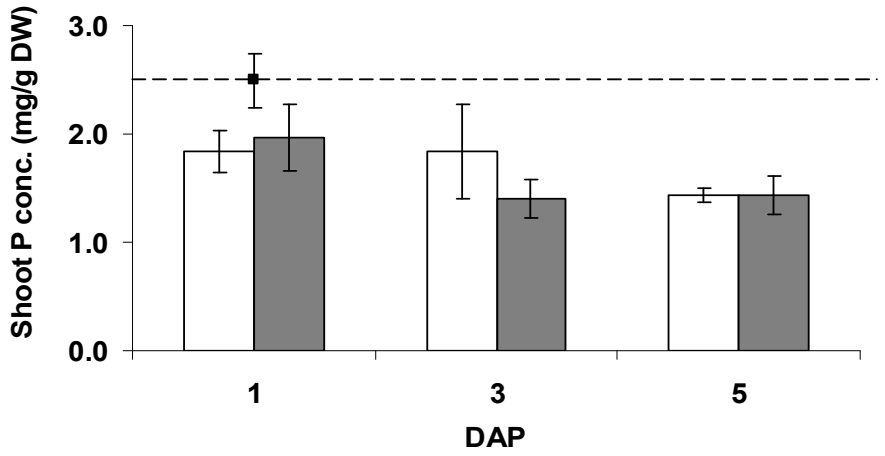

Figure 5.2. Shoot P concentration (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. Plants were grown with (dark grey bars) or without (white bars) *G. caledonium*. The black square and dashed line represent the "background control" plants. Means (n=4) and SE are presented. There were no significant differences between treatments.

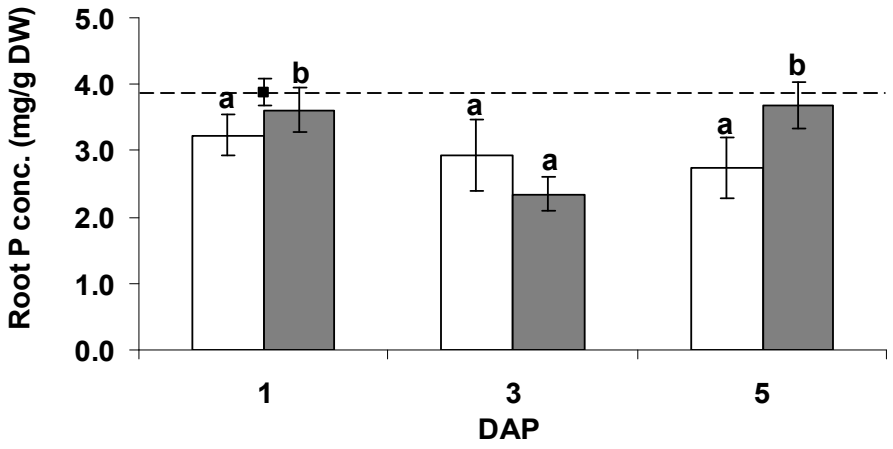

Figure 5.3. Root P concentration (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. Plants were grown with (AM, dark grey bars) or without (NM, white bars) *G. caledonium*. The black square and dashed line represent the "background control" plants. Means (n=4) and SE are presented. For AM or NM plants, different letters indicate significant differences between DAP according to Tukey's test (p=0.05). There were no significant differences within each DAP.

Root P concentrations were also matched between AM and NM plants and there were no significant differences between AM and NM treatments. At 3 DAP, AM plants had significantly lower root P concentrations than AM plants at 1 and 5 DAP (Fig. 5.3). The background control plants had slightly higher root P concentrations than the 3 DAP plants. Plant P contents were similar in AM and NM plants at each time point, and were also similar to those of the background controls (Fig. 5.4).

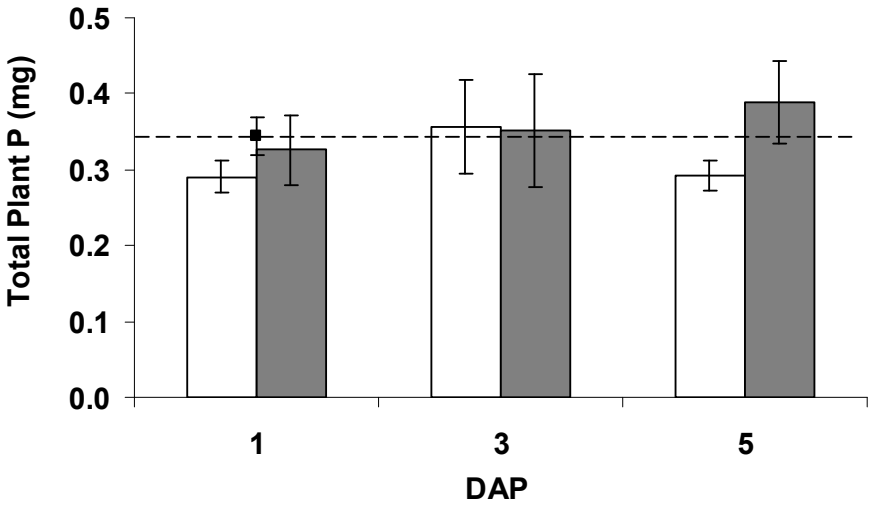

Figure 5.4. Total plant P content (mg) in *O. basilicum* harvested at 1, 3 or 5 DAP. Plants were grown with (dark grey bars) or without (white bars) *G. caledonium*. The black square and dashed line represent the "background control" plants. Means (n=4) and SE are presented. There were no significant differences between treatments.

Although shoot RA concentrations in NM plants appeared to increase at each DAP, this was not significant. In AM plants, shoot RA concentrations increased significantly ($p=0.011$) with DAP (Fig. 5.5). Shoot RA concentrations were somewhat higher than the background controls at 3 and 5 DAP in both AM and NM plants.

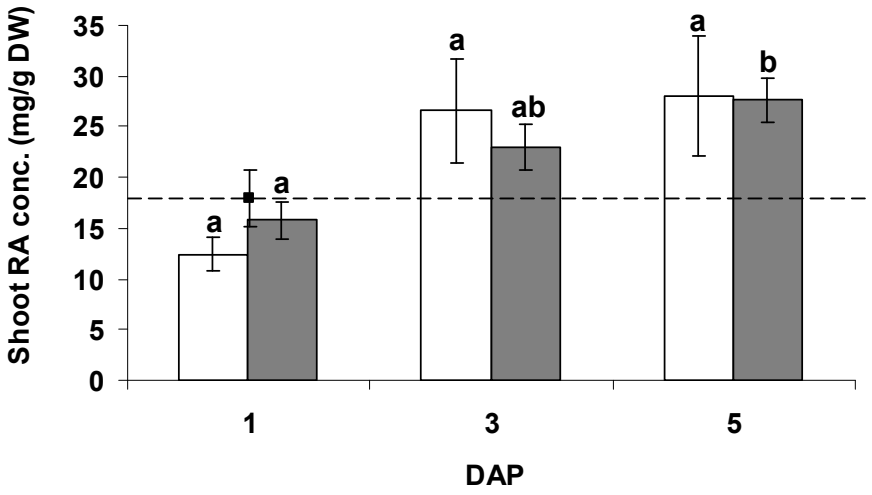

Figure 5.5. Shoot RA concentrations (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. Plants were grown with (dark grey bars) or without (white bars) *G. caledonium*. The black square and dashed line represent the "background control" plants. Means (n=4) and SE are presented. Different letters indicate significant differences between DAP according to Tukey's test (p=0.05). There were no significant differences within each DAP.

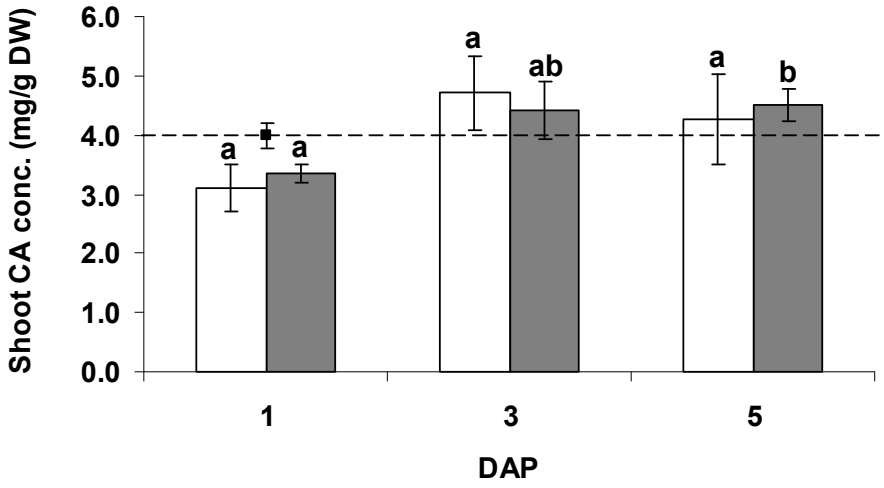

Figure 5.6. Shoot CA concentrations (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. Plants were grown with (AM, dark grey bars) or without (NM, white bars) *G. caledonium*. The black square and dashed line represent the "background control". Means (n=4) and SE are presented. Different letters indicate significant differences between DAP according to Tukey's test (p=0.05). For AM plants, there was a marginally significant difference between 1 and 5 DAP (p=0.08). There were no significant differences within each DAP.

Shoot CA concentrations were not significantly different between NM and AM plants for each time point. AM plants harvested 5 DAP had higher (marginally significant; p=0.08) shoot CA concentrations than those harvested 1 DAP (Fig. 5.6). Shoot CA concentrations of all treatments were similar to those of the background controls.

5.2. Second time course experiment

Materials and methods

The experimental set-up was the same as described in Section 5.2 but there were three mycorrhizal treatments (NM, *G. intraradices* and *G. mosseae* nurse pots). The nurse pots were set up as for the first experiment but were grown for 23 wks before being used (reaching colonisation levels in leek roots of ~60 and 80% for *G. mosseae* and *G. intraradices*, respectively). The experiment was carried out in summer, 7 wks after the one described in Section 5.1, and the same parameters were recorded.

Results

No colonisation was observed in background control plants or plants grown in NM nurse pots. Plants harvested 1 DAP had low colonisation levels in both *G. intraradices* and *G. mosseae* nurse pots, and no or very few arbuscules were observed at this stage (Table 5.2). At 3 DAP, plants transferred into *G. intraradices* nurse pots had much higher colonisation than those transferred into *G. mosseae* nurse pots. At 5 DAP, plants colonised by *G. intraradices* had higher colonisation levels than those colonised by *G. mosseae*, but all plants had some arbuscules, although these were sparse.

Table 5.2. Percent (%) total colonisation (Total) internal hyphae (IH), external hyphae (EH) and arbuscules (Arbs) in *O. basilicum* grown with *G. intraradices* or *G. mosseae* and harvested at 1, 3 or 5 DAP. Means (n=4) and SE are shown.

Fungus	Time course	Total	IH	EH	Arbs
G. intraradices	**1 DAP**	2 ± 0.9	0.04 ± 0.02	0.01 ± 0.01	0.03 ± 0.03
	3 DAP	27 ± 9	6 ± 2	5 ± 2	0
	5 DAP	39 ± 12	14 ± 7	14 ± 8	2 ± 0.9
G. mosseae	**1 DAP**	0	0	0	0
	3 DAP	0.8 ± 0.3	0.01 ± 0.003	0	0
	5 DAP	6 ± 0.6	0.1 ± 0.1	0.2 ± 0.09	0.08 ± 0.03

Plant dry weights were similar to those observed in the first experiment (Section 5.1) but did not vary significantly between treatments (Fig. 5.7). Most plants had higher biomass than the background controls. Shoot P concentrations decreased significantly with DAP in NM plants, but remained the same in AM plants (Fig. 5.8). There were no significant differences between AM and NM plants within each DAP, although plants colonised by *G. intraradices* appeared to have higher shoot P concentrations than other treatments at 3 DAP. Shoot P concentrations were similar to those of the background controls.

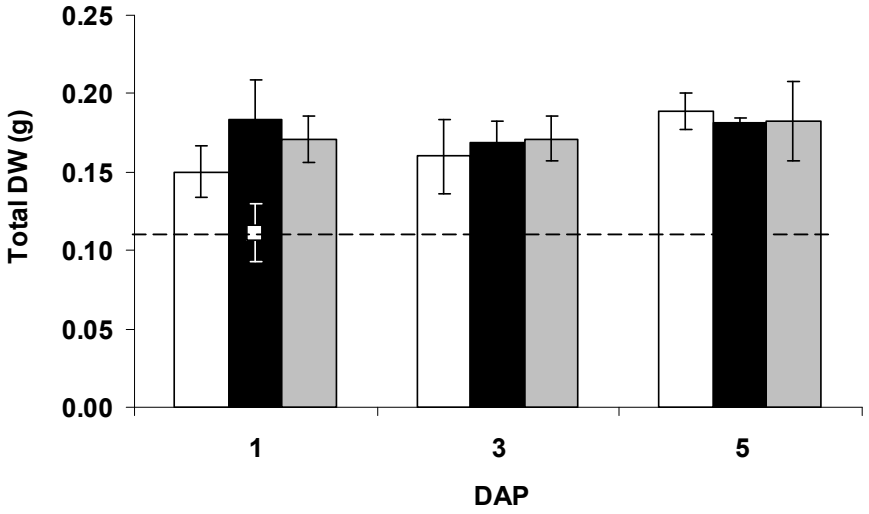

Figure 5.7. Total plant dry weight (DW, g) of *O. basilicum* harvested at 1, 3 or 5 DAP. The plants were colonised by *G. intraradices* (black bars), *G. mosseae* (light grey bars) or NM (white bars). The white square and dashed line represent the "background control". Means (n=4) and SE are presented. There were no significant differences between treatments.

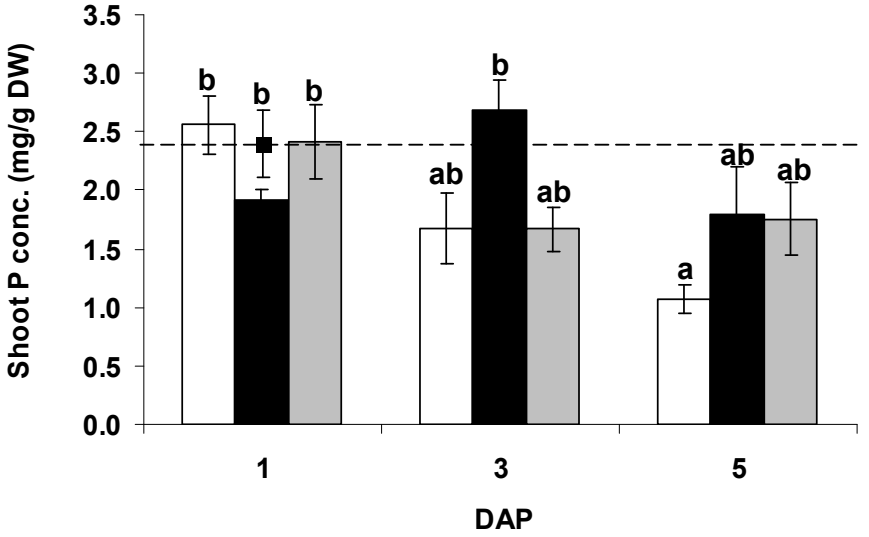

Figure 5.8. Shoot P concentration (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. The plants were colonised by *G. intraradices* (black bars), *G. mosseae* (light grey bars) or NM (white bars). The black square and dashed line represent the "background control". Means (n=4) and SE are presented. Different letters indicate significant differences between DAP according to Tukey's test (p=0.05). There were no significant differences within each DAP.

Root P concentrations did not vary significantly between treatments, but were lower than those of the background controls (Fig. 5.9). Total plant P content did not vary significantly either between treatments, which had somewhat higher P contents than those of the background controls (Fig. 5.10).

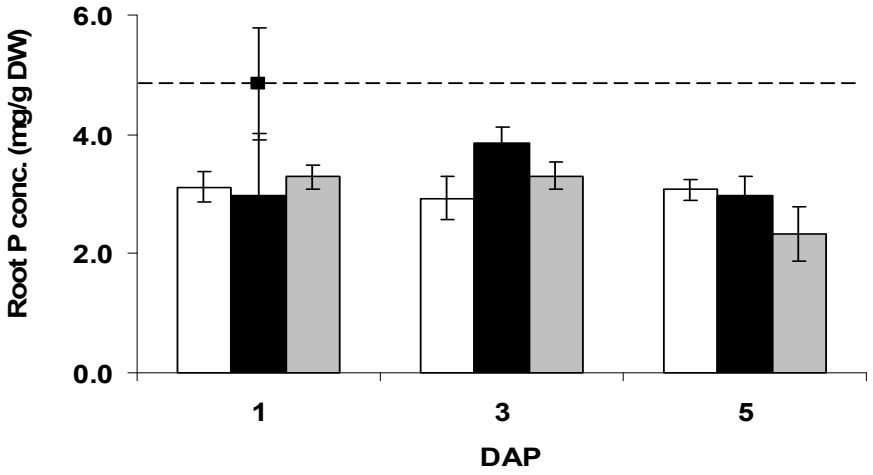

Figure 5.9. Root P concentration (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. The plants were colonised by G. intraradices (black bars), *G. mosseae* (light grey bars) or NM (white bars). The black square and dashed line represent the "background control". Means (n=4) and SE are presented. There were no significant differences between treatments.

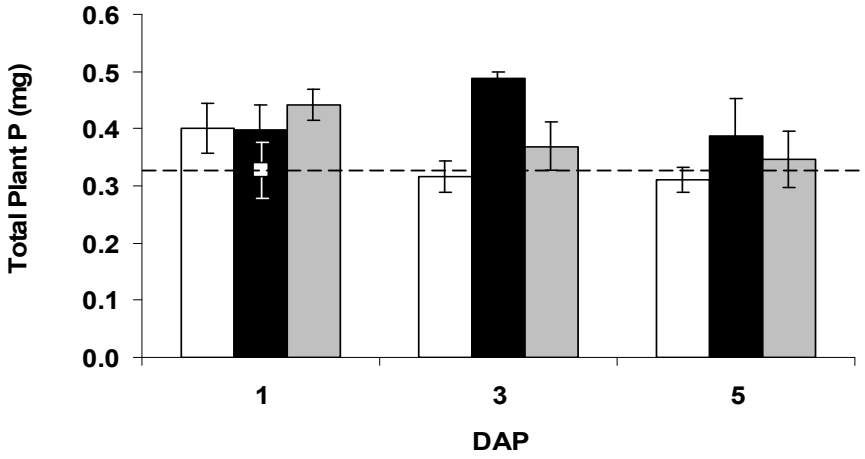

Figure 5.10. Total plant P content (mg) in *O. basilicum* harvested at 1, 3 or 5 DAP. The plants were colonised by *G. intraradices* (black bars), *G. mosseae* (light grey bars) or NM (white bars). The white square and dashed line represent the "background control". Means (n=4) and SE are presented. There were no significant differences between treatments.

There were no significant differences between treatments in terms of shoot RA concentrations, which were also similar to those of the background controls (Fig. 5.11). Shoot CA concentrations did not vary significantly between treatments either, and the values were similar to that of the background controls (Fig. 5.12).

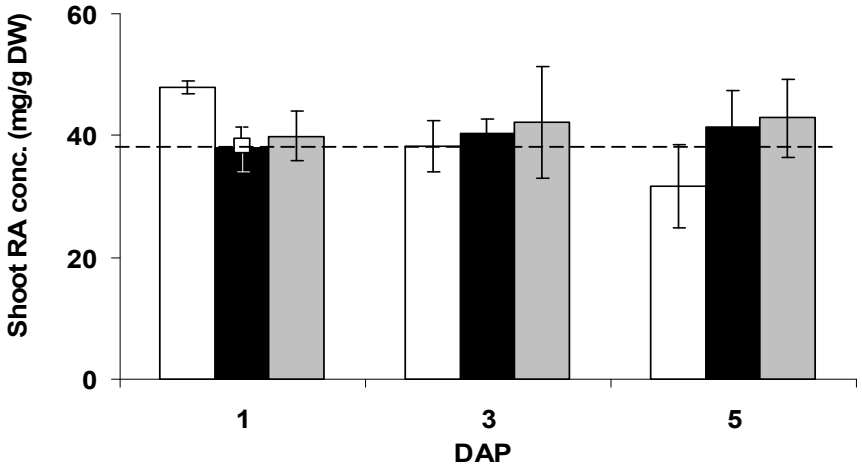

Figure 5.11. Shoot RA concentration (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. The plants were colonised by *G. intraradices* (black bars), *G. mosseae* (light grey bars) or NM (white bars). The white square and dashed line represent the "background control". Means (n=4) and SE are presented. There were no significant differences between treatments.

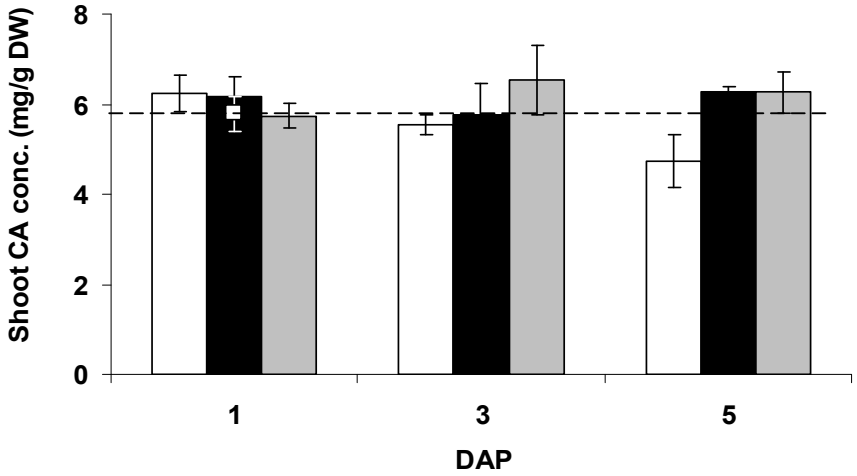

Figure 5.12. Shoot CA concentration (mg/g DW) in *O. basilicum* harvested at 1, 3 or 5 DAP. The plants were colonised by *G. intraradices* (black bars), *G. mosseae* (light grey bars) or NM (white bars). The black square and dashed line represent the "background control". Means (n=4) and SE are presented. There were no significant differences between treatments.

5.3. Discussion

Although the two time course experiments were carried out at different times, the results obtained for most of the variables were similar and in the same range to those observed in Chapters 3 and 4. However, shoot RA concentrations of most treatments were almost two-fold higher in this experiment compared to those reported in Section 5.1. Shoot RA concentrations were also much higher in plants colonised by *G. intraradices* than those reported in Chapter 4. The disparity in plant dry weight and P uptake observed between the background control plants and those transplanted into AM or NM nurse pots is likely to be the result of differences in nutrient availability in the growth media. After 5 weeks and 5 days, the background control plants must have been nutrient deprived, whereas the plants that were transplanted into the nurse pots (AM or NM) would have had access to more available nutrients (other than P), as the pot cultures received 10 ml of modified Long Ashton solution once a week (see materials and methods, Section 5.1). Hence, the difference in nutrient availability in the growth media might explain the discrepancy in plant dry weights observed between the background control plants and those transplanted in the nurse pots (see Fig. 5.1 and 5.7).

Shoot RA and CA concentrations in basil did not change significantly in relation to the developmental stage of the three AMF tested, even though colonisation levels varied considerably. Although the increase of shoot RA concentrations in plants colonised by *G. caledonium* seemed to correspond to the presence of arbuscules, shoot RA concentrations also appeared to increase (though not significantly) with time after transplanting into NM nurse pots, indicating that the

effect was not specific to the presence of arbuscules. This observation is contrary to what was hypothesised and discussed in Chapter 4. The increase of shoot RA concentrations in plants colonised by *G. caledonium* is unlikely to be due to improved P uptake, as P content and shoot P concentrations were the same in NM plants and those colonised by *G. caledonium*. However, both NM plants and those colonised by *G. caledonium* had higher shoot RA concentrations than the background controls (at 3 and 5 DAP), which suggests that transferring the plants to the nurse pots might have triggered the production of this phytochemical as a stress-induced response (Bais *et al.* 2002). Similarly, the changes observed in most treatments in terms of shoot and root P concentrations (Figs. 5.2 and 5.9) and shoot RA concentrations (Fig. 5.5) compared to those of the background controls are likely to have been the result of a stress-induced response of the transplanting, as all plants were harvested on the same day and were the same age.

Contrary to what has been mostly reported in the literature (Garcia-Garrido and Ocampo 2002), the results reported here indicate that there is no transient effect of the developmental stage of different AMF on the production of RA and CA in the shoots of basil over the period of time tested. In conventional pot systems (as described in Section 2.4), there might be continuous and localised transient effects of AM colonisation on RA and CA production as the root system becomes progressively colonised over time. However, the current results do not indicate that such effects happen in the shoots of basil. Bais *et al.* (2002) demonstrated that RA can be accumulated within 3 days in hairy root cultures of basil when in the presence of a fungal elicitor. However, to my knowledge, there is no information on how quickly RA can accumulate in the shoots of basil following elicitation by

AMF. It is therefore possible that the current experiments were not long enough to allow detection of a response in RA or CA production in the shoots of basil after the onset of AM colonisation.

Factors other than those tested here must be involved in the increase of RA and CA concentrations in basil when mycorrhizal. Environmental factors such as light intensity, temperature and seasonal change (as discussed in Chapters 1 and 3) might have a greater influence than suspected at first on the production of phytochemicals such as RA and CA. Hence, the formation of the AM symbiosis in conjunction with the growth conditions might greatly affect the production of such compounds and requires further investigation. Other influences, including N content and phytohormone concentration changes in the host plants through AM colonisation, could also affect the production of RA and CA in basil plants. These avenues will be further investigated in the next chapter.

6. Does nitrogen supply influence the production of RA and CA in basil and do phytohormones play a role in this process?

The part of the work related to phytohormone determination described in this chapter was done in collaboration with Dr. Eloise Foo and Dr. John Ross from the University of Tasmania.

There have been several reports on the uptake and transfer of N to host plants through AMF (Johansen *et al.* 1994, Subramanian and Charest 1999, Toussaint *et al.* 2004, Govindarajulu *et al.* 2005). It has also been reported that both the form and amount of N can affect the concentration of essential oils in basil (Adler *et al.* 1989, Sifola and Barbieri 2006). Therefore, a possible explanation for the increase of RA and CA concentrations in basil observed in Chapter 4 could be through improved N uptake via AMF. There have also been several reports indicating that AMF can alter cytokinin, auxin and abscisic acid concentrations in host plants, which can lead to changes in physiological processes such as the production of secondary compounds (Allen *et al.* 1980, 1982, Esch *et al.* 1994, Kaldorf and Ludwig-Muller 2000, Torelli *et al.* 2000, Fitze *et al.* 2005, Herrera-Medina *et al.* 2007). It has been reported that auxins can increase the production of RA in cell suspension cultures of *Anchusa officinalis* (Deeknamkul and Ellis 1985). It is therefore possible that phytohormones might be involved in the regulation of RA. However, the roles of phytohormones in the physiology of AM plants are still poorly understood (Barker and Tagu 2000, Hause *et al.* 2007).

On the basis of the above information, two main hypotheses were postulated: 1) higher N concentrations in AM basil might lead to higher shoot RA and CA

concentrations (possibly through the production of tyrosine and phenylalanine (substrate) and PAL (enzyme), three metabolic constituents that are required for the biosynthesis of CA and RA; see Section 1.5.1) and 2) different phytohormone concentrations in AM compared to NM plants might contribute to differences in RA and CA concentrations, as suggested by other authors (Copetta *et al.* 2006, Kapoor *et al.* 2007).

Aims

The main objective of the work described in this chapter was to investigate the effect of different N supplies (as ammonium nitrate) and different AMF on shoot RA and CA concentrations in basil. A second objective was to explore the influence of AM symbioses on the concentrations of indole acetic acid (IAA; auxin) and abscisic acid (ABA) in basil under the various N and AMF combinations, and to determine any correlation with the production of RA and CA.

Materials and methods

The experiment consisted of three N supplies, four inoculation treatments (three AMF species and one NM control), with seven replicates (one plant per pot) per treatment, all set up in a complete randomised design.

Basil seeds were sterilised and germinated directly into pots (1 seedling per pot) as described in Section 2.1. The pots contained 1400 g of the soil/sand mixture (1:3, w/w) as determined in Section 3.4. Nutrients were mixed with the soil as described in Section 3.2 with the exception of N, which was added at three

supplies (mg/kg dry soil NH_4NO_3): 85.7 (N1); 171.4 (N2); or 257.1 (N3). These supplies corresponded to approximately 20, 40 and 60 mg/kg of N added. Phosphorus (P) was added in the soil/sand mix as 0.2 g/kg of $CaHPO_4$ for all treatments, as modified following the results described in Section 3.4. NM and AM treatments were prepared as described in Section 2.3, with 25% inoculum (w/w, *i.e.* 350 g per pot, dry soil) of one of the following AMF: *G. caledonium*, *G. intraradices*, *G. mosseae* or NM inoculum. The inocula came from new pot cultures free of BNR as discussed in Sections 3.3 and 3.4. The plants were grown in a glasshouse during summer as described in Section 2.4 and the number of leaves was monitored in the course of the experiment to help choose a harvest time according to the estimated biomass (~40 leaves/plant; Section 3.1). However, after 5 wks growth there was a failure of the cooling system of the glasshouse and the plants were subjected to temperatures ranging from 40^{o}C to 50^{o}C for several hours. The plants were transferred to another glasshouse with controlled conditions (25 – 28^{o}C) but with light intensities averaging 250 μmol/m^2/sec (due to the position of the pots in the glasshouse). After 7 wks growth, the plants were harvested and the following parameters were recorded: shoot and root fresh and dry weights, plant P concentration, RA and CA concentrations and percent colonisation, as described in Chapter 2. In addition to these parameters, shoot carbon (C) and N concentrations, as well as phytohormone concentrations (IAA and ABA) were determined (see below).

Shoot C and N concentrations were determined in the laboratory of Dr Jeffrey A Baldock (CSIRO, Land and Water, Adelaide) by combusting dried samples (~50 mg) in an elemental analyser (ECS 4010, Costech Analytical Technologies Inc.,

USA) consisting of a combustion system coupled with an autosampler, and a detector system using gas chromatography to analyse the different elements (C and N).

IAA and ABA were assayed in the laboratory of Dr Eloise Foo and Dr John Ross (University of Tasmania, School of Plant Science, Hobart), according to the methods of Batge *et al.* (1999) and Jones *et al.* (2005). On the harvest day, young expanding leaves (100 – 250 mg) were cut and immediately frozen in liquid nitrogen before being stored at -80°C until analysed. Unfortunately, some samples were lost between harvest and analysis. As a result, only one replicate of plants colonised by *G. mosseae* at N2 was left. On the day of analysis, the frozen material was immersed in ice cold 80% methanol (MeOH) containing butylated hydroxytoluene (BHT), and stored at -20°C overnight. The material was then homogenised and hormones extracted overnight at 4°C. The samples were filtered and the internal standards $^{2}H_3$-ABA and $^{13}C_6$-IAA were added in appropriate amounts. The extraction methods for IAA and ABA were similar, with a few additional steps for IAA (see below).

The samples were dried, then resuspended in 2 ml 0.4% (v/v) acetic acid (AA) in MeOH before being filtered through a Sep-Pak C_{18} cartridge that was preconditioned with 100% MeOH, followed by 0.4% AA in distilled water (dH_2O). The samples were filtered through the Sep-Pak with three 2 ml washes of 0.4% AA in dH_2O. The samples were then eluted from the Sep-Pak with 2 ml of 0.4% AA in 80% MeOH and the eluates were reduced overnight to dryness under vacuum. They were dissolved in 200 ml MeOH and methylated with 750 µl

diluted ethereal diazomethane (1:10, v/v diazomethane/ether), and dried under a stream of nitrogen gas (N_2). The samples were resuspended in 1 ml dH_20 to which 800 µl ether was added to obtain separate phases. The top phase (ether) was transferred to vials with 400 µl ether (repeated twice) before the samples were dried once again under N_2. The residual dH_20 was dried in vacuum for 20 min and the samples were resuspended in 30 µl chloroform (twice) before being analysed by gas-chromatography and mass-spectrometry (GC-MS; see below). After detecting ABA by GC-MS, the samples were further processed for IAA determination. They were dried under N_2, resuspended in 10 µl pyrimidine and 40 µl N,O-bis (trimethylsilyl) trifluoroacetamide (BFTSA) and heated at 80°C for 20 min. The samples were further dried under N_2 before adding 20 µl BSTFA and incubated at 80°C for 20 min. After drying down with N_2, 40 µl chloroform was added to each sample before being transferred into autosampler vials and analysed by GC-MS.

Quantification of endogenous ABA and IAA was performed by GC-MS, using tandem MS on a triple quadrupole mass spectrometer (Varian 3800 GC coupled to a Varian 1200 triple quadrupole MS, a Varian 8400 autosampler, and a Varian 1177 injector; Varian Analytical Instruments, CA, USA) according to the method of Jones *et al.* (2005). The injector was held at 250^{o}C (splitless mode). The column was a Varian Factor Four VF-5ms (30 m x 0.25 mm x 0.25 µm film thickness). The carrier gas was helium at 1.4 ml/min in constant flow mode. The oven temperature was held at 50^{o}C for 2 min, then ramped to 190^{o}C at 30^{o}C/min, then to 270^{o}C at 10^{o}C/min and held at 270^{o}C for 5 min. The transfer line temperature was 280^{o}C and that of the ion source was 200^{o}C. The gain was 1450

V. The MS was operated in selected reaction monitoring mode, with the peak width at 0.9 m/z units, the collision energy at 4 V, and with the collision gas argon at 1 mTorr. For endogenous IAA (methyl ester TMS ether) the selected precursor ion (MS1) was m/z 261 and the selected product ion (MS3) was m/z 202. For $^{13}C_6$-IAA (methyl ester TMS ether) the ions were 267 (MS1) and 208 (MS3). The ions monitored for quantification of endogenous ABA were m/z 193.0817 and 190.0629. Identification was confirmed by monitoring additional ions 165.0868 and 162.0680, and by retention time. Endogenous concentrations of ABA and IAA were calculated on the basis of peak area, following correction for the presence of unlabelled ABA and IAA in the internal standards.

Results

NM plants were not colonised. There were significant differences in colonisation levels between AMF within each N treatment (Table 6.1). However, the colonisation levels of each AMF were not significantly different between N treatments. The percentage colonisation in plants inoculated with *G. caledonium* was significantly lower than plants colonised by the other two AMF at all N treatments. Plants colonised by *G. intraradices* and *G. mosseae* had similar and high colonisation levels, except at N3 where plants colonised by *G. intraradices* had significantly higher percent colonisation than those colonised by *G. mosseae*.

Table 6.1. Percent total colonisation (%) in *O. basilicum* colonised by *G. intraradices*, *G. caledonium* or *G. mosseae* and grown under three N supplies. Means (n=3-7) and SE are shown. For each N supply different letters indicate significant differences according to Tukey's test (p=0.05).

Fungus	N1	N2	N3
G. caledonium	7 ± 2 a	9 ± 3 a	7 ± 2 a
G. intraradices	69 ± 9 b	72 ± 10 b	80 ± 9 c
G. mosseae	69 ± 7 b	65 ± 7 b	40 ± 14 b

There was a trend for plant dry weight to decrease as N supply increased. This trend was more pronounced for NM plants, though not significant (Fig. 6.1). However, the reduction was significant for plants colonised by *G. caledonium* and *G. mosseae* (Fig. 6.1). At N3, plants colonised by *G. caledonium* and *G. intraradices* had significantly (p=0.01) higher dry weight than NM plants.

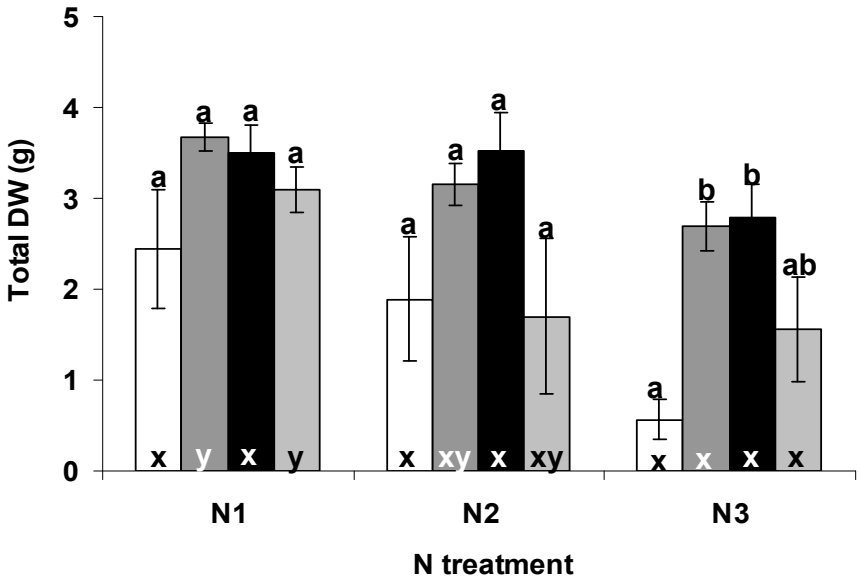

Figure 6.1. Total plant dry weight (DW, g) of *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within and across N treatments, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05).

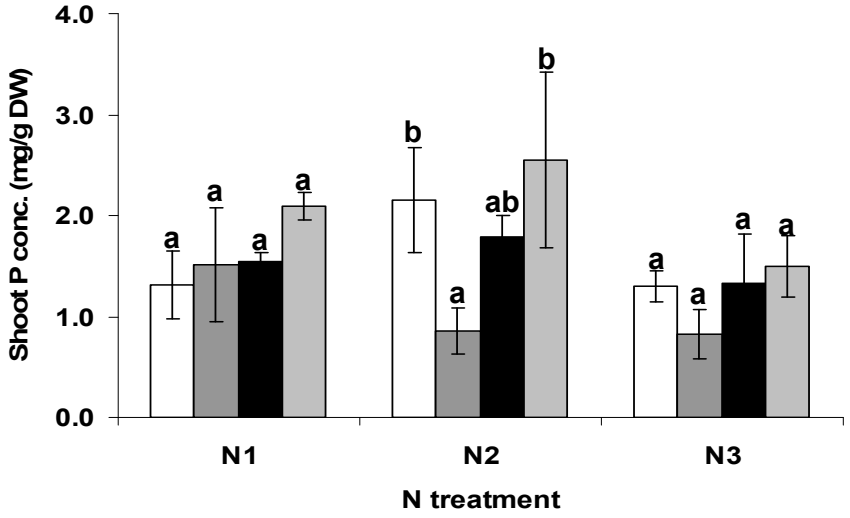

Figure 6.2. Shoot P concentration (mg/g DW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within each N treatment, different letters indicate a significant difference according to Tukey's test. There were no differences across N treatments for AM and NM plants.

There were generally no significant differences in shoot P concentrations with increasing N supplies (Fig. 6.2). However, there were significant differences between mycorrhizal treatments at N2, where plants colonised by *G. caledonium* had marginally ($p=0.064$) lower shoot P concentrations than NM plants and those colonised by *G. mosseae*. Root P concentrations did not change significantly in any treatments between N supplies (Fig. 6.3). However, there were significant differences between NM and AM plants within each N treatment. Plants colonised by *G. intraradices* had significantly higher root P concentrations than those colonised by *G. caledonium* at all N supplies. At N1, plants colonised by *G. intraradices* had also significantly higher root P concentrations than NM plants. At N2, plants colonised by *G. mosseae* had significantly higher root P concentrations than NM plants and those colonised by *G. caledonium*. At N3, all

NM plants and those colonised by *G. mosseae* and *G. intraradices* had significantly higher root P concentrations than those colonised by *G. caledonium*.

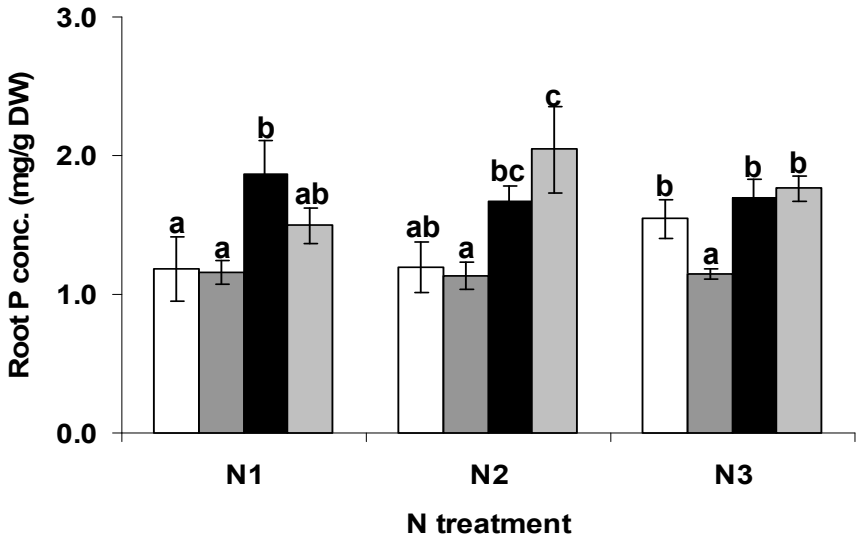

Figure 6.3. Root P concentration (mg/g DW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within each N treatment, different letters indicate significant differences according to Tukey's test (p=0.05). There were no differences across N treatments for AM and NM plants.

Plant P uptake decreased significantly with increasing N supply in NM plants and those colonised by *G. mosseae* (Fig. 6.4). Although there was a trend for plant P content to decrease with increasing N supply in plants colonised by *G. caledonium*, this was not significant. Plants colonised by *G. intraradices* took up the same amount of P at all N supplies. There were also significant differences in total plant P between mycorrhizal treatments, within N treatments. At N1, plants colonised by *G. mosseae* took up significantly more P than NM plants. At N2, there were no differences between mycorrhizal treatments, but at N3 plants colonised by *G. caledonium* and *G. intraradices* had significantly higher P contents than NM plants.

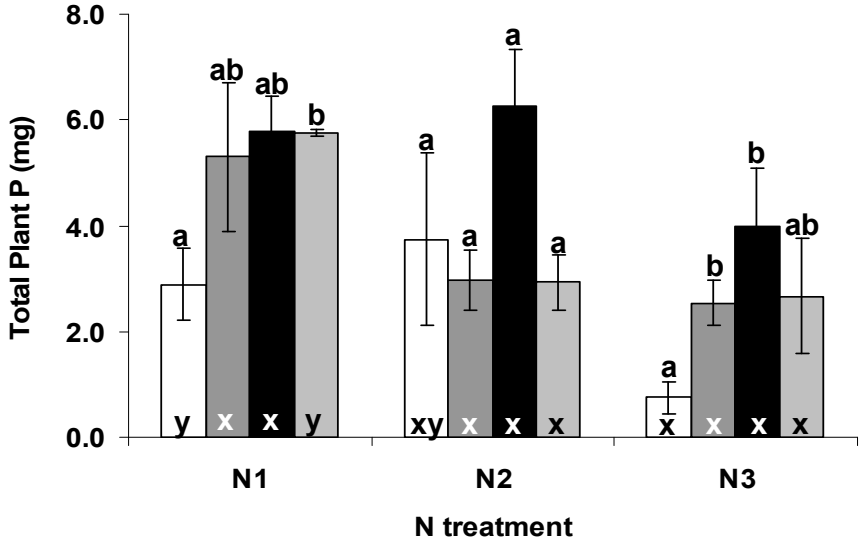

Figure 6.4. Total P content (mg) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within each N treatment and for each mycorrhizal treatment, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05).

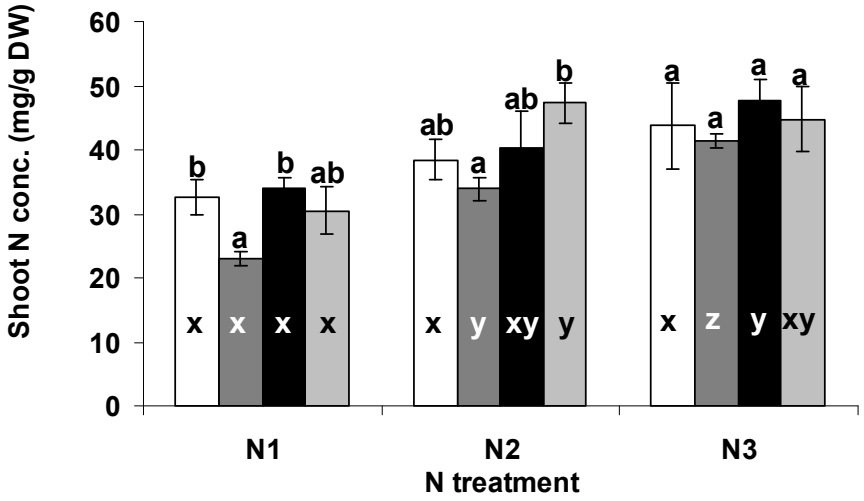

Figure 6.5. Shoot N concentration (mg/g DW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within and across N treatments, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05).

Shoot N concentrations of NM plants did not change significantly with increasing N supply, whereas those of all AM plants increased significantly (Fig. 6.5). At N1, plants colonised *G. caledonium* had significantly lower shoot N concentrations than NM plants and those colonised by *G. intraradices*. At N2, plants colonised by *G. caledonium* had marginally (p=0.08) lower shoot N concentrations than those colonised by *G. mosseae*, whereas at N3 all plants had similar shoot N concentrations. There were no significant changes in shoot N content with increasing N supply for both NM and AM plants (Fig. 6.6). At N1 and N2, there were no significant differences either between NM and AM plants. However, at N3, plants colonised by *G. caledonium* and *G. intraradices* had significantly higher shoot N content than NM plants.

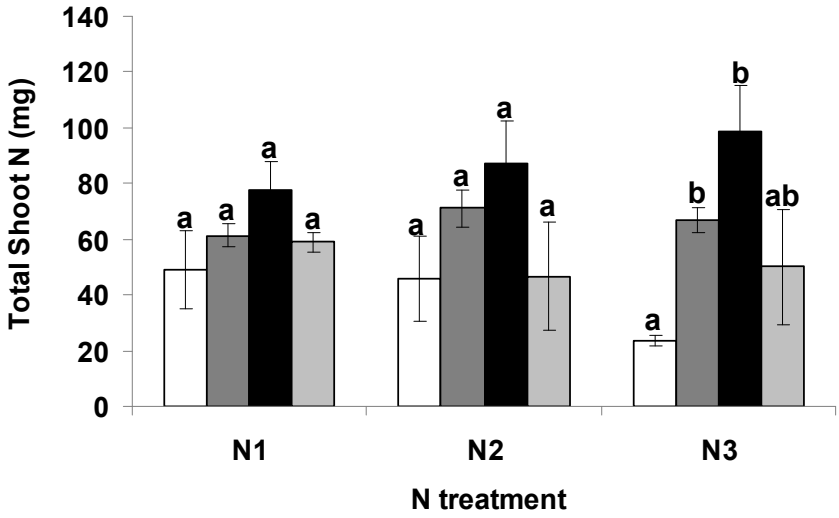

Figure 6.6. Total shoot N content (mg) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within each N treatment different letters indicate significant differences according to Tukey's test (p=0.05). There were no differences across N treatments for AM and NM plants.

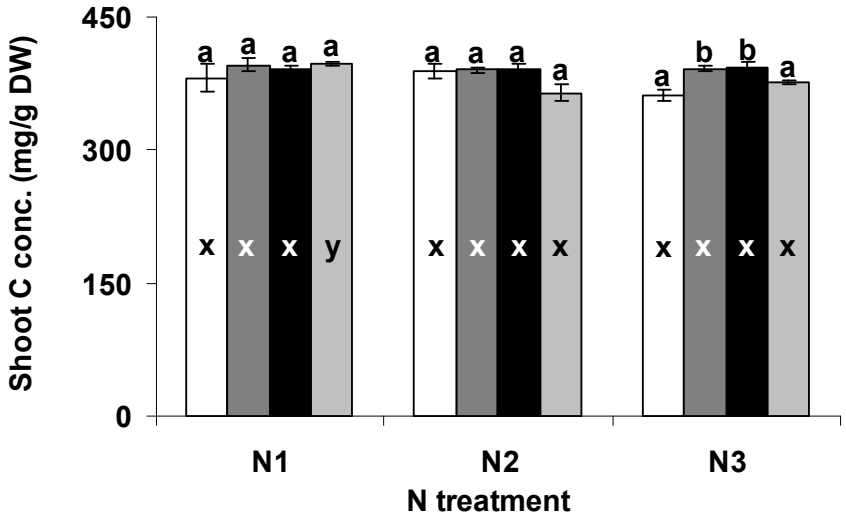

Figure 6.7. Shoot C concentration (mg/g DW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within and across N treatments, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05).

For most treatments, there were no significant differences in shoot C concentrations between N supplies (Fig. 6.7). However, plants colonised by *G. mosseae* had marginally (p=0.078) higher shoot C concentrations at N1 compared to the same plants at N2 and N3. There were no significant differences at each N supply between mycorrhizal treatments, except at N3 where plants colonised by *G. caledonium* and *G. intraradices* had slightly but significantly higher shoot C concentrations than NM plants and those colonised by *G. mosseae*. Shoot N/P ratios were not significantly different between NM and AM plants at any N supply. However, shoot N/P ratios of plants colonised by *G. mosseae* at N1 were significantly lower than those at N3 (Fig. 6.8).

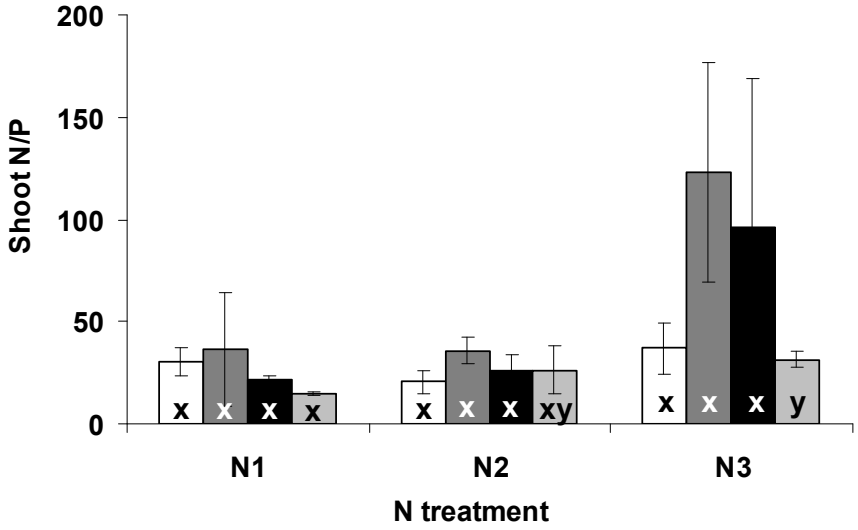

Figure 6.8. Shoot N/P ratio in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. For each mycorrhizal treatment, different letters indicate significant differences according to Tukey's test (p=0.05). There were no differences between NM and AM plants within each N treatment.

Shoot RA concentrations decreased significantly with increasing N supply for plants colonised by *G. intraradices* and *G. mosseae* (Fig. 6.9). Despite a decreasing trend, NM plants had similar shoot RA concentrations at all N supplies. Plants colonised by *G. caledonium* also had similar shoot RA concentrations at all N supplies. At N1, plants colonised by *G. intraradices* had significantly higher shoot RA concentrations than those colonised by *G. caledonium*. At N2, all AM and NM plants had similar shoot RA concentrations, whereas at N3, plants colonised by *G. caledonium* had significantly higher shoot RA concentrations than NM plants. There was also a significant correlation between shoot RA concentrations and shoot N content for NM plants (R^2=0.43; y=0.64x + 3.7; p=0.038). Shoot CA concentrations were not significantly different between any treatments (Fig. 6.10).

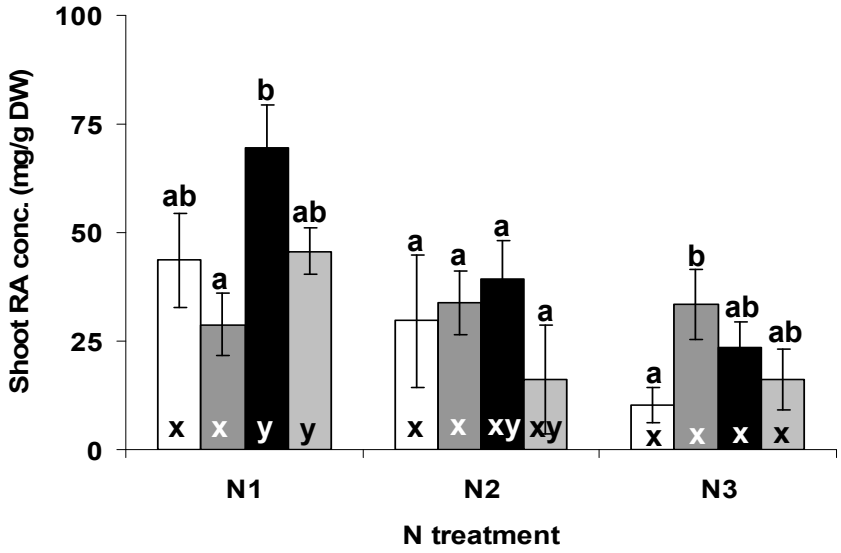

Figure 6.9. Shoot RA concentration (mg/g DW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. Within and across N treatments, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05).

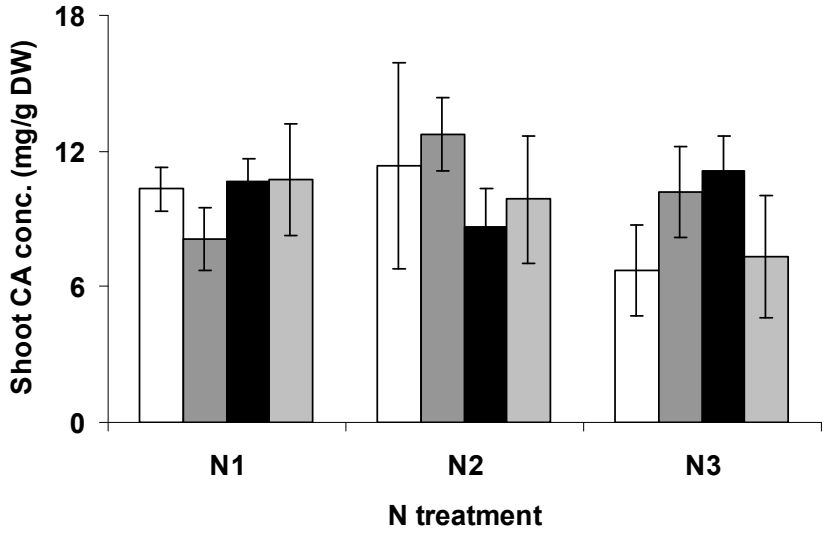

Figure 6.10. Shoot CA concentration (mg/g DW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=3-7) and SE bars are shown. There were no significant differences between treatments.

In general, there were no changes in ABA concentrations with increasing N supply, except for NM plants that had significantly higher shoot ABA concentrations at N1 compared to those at N2 (Fig. 6.11). There were no significant differences in shoot ABA concentrations between NM and AM plants at N1 and N3 (Fig. 6.11). However, at N2, plants colonised by *G. caledonium* and *G. intraradices* had significantly higher shoot ABA concentrations than NM plants (the comparison could not be made with plants colonised by *G. mosseae* as only one replicate was available). For AM plants, there was a significant correlation between plant biomass and shoot ABA concentrations (R^2=0.43; y=112x + 72.1; p=0.001).

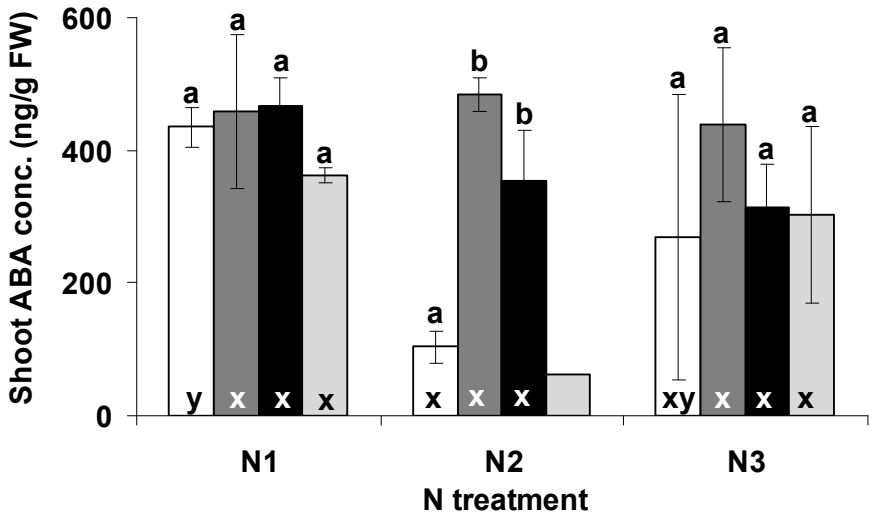

Figure 6.11. Shoot ABA concentration (ng/g FW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=2-3, except for *G. mosseae* at N2 were n=1) and SE bars are shown. Within and across N treatments, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05). For plants colonised by *G. mosseae* at N2, no statistical comparisons were made as only one replicate was available.

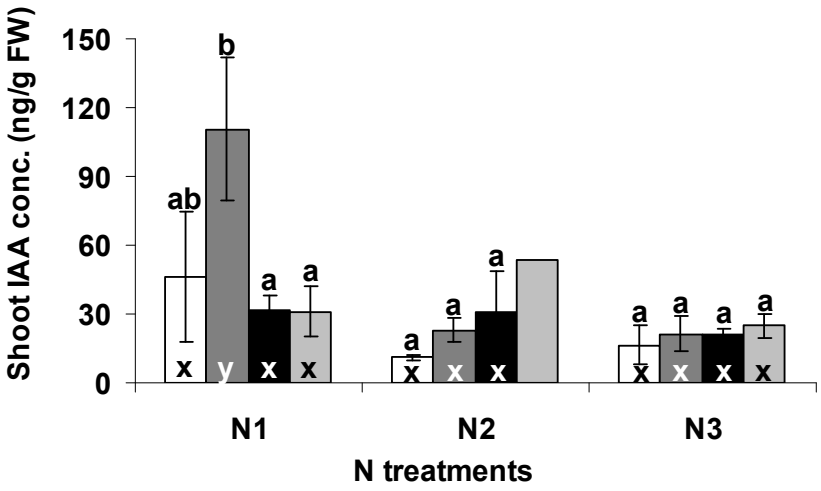

Figure 6.12. Shoot IAA concentration (ng/g FW) in *O. basilicum* grown under three different N supplies. The plants were NM (white bars) or colonised by *G. caledonium* (dark grey bars), *G. intraradices* (black bars) or *G. mosseae* (light grey bars). Means (n=2-3, except for *G. mosseae* at N2 were n=1). Within and across N treatments, different letters (abc and xyz, respectively) indicate significant differences according to Tukey's test (p=0.05). For plants colonised by *G. mosseae* at N2, no statistical comparisons were made as only one replicate was available.

Shoot IAA concentrations of plants colonised by *G. caledonium* were significantly higher at N1 compared to those at N2 and N3 (Fig. 6.12). There were no significant differences in shoot IAA concentrations between mycorrhizal treatments within most N treatments. However, at N1, plants colonised by *G. caledonium* had significantly higher shoot IAA concentrations than those colonised by *G. intraradices* or *G. mosseae*. For AM plants, there was a negative correlation between shoot N and shoot IAA concentrations (R^2=0.33; y=-2.3x + 126.5; p=0.004), which was most probably affected by the trend observed in shoot IAA concentrations for plants colonised by *G. caledonium*.

Discussion

Despite the stress that the plants might have undergone due to the failure of the cooling system of the glasshouse, the colonisation levels obtained in this experiment were consistent with those obtained in Chapters 4 and 5 for all AMF. Despite *G. caledonium* being a consistently poor coloniser, basil plants colonised by this fungus behaved differently than NM plants (*i.e.* plant dry weight, P content, shoot IAA concentrations). The growth patterns of AM and NM plants at N1 were also similar to those observed previously under similar experimental conditions (see Fig. 4.2, Chapter 4). Most NM and AM plants were matched for shoot P concentrations under all N supplies, also consistent with the results obtained in previous chapters. However, with the highest N supply, NM plants had low biomass and low shoot P concentrations, which resulted in low P uptake, indicating something was limiting growth. Higher N supplies did not increase the growth of basil and even resulted in reduced growth of plants colonised by *G. caledonium* and *G. mosseae* (as well as NM plants to some extent). Plants colonised by *G. intraradices* were the least affected by the different N supplies as their growth, shoot P concentration and total P uptake did not change significantly under the various N treatments.

With a few exceptions (Anwar *et al.* 2005, Sifola and Barbieri 2006), there is little information in the literature about the responsiveness of basil to N application. Anwar *et al.* (2005) showed that N concentrations in basil increased with N supply (NM plants). In the present study, shoot N concentrations also increased with N supply in AM plants, but were higher than those obtained by Anwar *et al.* (2005) by almost two-fold. It has been shown that AM plants are more efficient at taking

up and utilising ammonium than NM plants (Smith *et al.* 1985, Villegas *et al.* 1996, Hawkins *et al.* 2000, Govindarajulu *et al.* 2005) and that not all AMF are equally efficient at transporting N (Frey and Schuepp 1993). The latter was partially corroborated in this experiment as there were differences in N uptake between different AMF/plant combinations at N3. Furthermore, with the exception of plants colonised by *G. intraradices*, AM plants that accumulated luxury N (high shoot N concentrations) with increasing N supplies were the ones significantly exhibiting growth reductions. This suggests that extra N accumulation might have resulted in toxicity effects. In the family Lamiaceae, sage is considered to be very sensitive to ammonium toxicity (Jeong and Lee 1992). Similarly, it is possible that basil may be sensitive to ammonium supply as well. The high N/P ratios observed for plants colonised by *G. caledonium* and *G. intraradices* at N3 also suggest that N toxicity might have hindered their growth. However, despite relatively high N/P ratios, the growth of plants colonised by *G. intraradices* was not affected by high N supplies. Despite the high variability in shoot N/P ratio observed in these plants, the overall range of values was similar to what has been reported before for *Leucaena leucocephala* inoculated with *G. aggregatum* (Onguene and Habte 1995).

The hypothesis that higher N supplies would result in higher shoot RA or CA concentrations was not upheld. In fact, the opposite trend was observed in most treatments. These results are in contradiction to those observed by Sifola and Barbieri (2006) who showed that N fertilisation increased the above-ground biomass and essential oil concentrations of different cultivars of basil (all plants were NM). In contrast, Adler *et al.* (1989) found that when basil was fertilised

with ammonium (versus nitrate), the essential oil concentrations decreased. The authors suggested that this effect was due to water stress induced by ammonium supply through reduced root hydraulic conductivity. Further evidence suggests that ammonium can lead to water stress by reducing the abundance of plasma-membrane aquaporins (Adler *et al.* 1989, Guo *et al.* 2007). As N was applied as ammonium nitrate in the current experiment, AM plants could have been more efficient at taking the ammonium form, which might have resulted in reduced growth at the highest N supply (possibly through water stress). In turn, reduced growth might have resulted in reduced antioxidant concentrations. NM plants might have also suffered from N toxicity as they had high shoot N concentrations, low growth and low shoot RA concentrations under high N supplies. However, despite this suggestion, it is known that protons (H^+) associated with ammonium assimilation are lost in the soil solution and unlikely to be stored in the shoot vacuoles in land plants (Raven and Smith 1976, Britto and Kronzucker 2002). Furthermore, no visual signs of N toxicity were observed in the course of the experiment (*i.e.* darkening of the leaves, necrosis spots).

Again contrary to the initial hypothesis, the changes in phytohormone concentrations (both ABA and IAA) in NM and AM plants were not correlated with changes in RA and CA concentrations in the shoots of basil. It has been reported that nitrate supply does not affect the concentration of ABA in roots or shoots of NM barley (*Hordeum vulgare* L.) (Brewitz *et al.* 1995), which is similar to what was observed in this experiment. Furthermore, the higher shoot ABA concentrations in plants colonised by *G. caledonium* and *G. intraradices* at N2 is in agreement with previous studies on maize (*Zea mays* L.) and soybean (*Glycine*

max L.) (Danneberg *et al.* 1993, Meixner *et al.* 2005), suggesting a systemic accumulation of ABA in AM plants compared to NM plants. It was recently demonstrated that ABA plays an important role in the development and functionality of arbuscules in tomato (*S. lycopersicum* L.) (Herrera-Medina *et al.* 2007). The authors showed that exogenous ABA application directly increases to the susceptibility of tomato to colonisation by AMF, whereas in the present experiment, plants colonised by *G. caledonium* had high shoot ABA concentrations but low colonisation levels. The cause of this shoot ABA increase is still unknown.

ABA acts as a growth regulator that is known to slow shoot growth and to reduce stomatal aperture, especially under drought conditions (Roberts and Hooley 1988, Finkelstein *et al.* 2002, Yang *et al.* 2007). However, the high shoot ABA concentrations measured in this experiment corresponded to higher plant biomass in plants colonised by *G. caledonium* and *G. intraradices*. This was further supported by the positive correlation between plant dry weight and shoot ABA concentrations in AM plants at N2. In the same context, although IAA is known to be a plant growth promoter (Roberts and Hooley 1988) its concentrations did not seem to affect the growth of basil, even in plants colonised by *G. caledonium* which had high shoot IAA concentrations at N1. Unfortunately, no explanation can be yet proposed to explain such contrasting results.

From the results presented here it can be concluded that, contrary to what was hypothesised, higher N supplies do not necessarily lead to higher concentrations of RA and CA in basil. Furthermore, neither IAA nor ABA concentrations were

correlated to changes in RA or CA concentrations. Therefore, it appears that neither soil N supply nor IAA or ABA concentrations were mechanistically linked to the synthesis of RA and CA under my experimental conditions. Other factors such as light and temperature (see Chapters 1 and 3) might influence the production of RA and CA in the shoots of basil. However, the role of plant hormones should not be entirely excluded with regards to their influence on the production of phytochemicals, as other hormones such as cytokinin and jasmonic acid are known to be influenced by AM colonisation (Danneberg *et al.* 1993, Barker and Tagu 2000, Hause and Fester 2005, Meixner *et al.* 2005, Hause *et al.* 2007). The next chapter will explore an alternative hypothesis, investigating how RA and CA concentrations vary in the presence of a pathogen. This investigation was carried out with a commercial scope for the project.

7. *Glomus mosseae* confers a bioprotective effect against *Fusarium oxysporum* f.sp. *basilici* in basil

The work presented in this chapter has been conducted jointly with Ms. Magdalena Kraml from the Universität für Bodenkultur (BOKU), Vienna, Austria, who recorded and acknowledged its contents in her M.Sc. thesis, recently submitted to that institution. I contributed to this work by planning and performing most of the experiments in close collaboration with Ms. Kraml in Vienna. I also instructed her in the techniques to work with AMF. The work was done in Vienna, under the supervision of Dr. Horst Vierheilig who is a co-supervisor for my candidature. I have included this work in my thesis with the permission of my co-workers who also agree that my contribution is as stated.

As discussed in Chapter 1, the antioxidants found in basil (RA and CA) are known to act as defence compounds. Basil also has high concentrations of two major essential oils, eugenol and linalool (Simon 1998, Jirovetz *et al.* 2003). These essential oils have been shown to have antimicrobial activity against bacteria such as *Escherichia coli* and *Lactobacillus plantarum* (Bouzouita *et al.* 2003). As discussed in Section 1.5.2. (Chapter 1), there have been increasing occurrences of *Fusarium oxysporum* f.sp. *basilici* (*Fob*) which affects the commercial production of basil worldwide (Gamliel *et al.* 1996). As a result, ways of reducing the damage done by *Fob* are currently sought after, which could include the use of AMF as biocontrol agents. As different AMF have been demonstrated to trigger the production of RA in basil (Chapter 4), it was hypothesised that they could also induce a systemic resistance against *Fob* through an increased production of RA, CA or essential oils.

Aims

The work described in this chapter had two main objectives: 1) to evaluate the potential of an AM fungus to reduce the disease severity of *Fob* in basil, and 2) to evaluate the interaction between the AM fungus and *Fob* to increase the production of RA, CA and essential oils in shoots of basil, which are the harvested products.

Materials and methods

There were five treatments with 15 replicates each: 1) NM plants as controls, 2) NM plants with added phosphorus (NM+P), 3) NM plants with *Fob* (NM+*Fob*), 4) AM plants, and 5) AM plants with *Fob* (AM+*Fob*). These treatments allowed us to assess the efficacy of an AM fungus to reduce the harmful effects of *Fob* on basil through mycorrhiza formation, but also to take into account a simple P-mediated effect (NM+P treatment) on RA, CA and essential oil accumulation.

The basil seeds used in this experiment were the same ones as used previously (Section 2.1, Chapter 2). The seeds were surface-sterilised by soaking in a 30% hypochlorite solution for 5 min, rinsed with distilled water and germinated in Perlite in a controlled temperature room (28°C). When the seedlings were 14 day-old, they were transferred into small free-draining pots (8 cm diameter x 7 cm height) containing a mixture of sand, expanded clay and soil (1:1:1; v/v/v; autoclaved 20 min at 121°C). They were planted into holes in the substrate where the AM fungal inoculum (5 g per pot) had been previously added. The inoculum consisted of dried root pieces containing fungal hyphae and spores of *G. mosseae* (BEG 12; International Bank of Glomeromycota; http://www.kent.ac.uk/bio/beg/),

mixed with a carrier material (silica sand and expanded clay). It was obtained from Biorize, France, and regularly used in the laboratory of Dr Vierheilig. NM, NM+P and NM+*Fob* plants received 5 g of the sand, clay and soil mixture instead of the inoculum powder.

Fusarium inoculation

Fusarium oxysporum f.sp. *basilici* (*Fob*) cultures were obtained from Dr M. Pasquali, University of Torino, Italy. The stock cultures of *Fob* were sub-cultured on potato dextrose agar (PDA, Sigma Aldrich) in Petri dishes (31.2 g PDA in 800 ml distilled water) for 7 days at 24^{o}C in the dark before using them for inoculation on the plants. *Fob* inoculum was prepared by washing the dishes with sterile water, which was then filtered through cheesecloth to remove any mycelium and obtain only conidia (spores). The resulting spore suspension was examined under a light microscope to determine the number of conidia and the final concentration was adjusted to 2.1 x 10^{6} spores/ml. This suspension was then used as the inoculum. On the day of application of *Fob*, the roots of the various plant treatments (5 wks old) were washed and the degree of AM colonisation was determined in 5 plants which had been inoculated with *G. mosseae* (colonisation: 60±7%). The roots of the plants in the *Fob* treatments were dipped in the *Fob* suspension, whereas roots of all other plants were dipped in sterile water. The plants were then transplanted into new pots containing the sand, clay and soil substrate, as previously described. The transplanting was done for all treatments, and the plants that were mycorrhizal did not received more inoculum as they were already well colonised.

Plant growth

Treatments were randomly arranged in a glasshouse in Vienna, Austria and grown for another 5 weeks (day/night cycle: 16 h 22°C / 8 h 19°C; relative humidity 50-70%; light intensity averaging 400 µmol/m^2/s with ~200 µmol/m^2/s additional lighting provided by Radium HRI-T4W/DH lamps) and watered regularly with a nutrient solution with or without P, according to treatment. The nutrient solution without P consisted of (mg/l) $Ca(NO_3)_2$, 472; K_2SO_4, 256; $MgSO_4$, 136; MoO_3, 70; NH_4NO_3, 8; $Fe_6H_5O_7$ x 3 H_2O, 50; $Na_2BO_4O_7$ x $4H_2O$, 1.3; $MnSO_4$ x 4 H_2O, 1.5; $ZnSO_4$ x 7 H_2O, 0.6; $CuSO_4$ x 5 H_2O, 0.54; $Al_2(SO_4)_3$, 0.028; $NiSO_4$ x 7 H_2O, 0.028; $Co(NO_3)_2$ x $6H_2O$, 0.028; TiO_2, 0.028; $LiCl_2$, 0.014; $SnCl_2$, 0.014; KI, 0.014; KBr, 0.014. The nutrient solution with P consisted of the same solution with added KH_2PO_4 (0.136 g/l).

Plants were monitored regularly in the first days after application of *Fob* and mortality was recorded throughout the experiment. Five weeks after inoculation with *Fob*, plants were harvested (10 wks old). The disease severity was visually assessed by estimating the percentage of root browning/decay, and the following parameters were recorded: shoot and root fresh and dry weights, percent AM colonisation, shoot P, shoot and root RA and CA concentrations, shoot essential oil composition and concentrations, shoot total phenolic content and total antioxidant activity (see below for methods). The methods for dry weight and percent colonisation determination were as described in Chapter 2. Phosphorus concentrations were determined with the ammonium-vanadate-molybdate method (Gericke and Kurmies 1952) on pooled samples as not enough plant material was available to conduct all analyses on all samples.

Rosmarinic and caffeic acid (RA and CA) as well as essential oil concentrations, total phenolic content and antioxidant activity were determined in the laboratory of Dr Johannes Novak (Institute for Applied Botany, Department of Public Health, University of Veterinary Medicine, Vienna, Austria).

Chemicals

Folin-Ciocalteu phenol reagent was obtained from Merck (Darmstadt, Germany). DPPH (2,2-Diphenyl-1-picrylhydrazyl), caffeic acid and (R)-(-)-Carvon were purchased from Sigma Aldrich (Vienna, Austria). Trolox (6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid) was obtained from Fluka (Vienna, Austria) and rosmarinic acid from Roth (Karlsruhe, Germany). Solvents for extract preparation were of analytical grade and solvents for HPLC analysis of HPLC-grade.

Sample extraction

100 mg dried ground leaves or roots (RA and CA) were extracted with 16 ml MeOH/water (50:50, v/v) at room temperature for one hour in a sonicator. The filtered methanolic sample extracts were used for determination of total phenolic content, antioxidant activity and HPLC analysis. An additional 100 mg dried leaves were extracted for 30 min in a sonicator with 2 ml dichloromethane containing Carvon (0.1 µl/ml) as an internal standard. The samples were filtered and subsequently used for GC analysis.

Determination of total phenolic content

The total phenolic content was determined according the Folin-Ciocalteu method (Singleton *et al.* 1999). 200 µl of sample extract were added to a flask which contained 0.5 ml Folin-Ciocalteu reagent and 10 ml water. After 3 min, 1 ml of a saturated Na_2CO_3 solution was added, vortexed and filled up with water to a final volume of 25ml. After incubation for one hour in the dark, the colorimetric reaction was recorded with a spectrophotometer (Hitachi Ltd., Tokyo, Japan) at 725 nm. The total phenolic content of the samples was expressed in mg CA/g DW.

Determination of total antioxidant activity (DPPH)

The radical scavenging activity was measured using the stable radical DPPH. Briefly, 50 µl sample and 950 µl methanol were added to 1 ml DPPH (0.015%, v/v in methanol). After 30 min incubation in the dark, the reductive discoloration of the DPPH radical, which is due to the active antioxidants found in the sample, was recorded. Measurements were done using a spectrophotometer (Hitachi Ltd., Tokyo, Japan) at 515 nm, and the results were expressed in Trolox equivalents.

HPLC analysis

The method was adapted from that of Cuvelier *et al.* (1996). The analysis was performed on a reversed phase C_{18} Symmetry® column (4.6 x 150 mm, 5µm pore size; Waters, USA) equipped with a guard column of the same stationary phase (Symmetry® C_{18}). The mobile phase was programmed with a linear gradient from 90% A (840 ml de-ionised water with 8.5 ml acetic acid and 150 ml acetonitrile), 10% B (MeOH), to 100% B in 30 min with a flow rate of 1.5 ml/min. 20 µl of

methanolic samples were injected, which were detected by a photodiode array detector (Waters, 996 PDA, USA) at 330 nm.

Essential oil analyses

Identification of the essential oil compounds was performed on a GC/MS HP 6890 chromatograph coupled to a 5972 MSD (HP, Palo Alto, CA, USA) and fitted with a DB-5MS (30 m x 0.25 mm, 0.25 μm film thickness, Agilent, USA). Operating conditions were helium as carrier gas (average velocity 42 cm/s), injection temperature 250°C with a split ratio if 20:1. The temperature program was 60°C for 4 min, 60-280°C at 5°C/min. The compounds were identified by comparison of retention indices (Adams 2001) and mass spectra from Wiley database (McLafferty 1989).

Results

After 10 wks growth, NM and NM+P plants were not colonised by the AM fungus nor infected by *Fob*. Percent colonisation in plants inoculated with *G. mosseae* was significantly ($p=0.009$) higher than in AM+*Fob* plants (Table 7.1). There was no mortality or root browning in plants not inoculated with *Fob*. The mycorrhizal status of the plants affected the disease severity of *Fob* (Table 7.1). NM+*Fob* plants had a higher mortality rate compared to equivalent AM plants. NM+*Fob* plants also had significantly ($p=0.05$) higher levels of root browning compared to AM plants under the same conditions.

Table 7.1. Percent AM colonisation, mortality and root browning in *O. basilicum* grown under various treatments. Values represent means (n=5) and for each parameter different letters represent significant differences according to Tukey's test (p=0.05).

Treatment	% AM colonisation	% Mortality	% Root Browning
NM	0	0	0
NM+P	0	0	0
AM	58 b	0	0
AM+*Fob*	40 a	20 a	6 a
NM+*Fob*	0	33 b	19 b

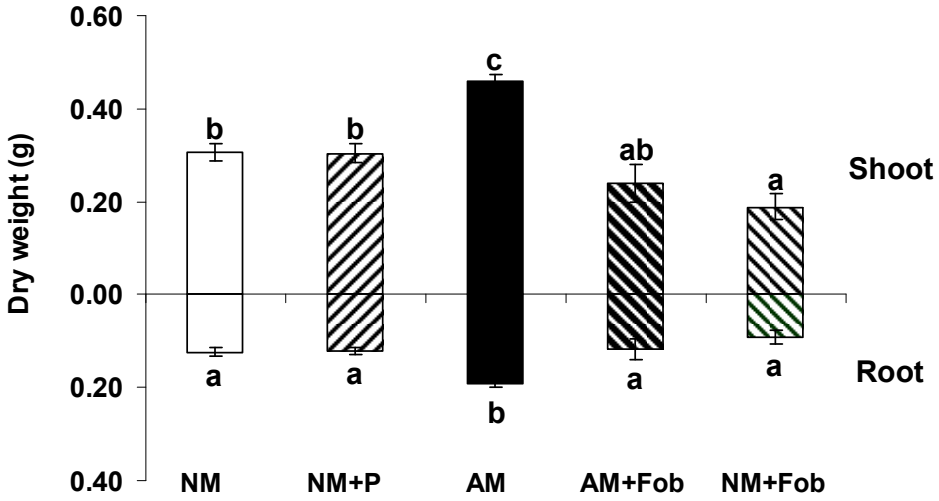

Figure 7.1. Shoot and root dry weight (DW, g) of *O. basilicum* grown under various treatments. Means (n=10-15) and SE bars are shown. Different letters represent significant differences according to Tukey's test (p=0.05).

Additional P had no significant effect on the growth of NM plants, but inoculation with *G. mosseae* significantly increased both shoot and root dry weights (Fig. 7.1). Inoculation with *Fob* significantly reduced shoot growth, but this effect appeared slightly reduced in AM plants, though not significantly so. AM+*Fob* plants grew as well as NM plants grown with or without P. Shoot P concentrations were similar in most treatments (Fig. 7.2). NM and AM plants had similar shoot P concentrations, while NM+P plants appeared to have slightly, but not significantly, higher concentrations. NM plants treated with *Fob* had significantly

higher (p=0.05) mean shoot P concentration compared to AM plants. Shoot P content was significantly lower in NM plants compared to NM+P and AM plants (Fig. 7.3). Inoculation with *Fob* did not affect shoot P uptake significantly compared to any other treatments.

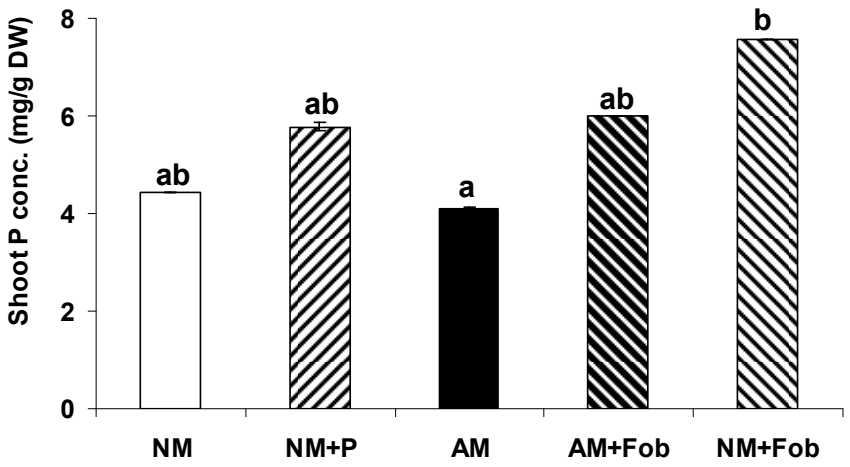

Figure 7.2. Shoot P concentration (mg/g DW) in *O. basilicum* grown under various treatments. Means (n=2; pooled samples) and standard deviation bars are shown. Different letters represent significant differences according to Tukey's test (p=0.05).

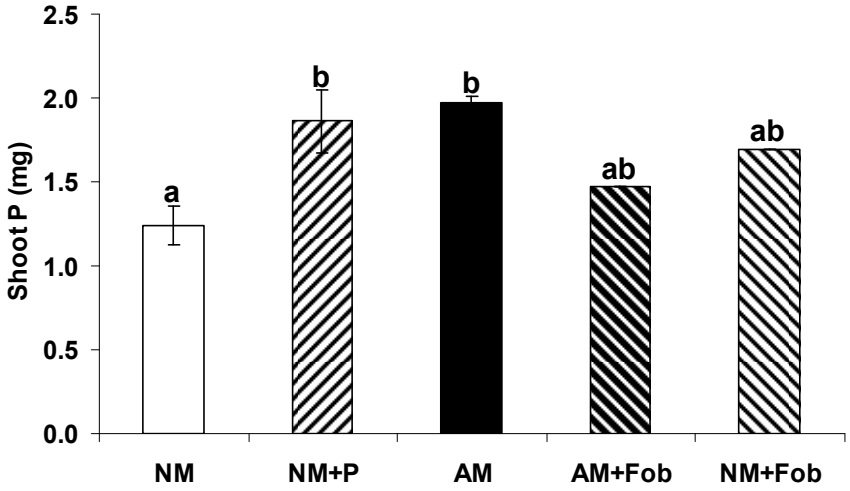

Figure 7.3. Shoot P content (mg) in *O. basilicum* grown under various treatments. Means (n=2; pooled samples) and standard deviation bars are shown. Different letters represent significant differences according to Tukey's test (p=0.05).

Shoot RA concentrations were significantly (p=0.001) affected by the treatments (Table 7.2). NM plants had the highest mean shoot RA concentration, but not significantly higher than NM+P and AM plants. NM plants treated with *Fob* had significantly lower RA concentrations than most other treatments. Mean shoot CA concentrations were lower than RA concentrations, but there were no significant differences between the treatments (Table 7.2). Shoot phenolic concentration was significantly (p=0.001) higher in NM plants than in other treatments, except AM plants (Table 7.2). The shoot antioxidant activity was also significantly (p=0.001) higher in NM plants than in all other treatments (Table 7.2). The antioxidant activity was significantly correlated to shoot RA concentration in all treatments (R^2=0.41; y = 0.0118x + 0.3904; p=0.001). There was also a significant relationship between shoot RA and phenolic concentrations for all treatments (R^2=0.55; y = 0.0257x - 0.0702; p=0.000).

Table 7.2. Shoot RA and CA concentrations (mg/g DW), phenolic concentration (mg CA/g DW) and antioxidant activity (mg Trolox/g DW) in *O. basilicum* grown under various treatments. Values represent means (n=5) and for each parameter different letters represent significant differences according to Tukey's test (p=0.05).

Treatment	Shoot RA (mg/g DW)	Shoot CA (mg/g DW)	Shoot Phenolic concentration (mg CA/g DW)	Shoot antioxidant activity (mg Trolox/g DW)
NM	16.8 c	0.52 a	69.48 b	111.60 b
NM+P	15.2 bc	0.50 a	55.22 a	81.46 a
AM	15.7 c	0.52 a	60.56 ab	89.86 a
AM+Fob	11.8 ab	0.75 a	53.10 a	80.25 a
NM+Fob	11.0 a	0.70 a	41.26 a	61.50 a

Root RA concentrations were higher than shoot RA concentrations, which is similar to the results reported in Section 3.1. Both NM+P and AM plants had significantly (p=0.000) higher root RA concentrations than other treatments (Table 7.3). Root CA concentrations were slightly lower than those observed in the shoots. AM plants had significantly (p=0.000) lower root CA concentrations than most other treatments, except NM plants (Table 7.3). AM+*Fob* plants had significantly (p=0.001) higher root CA concentrations than both NM and AM plants.

Table 7.3. Root RA and CA concentrations in *O. basilicum* grown under various treatments. Values represent means (n=10-15). Different letters within columns represent significant differences according to Tukey's test (p=0.05).

Treatment	Root RA (mg/g DW)	Root CA (mg/g DW)
NM	24.7 a	0.36 ab
NM+P	34.3 b	0.39 bc
AM	37.8 b	0.33 a
AM+Fob	27.6 a	0.44 c
NM+Fob	23.9 a	0.40 bc

The most abundant essential oil compounds found in the shoots were eugenol, linalool, methyleugenol and 1,8-cineole. The patterns of essential oil concentration were quite similar in NM, NM+P, AM and AM+*Fob* plants (Fig. 7.4). However, NM+*Fob* plants showed a different pattern. In these plants, methyleugenol concentration was much higher than eugenol. Methyleugenol concentration in NM+*Fob* plants was also significantly higher (p=0.000) than in all other treatments. Concentrations of other essential oils did not vary significantly between treatments.

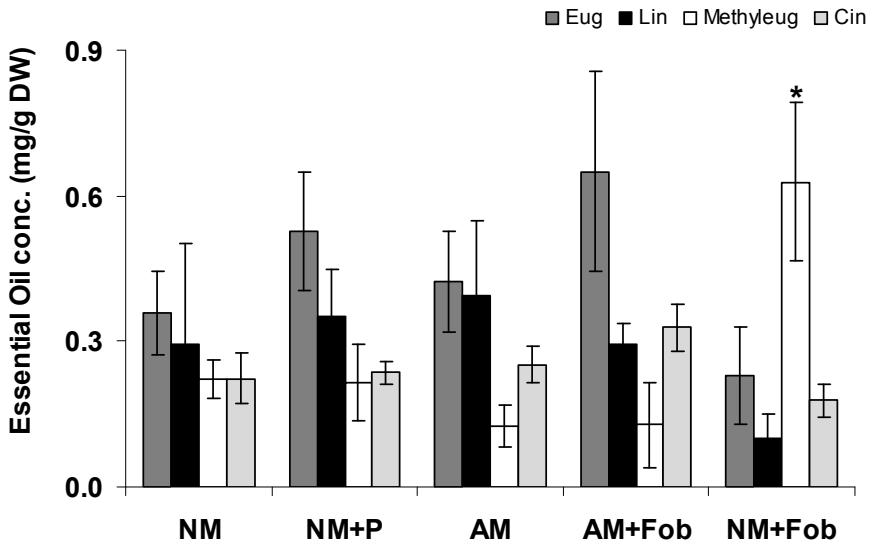

Figure 7.4. Essential oil concentrations (mg/g DW) in the shoots of *O. basilicum* grown under various treatments. The oils are: eugenol (Eug), linalool (Lin), methyleugenol (Methyleug) and 1,8-cineole (Cin). Means (n=3-5) and SE bars are shown. * Indicates a significant difference in methyleugenol concentration between the different treatments according to Tukey's test (p=0.05).

Discussion

The results indicate that increasing P supply to NM plants did not significantly affect their growth under the conditions of this experiment. AM plants grew better than NM+P plants, even though both took up similar amounts of P into their shoots. Furthermore, AM plants had similar shoot P concentration to NM and NM+P plants, which suggests that something other than P was limiting the growth of NM plants. Perhaps N was a limiting factor, but this is unlikely to be the case as the nutrient solution applied in this experiment corresponded to ~85 mg/kg of actual N, which is close to the highest N supply that was used in the previous experiment (Chapter 6). However, plant N concentrations and contents were not determined in this experiment. It was also clear that *Fob* caused growth reductions in basil, regardless of the mycorrhizal status. *G. mosseae* provided a certain

bioprotection to basil against *Fob*, which reduced the damage caused by the pathogen; AM+*Fob* plants had lower mortality and root decay than NM+*Fob* plants. This protective effect was not an indirect effect of AM on P uptake as NM and AM plants treated with *Fob* had similar shoot P concentrations and total P uptake.

In contrast to the results obtained in Chapter 4, AM plants did not have higher shoot RA or CA concentrations. As shoot CA concentrations were not affected by any of the treatments, this could imply that it was all converted into RA. The discrepancy between these results and those obtained in Chapter 4 is possibly due to differences in fungal inoculum, growth conditions and nutrient solution used in this experiment. It was originally hypothesised that RA production could be systemically triggered in the shoots following infection by *Fob*. However, this hypothesis was refuted in this experiment. Shoot RA concentrations were reduced by *Fob* and, contrary to expectations, AM+*Fob* plants did not have higher shoot RA concentrations than AM or NM+*Fob* plants. In fact, *Fob* inoculation was associated with reductions in shoot RA concentrations.

RA has been speculated to serve as a defence compound and feeding deterrent against herbivores, mainly in roots (Bais *et al.* 2002). Therefore, it was hypothesised that RA could have been mainly produced in the roots of basil following elicitation by *Fob*, or translocated from shoots to roots as a defence response against the pathogen. There is some evidence that RA can be transported from leaves to flowers in the shoots of rosemary (*Rosmarinus officinalis* L.) (del Bano *et al.* 2003). However, although RA can be produced in roots (Bais *et al.*

2002), there is no evidence for its transport from shoots to roots (M. Petersen personal communication). The above hypothesis was rejected as *Fob* did not increase root RA concentrations in this experiment, although both P supply (NM+P) and colonisation with *G. mosseae* (AM) did increase these concentrations. As NM+P and AM plants had the highest shoot P uptake, it is therefore possible that root RA concentrations were also affected by P uptake in those plants (P could not be measured in roots due to lack of material). The latter hypothesis would be in accordance with the results reported previously in NM roots (Chapter 4). Furthermore, as AM+*Fob* and NM+*Fob* had similar root CA concentrations, we cannot conclude that either RA or CA concentrations are correlated to the bioprotection effect conferred by *G. mosseae* against *Fob*. Therefore, the mechanisms by which *G. mosseae* protects basil against *Fob* remain unclear.

Total phenolic concentrations and shoot antioxidant activity followed a similar trend to that of shoot RA concentrations. These findings suggest that under our experimental conditions, RA could have been the predominant phenolic compound conferring antioxidant activity in basil, as proposed by other authors (Juliani and Simon 2002, Jayasinghe *et al.* 2003). NM plants, NM+P plants and AM plants all had similar shoot P, RA and CA concentrations, as well as patterns of accumulation of essential oils, regardless of P nutrition. This is consistent with previous observations (Chapter 4) that P nutrition does not necessarily contribute to higher production of phytochemicals in basil (Copetta *et al.* 2006).

Eugenol and linalool were the most abundant essential oils in most treatments, consistent with previous reports (Copetta *et al.* 2006). Eugenol is considered to be an antibacterial and antifungal agent (Gang *et al.* 2001), and is converted to methyleugenol by an *O*-methyltransferase. In this study, NM+*Fob* plants had substantial concentrations of methyleugenol, which suggests that under the sole influence of the pathogen there was an increased conversion of eugenol into methyleugenol compared to other treatments. This result is consistent with reports that showed induction of *O*-methyltransferases as a defense response to the presence of fungal elicitors (Lewinsohn *et al.* 2000). The latter results are also consistent with the observations of Miele *et al.* (2001) that basil plants with reduced growth show greater concentrations of methyleugenol. As the presence of *Fob* caused growth reductions, it is therefore likely that the pathogen triggered the production of methyleugenol via an increased activity of *O*-methyltransferase. However, the fact that there was no significant increase in methyleugenol in AM+*Fob* plants suggests that the presence of *G. mosseae* can contribute to reduce the formation of methyleugenol in *Fob*-infected plants. This result is somewhat consistent with those of Copetta *et al.* (2007) who observed slight reductions (although not significant) in methyleugenol concentrations in basil plants colonised by the same strain of *G. mosseae* (BEG 12, Biorize) compared to NM plants.

The lower level of methyleugenol in shoots of *AM+Fob* plants compared to NM+*Fob* plants is of dietary importance as the intake of considerable doses (*e.g.* through consumption of fresh basil or pesto) is considered harmful (Miele *et al.* 2001). The chemical structure of methyleugenol is very close to that of estragole

(Gang *et al.* 2001, Miele *et al.* 2001), a known genotoxic carcinogen (http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf; European Commission report). Thus, mycorrhization could reduce the accumulation of a potentially harmful compound in *Fob* infected plants.

In conclusion, although the interaction between *G. mosseae* and *Fob* did not contribute to higher concentrations of phytochemicals (RA, CA or essential oils) than in NM plants, it was shown that this AM fungus can 1) promote the growth of basil; 2) yield similar RA and CA concentrations as NM plants under high P supply, consistent with previous results (Chapter 4); 3) confer a protective effect against *Fob*; and 4) alter the essential oil composition of basil when in the presence of *Fob*. The protective effect of *G. mosseae* against *Fob* was not associated with an increase in P content, shoot RA or CA concentrations nor an increase in essential oil concentrations. Further investigations are therefore required to elucidate the mechanisms responsible for this protective effect. Root RA and CA concentrations may shed some light in understanding the mechanisms described above, when the data become available.

8. General discussion

As identified in Chapters 1 and 3, there have been few investigations on basil as a host plant in AM symbioses, particularly with respect to growth and nutrition (Giovannetti 1997, Dickson 2004, Copetta *et al.* 2006, Copetta *et al.* 2007). This thesis provides one of the first comprehensive studies on the effect of the AM symbiosis on phytochemical production in the shoots of basil. The work presented had four main objectives: 1) to identify suitable growth conditions in which AM and NM plants were matched for tissue P concentrations (Chapter 3), and under these conditions; 2) to explore the effect of AMF on the production of phytochemicals (mainly RA and CA) (Chapter 4); 3) to investigate the mechanisms by which AMF can affect RA and CA concentrations in basil compared to NM plants (Chapters 5 and 6); and finally 4) to explore the combined effects of an AM fungus and a specific pathogen of basil on phytochemical production (Chapter 7). These objectives were set in order to answer the questions raised at the beginning of this project:

- does the AM symbiosis affect the production of phytochemicals in the shoots of basil?
- if so, what are the mechanisms involved?

Based on the findings and outcomes of the previous chapters, this last section will address how successful this project was at answering these two questions and at addressing the objectives outlined above. Furthermore, suggestions for future research avenues will be discussed.

Phosphorus supply and AM symbiosis greatly affect the growth of basil

In order to answer the first question, it was essential to obtain AM and NM plants with similar growth rates and shoot P concentrations, so that any nutritional effect of the fungi (*e.g.* improved P uptake) on the production of RA and CA could be eliminated. The experiments described in Chapter 3 aimed at doing this and yielded interesting results. Firstly, it was shown that basil is highly responsive to P, as its growth increased with increasing $CaHPO_4$ supply (Sections 3.1 – 3.4). Secondly, the growth of basil was also shown to be dependent on the AM symbiosis when very little P (Section 3.1) or no P is supplied (Sections 3.2 and 3.3). Indeed, all NM plants that did not receive any P supply grew very poorly and/or died within the first few weeks of the experiments, whereas AM plants survived under these limiting P conditions. Similarly, it has been shown that lavender (*Lavandula spica* L.) and mint (*Mentha arvensis* L.), which are also members of the family Lamiaceae, are highly dependent on the AM symbiosis in order to thrive in nutrient-poor Mediterranean habitats (Azcón and Barea 1997, Freitas *et al.* 2004).

Basil was also shown to grow poorly when the experiments were carried out in winter (*i.e.* under low light and temperatures), which highlighted the need to cultivate it in summer (as basil is a Mediterranean plant). Furthermore, the combination of growing basil in winter and forming a symbiosis with *G. intraradices* resulted in growth depressions. As suggested in Chapter 3, such results may have been due to the fungus being a rather large sink for C, which would use a larger proportion of total photosynthates in winter when light intensity is low (Son and Smith 1988, Baon 1994, Smith and Read 1997).

However, the growth depressions observed in AM plants also coincided with low colonisation levels. Hypothetically, despite basil being quite poorly colonised by *G. intraradices,* the fungus might not have been contributing at all to P uptake through the mycorrhizal pathway (Smith *et al.* 2003). Thus, *G. intraradices* might have been "cheating" its host by utilising more C rather than benefiting it through improved P delivery. To overcome growth depressions, the remainder of the experiments were carried out in summer, together with further adjustments to experimental conditions (see below).

Altogether, the experiments described in Chapter 3 led to the conclusion that supplying 0.2 g/kg $CaHPO_4$ to the plants improved their growth (both AM and NM), while allowing satisfactory colonisation levels by *G. intraradices*. The results obtained in Section 3.4 further indicated that growing basil in a mixture of soil/sand at a ratio of 1:3 (w/w) contributed to obtaining AM and NM plants with similar growth rates. Finally, increasing the amount of AM inoculum added to the soil to 25% (w/w, dry weight) instead of 10% also contributed to obtain high colonisation levels, improved plant growth and alleviating growth depressions. Hence, all of these amendments resulted in AM and NM plants with matched growth rates and shoot P concentrations (see Section 3.4, Chapter 3). Therefore, the first objective of the project was achieved.

The AM symbiosis can increase the production of phytochemicals in basil, which is not mediated by improved P uptake

During the course of this project several reports were published relating to the effect of the AM symbiosis on the production of phytochemicals in medicinal

herbs. Hence, it has now been shown that AMF can increase the concentrations of phytochemicals (*e.g.* essential oils) in basil, oregano and wormwood (Copetta *et al.* 2006, Khaosaad *et al.* 2006, Copetta *et al.* 2007, Kapoor *et al.* 2007). However, such information has not been reported with respect to the production of RA and CA in basil. This is a new outcome from the project.

Under the amended growth conditions reported above, it was shown that both *G. caledonium* and *G. mosseae* can increase the concentrations of RA and CA in the shoots of basil (Chapter 4). These increases were not an indirect effect of improved P nutrition in AM plants, as all NM and AM plants were matched for shoot P concentrations and P uptake. Increases in RA and CA concentrations in AM plants were also obtained in Chapter 6 under high N supply (see Fig. 6.9). These results provided evidence that an improved P uptake in AM plants is not the main mechanism by which AMF can increase the production of antioxidants in basil, contrary to what had been suggested previously (Sirohi and Singh 1983, Kapoor *et al.* 2002a, b). Such results had not been demonstrated previously in basil.

The concentrations of RA and CA in the shoots of basil were quite variable among mycorrhizal treatments in the experiments (Chapters 3 to 6). This variability is likely to have been the result of different growth conditions (*i.e.* light intensity, temperature) between the various experiments. As a consequence, it cannot be concluded that *G. caledonium* (or other AMF) consistently increases the production of antioxidants in basil. Hence, the experiments (mainly those of

Chapters 4 and 6) were only partly successful at addressing the main question of this thesis.

The degree of AM colonisation does not reflect the influence of the symbiosis on the physiology of the host plant

Although *G. caledonium* was the fungal partner that induced the largest increase in shoot RA and CA concentrations (Chapter 4), it was also the one that had the lowest colonisation level in basil. On the other hand, *G. intraradices* always heavily colonised the roots of basil, but did not significantly contribute to the increase of RA and CA concentrations. These results emphasise that AMF can greatly influence the physiology of their host plants regardless of colonisation levels. Such results also underline the functional diversity that exists between AM symbioses (as addressed in Chapter 4) involving different fungal partners belonging to the same genus. In a similar study, Copetta *et al.* (2006) observed that, despite low colonisation levels, *Gigaspora margarita* can significantly alter the essential oil pattern in basil compared to equivalent NM plants. The poor ability of *G. caledonium* to colonise basil highly compared to *G. intraradices* or *G. mosseae* was observed consistently in the course of this project (Chapters 4 to 6). This was also consistent with the percent colonisation observed in pot cultures from which the AM inocula came from (colonisation levels in leek roots of approx. 40% for *G. caledonium*, 75% for *G. intraradices* and 60% for *G. mosseae*). These observations led to the hypothesis that the strain of *G. caledonium* used in my experiments is an "incompatible fungus" with basil, at least under the conditions used here. It was also hypothesised that *G. caledonium* might trigger the equivalent of a defence response in basil, as very few arbuscules

were formed (see Harrison (2005) and the discussion in Chapter 4). Such responses might explain the increase in shoot RA and CA concentrations obtained in the experiments described in Chapters 4 and 6. These hypotheses were further investigated while addressing the second question of this project.

The developmental stage of the AM symbiosis, as well as N uptake and phytohormone concentrations are not involved in the increase of phytochemical production in the shoots of basil

Chapter 5 aimed to elucidate how the developmental stage of different AMF affects the production of RA and CA in the shoots of basil. It was hypothesised that the onset of the colonisation might trigger the equivalent of a defence response in the host plant, which would be more pronounced in plants colonised by *G. caledonium* (as discussed above). However, under my experimental conditions, this hypothesis was ruled out by the results obtained. The results of Chapter 5 might have been confounded by the short duration of the experiments – *i.e.* a maximum of 5 days of colonisation might not have been long enough to induce the production of RA and CA. Unfortunately, there is almost no evidence on the production of RA in the shoots of basil following microbial elicitation in the roots. Furthermore, there is no information in the scientific literature to support whether or not RA can be transported from roots to shoots and the speed at which this transport might occur (M. Petersen personal communication).

Following these experiments, it was hypothesised that AMF might affect the production of antioxidants in basil through improved N uptake. However, the results of Chapter 6 suggested that high N supplies do not contribute to higher

shoot RA or CA concentrations. In fact, increasing N supply resulted in reduced growth and shoot RA concentrations in both AM and NM plants. It was then suggested that the reduced growth might have been the result of N toxicity, although this hypothesis remains unresolved. Furthermore, as AMF have been demonstrated to induce changes in phytohormone concentrations (Hause *et al.* 2007), IAA and ABA were also investigated for their potential involvement in the production of RA and CA in basil.

Under my experimental conditions, neither an increase in N uptake via AMF nor changes in IAA and ABA concentrations though mycorrhizal colonisation were shown to be correlated to increases in RA and CA concentrations in the shoots of basil. However, such results do not entirely rule out a possible role of other phytohormones in this process, as suggested in Chapter 6. Hence, no clear mechanism could be identified to explain the changes in antioxidant concentrations obtained between AM and NM plants in Chapters 4 and 6. Other factors such as light intensity and seasonal conditions have been proposed to influence the production of RA and CA in basil, both in the literature (Johnson *et al.* 1999, Keita *et al.* 2000, Javanmardi *et al.* 2002) and as suggested by the results obtained in this project.

Difficulties and challenges

One of the main difficulties arising when conducting experiments with basil is that it has not been well studied or characterised (*i.e.* compared to other crops such as barley, rice, tomato, wheat, or a model plant like *Arabidopsis thaliana*). Basil seeds do not germinate synchronously and uniformly, which made comparisons

between AM and NM plants quite difficult when trying to obtain plants with matched growth rates. Furthermore, as basil has an initial slow growth rate, it is more vulnerable to environmental conditions (light intensities, water-stress, disease, etc.). As emphasised several times, another major constraint in this project was the difficulty of growing basil in winter. Because basil is a Mediterranean plant, it requires high light intensities and warm temperatures to grow well. However, under the time constraints of the Ph.D., experiments had to be carried out all year-round, including in winter (Chapter 3). The semi-controlled conditions of the glasshouse were not sufficient to grow basil well in winter, especially under the AM symbiosis.

Generally speaking, the experimental designs described in each chapter were suitable to address the main objectives. However, in Chapter 5, the length of the experiments was insufficient to detect any mycorrhizal response in the production of RA and CA. This problem was difficult to circumvent as I wanted to relate the onset of the different developmental stages of the AM symbiosis to the production of antioxidants in the shoots of basil. Therefore, more thought ought to be given on how to get around this issue in future work.

Outcomes

Based on the results obtained from this project, it was shown that AMF can increase the production of RA and CA in the shoots of basil, but that this response can be quite variable (see Chapters 3 to 7). Therefore, from a practical and commercial point of view, one must be cautious about the “miraculous virtues” of AMF in improving the growth and phytochemical production in basil. The results

obtained here suggest that growing basil with AMF does not necessarily represent an advantage over growing it without AMF if the main concern is to obtain increased and standardised concentrations of phytochemicals (as is currently sought after in the herbal industry; see Section 1.2, Chapter 1).

On the other hand, the results also pointed toward some benefits of growing basil with AMF. The results of Chapter 3 strongly suggest that under low P supply, the AM symbiosis is almost "essential" in order for basil to grow. In addition, the results of Chapters 4, 5 and 6 indicate that AM plants can grow as well as – if not better than – NM plants under similar P supplies, and can even increase the concentrations of RA and CA. Thus, from a practical perspective, if a grower wishes to cultivate basil organically (considered "healthier"; see Section 1.1, Chapter 1), the AM symbiosis might be beneficial. AMF can help establishing basil seedlings under low P conditions, as well as contributing to reasonable yields of the harvested product (*i.e.* high shoot biomass and antioxidant concentrations).

Furthermore, the AM symbiosis was shown to confer a protective effect against *Fusarium oxysporum* f.sp. *basilici* (*Fob*), a pathogen specific to basil (Chapter 7). Under such experimental conditions, *G. mosseae* was shown to promote the growth of basil and to yield similar RA, CA and essential oil concentrations to NM plants with matched shoot P concentrations. Such results are of commercial (and economic) importance because *Fob* is the major disease limiting the production of basil worldwide (Gamliel *et al.* 1996). Moreover, the inoculation of basil with *G. mosseae* was shown to reduce the accumulation of a potentially harmful (to humans) essential oil, methyleugenol, in *Fob*-infected plants. In the

light of these results, and despite high variability in obtaining standardised phytochemical concentrations in AM basil plants, the many pros of inoculating basil with AMF seem to outweigh the cons.

Finally, there is a growing interest in the use of commercial AM inocula (Gianinazzi and Vosátka 2004), which are promoted as being a "natural choice" for fertilising horticultural plants such as basil. Such approaches are becoming increasingly popular as a way to reduce large inputs of inorganic fertilisers. However, as was briefly emphasised in Chapter 2 (see also Appendix 2), and with few exceptions (see Chapter 7), some commercial inocula are not reliable. The one tested in this project proved to be unsuitable for thorough scientific experiments, as the levels of AM colonisation were inconsistent and growth benefits quite poor compared to uninoculated plants. Thus, I would recommend caution when considering using such inocula for both scientific and commercial purposes. Inocula need to be tested prior to extensive application and quality-assured (with respect to infectivity, effectiveness and lack of pathogens).

Conclusions and future directions

Basil is a "newly investigated" plant in the field of mycorrhizal research. Therefore, many aspects of the work presented in this thesis had not been reported before and bring forth some interesting results; here are the main conclusions of this project:

- basil is highly responsive to P supply, but also to mycorrhizal colonisation when little P is available;

- when cultivated in winter, basil does not grow well and can be subjected to growth depression when mycorrhizal; however, when cultivated in summer, AM basil plants can grow as well, or better than, NM plants under similar P supply and P tissue concentrations;
- AMF can significantly increase the concentrations of RA and CA in the shoots of basil compared to NM plants when matched for shoot P concentrations;
- the mechanisms underlying such results remain unclear, although neither improved P nor N uptake were found to be the main drivers for the increase in phytochemical concentrations in basil;
- the changes in auxin (IAA) and abscisic acid (ABA) concentrations in AM compared to NM plants are not correlated to changes in RA or CA concentrations, nor are the early events of the developmental stages of the AM symbiosis;
- *G. mosseae* (BEG 12) confers a bioprotective effect against *Fob* and reduces the production of methyleugenol, an essential oil that is considered harmful to human health;
- the use of commercial AM inocula is questionable as they are not all reliable, although they might represent a cost-effective way of inoculating medicinal herbs for large-scale production (as opposed to producing the inoculum as described in Chapter 2).

There is an increasing interest in AM research on the systemic and localised (*e.g.* shoots) effects of the AM symbiosis in the production of secondary compounds in host plants of commercial importance (Kapoor *et al.* 2002a, b, Copetta *et al.* 2006,

Khaosaad *et al.* 2006, Copetta *et al.* 2007, Kapoor *et al.* 2007, Toussaint 2007). As highlighted by the results of this thesis, some of the gaps that need to be filled in future work in this area of research include the following:

- there is a need for further work to understand the mechanisms underlying the increase of phytochemicals in host plants (such as basil) when mycorrhizal;
- the role of phytohormones should not be entirely discarded regarding their influence on the production of RA and CA in the shoots of basil. Other phytohormones should be investigated for their involvement in such processes (*e.g.* cytokinin, gibberellin and jasmonic acid);
- environmental conditions such as light intensity and temperature ought to be controlled in order to examine (or eliminate) their influence on the production of phytochemicals in basil;
- it would be worthwhile to further explore the effect of the AM symbiosis on the production of phytochemicals in different varieties of basil, but also in other members of the Lamiaceae or other medicinal herbs/plants (see Section 1.5, Chapter 1 and Appendix 1);
- in the same line of thoughts, more AMF should be tested for their influence on phytochemical production in basil, as only a few species belonging to one genus were investigated in this study;
- field trials would allow assessment of the effect of AM colonisation on the growth of and phytochemical production in basil in a more practical and commercial approach. In order to achieve this, proper NM controls would have to be established in the field in which existing AMF would be eliminated;

- finally, in order to use commercial inocula for AM research or in horticulture, thorough testing and quality control should be achieved to assure the effectiveness of such inocula.

Appendix 1. Information on medicinal plants

As briefly discussed in Section 1.5 (Chapter 1), this section presents the information that was gathered when deciding which medicinal plant(s) to investigate for the purpose of this project. The plants described in this section are sage, thyme, St. John's Wort and echinacea. Table 1 found at the end of this appendix summarises the main information about each plant, including basil, the main plant of interest for this project.

Sage (Salvia officinalis L.)

"Cur moritur, qui salvia crescit in horto" – "Why should he die who has sage in his garden?" is a saying that was used to portray the importance of *Salvia fruticosa* L. (Gali-Muhtasib *et al.* 2000). The genus *Salvia* includes approximately 900 shrubs and herb species that range from tropical to temperate climates, many of them being endemic to the Mediterranean region (Mabberley 1989, Grieve 1995, Gali-Muhtasib *et al.* 2000). The name *Salvia* has Latin origins, having slight variations in its meanings according to different authors; Mabberley (1989) says it comes from "*salveo*" meaning "I heal", whereas Grieve (1995) and Miura *et al.* (2002) refer to "*salvare*" as "to be saved" or "to cure". Nevertheless, all meanings clearly relate to the reputation of sage as having healing properties in folk medicines (Grieve 1995, Lu and Foo 1999). As for the term "*officinalis*", it means "medicinal" (Miura *et al.* 2002). Sage has long been used in Ayurvedic and Traditional Chinese medicines (Perry *et al.* 1999), which attests to its wide use and recognition. It is principally the aerial parts of sage that are used for medicinal purposes, generally as leaf infusions or tea, which are used for the relief of abdominal pain, stomach aches and headaches, as well as other ailments (Grieve

1995, Gali-Muhtasib *et al.* 2000, Lu and Foo 2000). Sage is also known for its use as a spice for food flavouring, but also for its carminative[1] and soothing effects (Akhondzadeh *et al.* 2003).

The chemical composition of sage is quite complex, but the active phytochemicals are mainly phenolics that are believed to contribute significantly to the antioxidant property of this plant (Wang *et al.* 1998). As with many other species in the Lamiaceae, plants belonging to the genus *Salvia* contain rosmarinic acid (RA) (Hippolyte *et al.* 1992, Nascimento *et al.* 2000) which is considered to be one of the major components that gives sage its antioxidant activity, alongside carnosic acid and carnosol (Cuvelier *et al.* 1994, Lu and Foo 1999, Wang *et al.* 1999). It has been recently suggested by a number of authors that the chemical components of sage could have some choligernic[2] binding properties and therefore potentially provide a natural treatment for Alzheimer's disease (AD) (Perry *et al.* 1999, Akhondzadeh *et al.* 2003, Howes *et al.* 2003). As AD involves oxidative stress, the antioxidant activity of some plants, including sage, might have relevance in order to treat this disorder (Howes *et al.* 2003). As reported by Perry *et al.* (1999) and more recently by Howes *et al.* (2003), sage has historically been known as being good for the memory. The authors cite the 16th century best-known English herbal "*The Herball or Generall Historie of Plantes*" (1597) from John Gerard [1545 – 1612 (Sumner 2000)], who said about sage that "It is singularly good for the head and brain and quickeneth the nerves and memory". The potential of sage as a phytomedicine to treat AD might have an important impact on the

[1] A substance that dispels gas from the intestines (referring to herbs and spices that are rich in aromatic volatile oils)
[2] Released or activated by acetylcholine (a neurotransmitter used for memory and the control of sensory input signals) or a related compound

pharmaceutical market as the Food and Drug Administration-approved drugs that are currently available on the market for the management of AD show unsatisfactory outcomes (Akhondzadeh *et al.* 2003). With its many culinary uses and its medicinal popularity, sage shows great economic potential. Indeed, in Australia, sage is one of the crops considered for its current or potential commercial value as a culinary and medicinal herb (RIRDC 1997).

Thyme (Thymus vulgaris L.)

Thyme is an aromatic perennial herb that has been used since ancient times for medicinal purposes, as have many other members of the Lamiaceae (Echeverrigaray *et al.* 2001). The genus *Thymus* comprises approximately 350 species usually found in temperate climates (Mabberley 1989). The name of this herb originates from the Greek and would derive from the word "*thumus*" meaning "courage", as the qualities of the plant were believed to inspire courage in ancient and medieval times (Grieve 1995). Thyme was also used in religious practices, being burnt to purge and fumigate magical rooms and spaces, as well as to bring good health (Waltz 1996). Thyme also shows strong antioxidant activity (Kahkonen *et al.* 1999, Dragland *et al.* 2003), as some flavones were isolated and reported to be antioxidants (Miura and Nakatani 1989, Zheng and Wang 2001). The essential oils of thyme, which represent 0.7 – 6.5% of the dry weight of leaves and flowers (Echeverrigaray *et al.* 2001), are known to have antioxidant, antibacterial, antispasmodic, antiseptic and antimicrobial activities (Grieve 1995, Essawi and Srour 2000, Zheng and Wang 2001). Thymol and carvacrol are generally recognized as the major components that show such properties (Zheng and Wang 2001, Badi *et al.* 2004). Light intensity and soil water availability have

been shown to influence the oil concentration of thyme; plants grown under supplemented light showed higher accumulation of oil in the leaves compared with those in natural light when the soil water was 70% of field capacity (Letchamo *et al.* 1995). Letchamo and Gosselin (1996) further suggested that the number of oil glands along with a simultaneous increase in light regime and soil water content might lead to increases in essential oil concentrations. Harvesting season might also influence oil concentrations in thyme (Badi *et al.* 2004), as it was reported that seasonal variations affect antioxidant concentrations in thyme (Dragland *et al.* 2003).

Thyme is of increasing economic importance as a culinary herb and medicinal plant in a number of countries in North America, Europe and North Africa (Badi *et al.* 2004), as well as Australia (RIRDC 1997). Thyme oil has been reported to be among the top ten essential oils for its many properties, ranging from culinary to medicinal (Letchamo *et al.* 1995). Due to the manifest interest toward natural herb products and considering the economic importance of thyme, it has been suggested that proper management programs could play a central role to provide a competitive alternative for growers (Letchamo *et al.* 1995).

St. John's Wort (Hypericum perforatum L.)

This well-known medicinal plant is named after St. John the Baptist, as its yellow flowers blossom on the days surrounding his feast day on June 24, at least in Europe (O'Hara *et al.* 1998, Gaster and Holroyd 2000). The term "wort" comes from the Old English, literally meaning "plant" (Gaster and Holroyd 2000) and the scientific name of St. John's Wort derives from the Greek *hyper* and *eikon*,

meaning "to overcome an apparition", relating to ancient beliefs that the plant could protect against evil spirits (O'Hara *et al.* 1998). *H. perforatum* is a member of the family Clusiaceae (Hypericaceae) (Collins *et al.* 1990) and is indigenous to Europe and Western Asia (Ganzera *et al.* 2002) but now grows wild in much of the world (O'Hara *et al.* 1998). Although considered a weed in 20 countries, including Australia and the United States (O'Hara *et al.* 1998, Mayo and Langridge 2003), this perennial herb has been widely used as a medicine since ancient times. Its above-ground parts (leaves and flowers) have been mainly used as medicine (O'Hara *et al.* 1998) for at least 2000 years (Saskatchewan Agriculture 2000). St. John's Wort is highly valued in Europe (Israelsen 1993), especially in Germany where it has been extensively used as an antidepressant since the late 1980s (Gaster and Holroyd 2000, von Eggelkraut-Gottanka *et al.* 2002). In the United States, this herb was considered one of the fastest growing in terms of sales in the late 1990s with increases up to 2800% in one year (Ernst 2000).

H. perforatum extracts have been used for a long time to cure insomnia and nervous conditions (Gaster and Holroyd 2000), and its red pigment is now also extracted commercially and used for dying wool and silk, as well as having some use in cosmetics (Saskatchewan Agriculture 2000). Today, St. John's Wort is widely used to treat mild to moderate depression (De Smet and Nolen 1996, Linde *et al.* 1996, O'Hara *et al.* 1998, Ganzera *et al.* 2002). In a meta-analysis[3], Linde *et al.* (1996) concluded that *H. perforatum* extracts are more effective than placebo. Moreover, not only are *H. perforatum* extracts believed to be as effective as

[3] Overview in which quantitative methods are used to summarize the results of several studies on a single topic; used in an attempt to gain greater objectivity on a specific topic.

conventional drugs such as tricyclic antidepressants, but they also seem to show significantly fewer side effects (Linde *et al.* 1996, Ernst 1998, O'Hara *et al.* 1998, Gaster and Holroyd 2000). The main active ingredient has long been thought to be hypericin, but it was demonstrated in recent years that many substances in St. John's Wort have biological activity such as hyperforin, pseudohypericin and quercetin, among others (Hansen *et al.* 1999, Gaster and Holroyd 2000, von Eggelkraut-Gottanka *et al.* 2002). Hypericin and pseudohypericin are known to be potent antiviral substances (Ganzera *et al.* 2002). Although it was assumed that extracts of *H. perforatum* inhibited monoamine oxidase (MAO) activity (O'Hara *et al.* 1998, Ernst 2000), a brain enzyme that denaturises neurotransmitters (monoamines such as serotonin, dopamine and norepinephrine[4]) associated with depressive symptoms, this could not be confirmed in recent studies (De Smet and Nolen 1996, Ganzera *et al.* 2002). Therefore, the mechanisms underlying the action of St. John's Wort are still only partially understood (von Eggelkraut-Gottanka *et al.* 2002). Other than antidepressant activities, the extracts of *H. perforatum* have been investigated for their use as antibacterial, anticancer, and antiviral substances (Kireeva *et al.* 1998, O'Hara *et al.* 1998, Ganzera *et al.* 2002). According to a number of authors (O'Hara *et al.* 1998, Ernst 2000, Gaster and Holroyd 2000), the increasing popularity of St. John's Wort might be attributed to its low adverse effects and is probably further strengthened by its low cost compared to standard drugs. However, no conclusive cost evaluation studies are available to that regard (Ernst 2003).

[4] When MAO is active and when these neurotransmitters are in low concentrations, depression symptoms are observed.

Kireeva *et al.* (1998) demonstrated that high hypericin concentrations in *H. perforatum* were observed at seed formation. It was also reported that the crude drug *Hyperici herba* consists of the aerial parts of St. John's Wort collected just prior or during blossom (von Eggelkraut-Gottanka *et al.* 2002). Therefore, harvest times might have to be taken into consideration in order to obtain optimum concentrations of the phytochemicals found in St. John's Wort.

Echinacea (Echinacea purpurea L.)

Echinacea is a herbaceous perennial plant member of the family Asteraceae (Compositae), that is native to western and south-western USA (Great Plains) as well as south-eastern Saskatchewan (Prairies), Canada (Porter 2000). It is well-known to the Aboriginal people of North America who have collected it for centuries to treat various illness such as colds, burns, snakebites and even cancer (O'Hara *et al.* 1998, Sumner 2000). Traditionally, the First Nations groups of the Great Plains regions mostly used the roots of *E. angustifolia*, but the flowering tops are also a source of medicinal compounds (Binns *et al.* 2002a). Today, Echinacea is being recognized for its immunostimulant[5] properties (Wagner 1995) in treating colds and flu, as well as for its antiseptic, antiviral and anti-microbial properties (Ernst 1998, Porter 2000). It is widely popular in Western Europe and North America as a natural product (Israelsen 1993, Sumner 2000) and is considered to be on the top ten herbs used in USA and Germany (RIRDC 1997). As reported by Brevoort (1996), a survey conducted by Whole Foods magazine classified echinacea as one of the top-selling herbal products in North America, representing 9.9% of herb sales, which in 1998 was estimated to generate more

[5] Defined as a drug capable of stimulating the function and efficiency of a non-specific immune system to counteract microbial infections or immunosuppressive states.

than $300 million in the USA (O'Hara *et al.* 1998). Echinacea is also marketed in Australia, where it was considered to be one of the largest selling herbs in 1997 (RIRDC 1997).

There are nine species in the genus *Echinacea* (Mabberley 1989), but the ones that are mainly used for their medicinal purposes are *E. purpurea*, *E. angustifolia* and *E. pallida* (Sumner 2000), *E. purpurea* being the most commonly used and extensively studied (O'Hara *et al.* 1998). Recent research has suggested that *E. angustifolia* should be considered a variety of *E. pallida*, *E. pallida* var. *angustifolia* (Porter 2000, Binns *et al.* 2002b). *Echinacea* spp. contain an array of phytochemicals, including caffeic acid (CA) derivatives such as chicoric acid, known to have *in vitro* and *in vivo* immunostimulatory properties. It also contains echinacoside, which is generally used as a marker for *Echinacea* preparations by the herbal industry (Bergeron *et al.* 2000). Other compounds include cynarin (another CA derivative), alkamides, ketoalkynes and ketoalkenes (present in the essential oil), and also polyacetylenes (Mazza and Cottrell 1999, WHO 2004). Usually, the products sold on the market contain a mixture of echinacea species, which might confer varying medicinal properties and potency as stated by Sumner (2000). This partly justifies the need for better quality control of echinacea products, as acknowledged by Bergeron *et al.* (2000). Furthermore, as the popularity of echinacea increased in the last decades, so has the loss of native echinacea species (Sumner 2000). As the demand for *E. angustifolia* has much exceeded the capacity for sustainable wild root harvesting, successful cultivation of all species of the genus is crucial (Binns *et al.* 2002a).

Table 1. Information about the medicinal plants reviewed

Plant	**Sweet Basil** (*O. basilicum* L.) Family: Lamiaceae	**Sage** (*S. officinalis* L.) Family: Lamiaceae	**Thyme** (*T. vulgaris* L.) Family: Lamiaceae
Uses	Antioxidant, antispasmodic, antibacterial, relief of headaches, coughs, carminative, aromatic, culinary uses	Antioxidant, highly antiseptic, treatment of Alzheimer's disease, relief of itching, sore throats, astringent, tonic, carminative, culinary uses.	Antioxidant, antibacterial, antispasmodic, antiseptic, culinary uses
Parts used	Leaves, flowering buds	Leaves, flowering buds	Leaves, flowering buds
Cultivation	Prefers light, loamy alkaline and well-drained soils, intolerant to water stress; slow initial growth, but increases rapidly after the 1st few leaves	Well drained soils; nitrogen rich soils; clay, pH 4.9 – 8.2; full sunlight; 14 –21 days germination; susceptible to root rot and fungal diseases.	Dry and well drained calcareous soils; pH 4.5 – 8.0
Chemicals	RA, CA, anthocyanins, essential oils: eugenol, linalool; free phenolic acids, flavonoids	RA, CA, Carnosic acid, essential oils, free phenolic acids, tannins, flavonoids	Essential oils: carvacrol, thymol, methylated flavones, mono - sesquiterpenes
Mycorrhizal	Yes *G. mosseae*; *Gigaspora margarita*; *Gi. rosea* (Camprubi *et al.* 1990, Giovannetti *et al.* 1996, Dickson 2004, Copetta *et al.* 2006)	Yes *G. mosseae*; (Camprubi *et al.* 1990)	Yes *G. mosseae*; (Camprubi *et al.* 1990)
Assay[6]	HPLC; steam distillation (oils)	HPLC; steam distillation (oils)	HPLC; steam distillation (oils)

[6] Most assays are well summarized in **Cimpan G, Gocan S. 2002.** Analysis of medicinal plants by HPLC: Recent approaches. *Journal of Liquid Chromatography & Related Technologies*. **25:** 2225-2292.

Table 1. Information about the medicinal plants reviewed (continued)

Plant	**St. John's Wort** (*H. perforatum* L.) Family: Clusiaceae (Hypericaceae)	**Echinacea** (*E. purpurea* L.) Family: Asteraceae
Uses	Treatment of depression problems, antiseptic, antibacterial, diuretic, analgesic, antispasmodic, astringent, expectorant	Increase immune system, antiseptic, antimicrobial, antifungal, anticancer, antitumor
Parts used	Aerial parts, flowers	Roots, flower buds
Cultivation	Prefers light and chalky soils; well-drained soils; pH 5.5 [7]; 1:2 steam-sterilized horticultural peat: fine sand	Prefers clay-type soils; full sunlight; good drainage; pH 5.5 –7.5, seeds slow to germ., need cold stress; prone to diseases[8]
Chemicals	Hypericin, hyperforin, glycosides, tannins, volatile oils	CA derivatives: chicoric acid, echinacoside, cynarin, polyacetylenes, volatile oils
Mycorrhizal	Yes Fungi? (Moora and Zobel 1998)	Yes G. *mosseae*; *Scutellospora. fulgida* (Lata *et al.* 2003)
Assay	HPLC	HPLC

Conclusions

The plants described in this appendix were not chosen for investigation for a few reasons. First, it proved to be too optimistic to investigate all of these plants under the time constrains of this Ph.D. As it was also time-consuming to find suitable growth conditions for basil (Chapter 3), it would have been rather difficult to do the same for all of the plants presented here. Basil was preferred as the principal plant of investigation because 1) it is of economic importance worldwide (as the project started with a commercial incentive); 2) the assay for its phytochemical

[7] **Moora M, Zobel M. 1998.** Can arbuscular mycorrhiza change the effect of root competition between conspecific plants of different ages. *Canadian Journal of Botany*. **76:** 613-619.
[8] Dr. Shanon Binns' personal communications (University of British Colombia)

determination (RA and CA) was already well established and quite simple, requiring relatively few steps, contrary to other assays for the other plants of interest. Furthermore, the main phytochemicals (essential oils) of sage and thyme required technical equipment that was not easily available. Nevertheless, it would be interesting to study the influence of the AM symbiosis on the production of phytochemicals in the plants presented in this appendix as this has been poorly, or simply not investigated.

Appendix 2. Testing of a commercial inoculum – Myco-Gro

The experiment described in this chapter was jointly conducted with fellow Ph.D. candidate Mr. Ashley Martin, who also wanted to investigate the effectiveness of the commercial inoculum "Myco-Gro". Mr Martin presented the outcome of this experiment in his thesis which was submitted in May 2007. Myco-Gro is a commercial inoculum that was provided to Mr. Martin and I by Mr Sandy Montague (New-Edge Microbials Pty. Ltd, Albury, Australia). The inoculum consisted of spores and "propagules" of the AM fungus *G. intraradices* and was available in bags of 0.5 g or 15 kg ($100/kg).

Aims

This experiment aimed at testing the efficacy of the commercial inoculum Myco-Gro, as an initial experiment failed at doing so (Section 2.2, Chapter 2). The failed attempt could have been the result of a low number of spores/propagules in the batch of inoculum (100 spores and propagules per g inoculum). Therefore, Mr Montague kindly agreed to provide Mr. Martin and myself with a new batch of inoculum containing a higher number of spores/propagules per unit weight (claimed to be 300 spores/propagules per g inoculum).

Materials and methods

As Mr. Martin wanted to test the inoculum on medic (*Medicago truncatula* L.) and as my project focused on basil (*O. basilicum*), we decided to use both species as "trap plants". The experiment consisted of three inoculation treatments (two AM and one NM) with three replicates per treatment, all set up in a complete randomised design.

Seeds of medic and basil were sterilised and germinated in 700 g pots containing a mixture of soil/sand (1:9, w/w) as described in Section 2.4. No P was added to the medium. Two rates of application of Myco-Gro were tested. The first was as recommended by the manufacturer, *i.e.* approx. 0.2 g Myco-Gro/pot, which corresponded to ~60 spores/pot. As this seemed quite a low number of spores, the second rate of application was ten times the recommended one. The inoculum was applied as a layer on top of the growth medium and then covered with more of the medium prior sowing the seeds (as recommended by the manufacturer). A control treatment was also set up which consisted of no inoculum added at all. One seedling of each species was planted per pot. The plants were grown in a glasshouse during summer as described in Section 2.4. The plants were watered three times per week and received 4 ml of a modified Long Ashton solution once a week as described in Section 2.3. Core samples were taken from the different treatments after 5 wks to assess for mycorrhizal colonisation. After 10 wks, both medic and basil were harvested and pooled together and the following parameters were recorded: shoot and root fresh and dry weights as well as percentage colonisation, as described in Chapter 2. At harvest, the roots were not separated, and therefore the percent colonisation was not specifically distinguished for both medic and basil individually.

Results

The levels of colonisation were quite low and variable in the core samples at 5 wks (Fig. A1). The control treatments were slightly colonised. Although plants treated with the regular application rate of Myco-Gro appeared to have higher colonisation than the ones treated with ten times that rate, this was not significant

(Fig. A1). One replicate of the regular rate of application had an exceptionally high percentage colonisation (63%), which might have been due to a manipulation error when adding the inoculum to the growth medium.

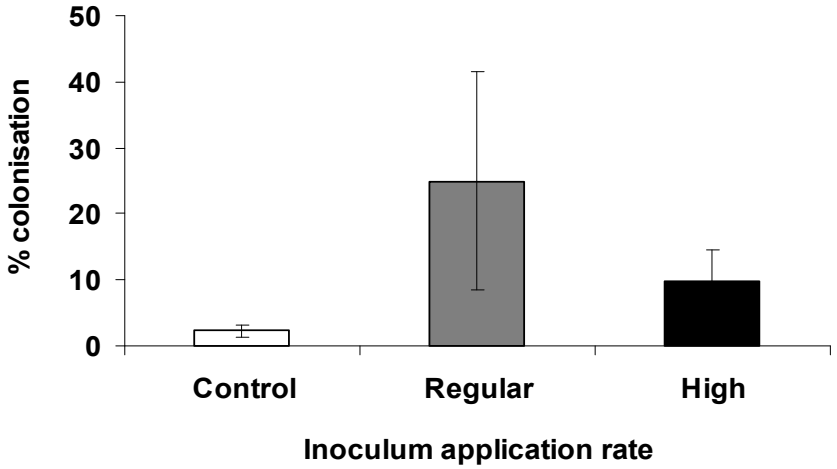

Figure A1. Percent colonisation at 5wks in *M. truncatula* and *O. basilicum* (pooled) grown with three different application rates of Myco-Gro. The plants were NM (Control; white bars) or received either the recommended application rate (Regular; dark grey bars) or ten times that rate (High; black bars). Means (n=3) and SE bars are shown. There were no significant differences between treatments.

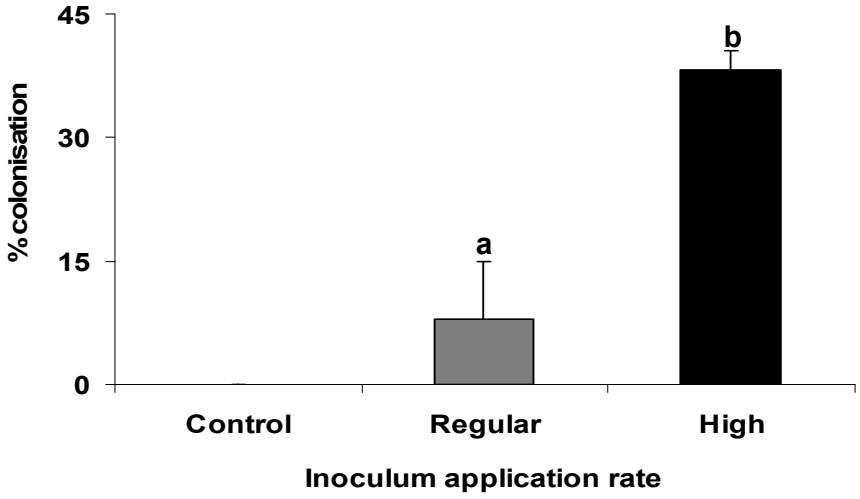

Figure A2. Percent colonisation at 10 wks in *M. truncatula* and *O. basilicum* (pooled) grown with three different application rates of Myco-Gro. The plants were NM (Control; white bars) or received either the recommended application rate (Regular; dark grey bars) or ten times that rate (High; black bars). Means (n=3) and SE bars are shown. Control plants were not colonised at all; different letters indicate significant differences according to Tukey's test (p=0.05).

At harvest, at 10 wks, the control plants did not show any sign of colonisation at all (Fig. A2). AM plants grown with the recommended rate of application were colonised, but significantly (p=0.003) less than those having received ten times the recommended rate of Myco-Gro. NM control plants grew significantly less well than the ones treated with both application rates of Myco-Gro (Fig. A3). At either rate of application, plants grown with Myco-Gro grew equally well.

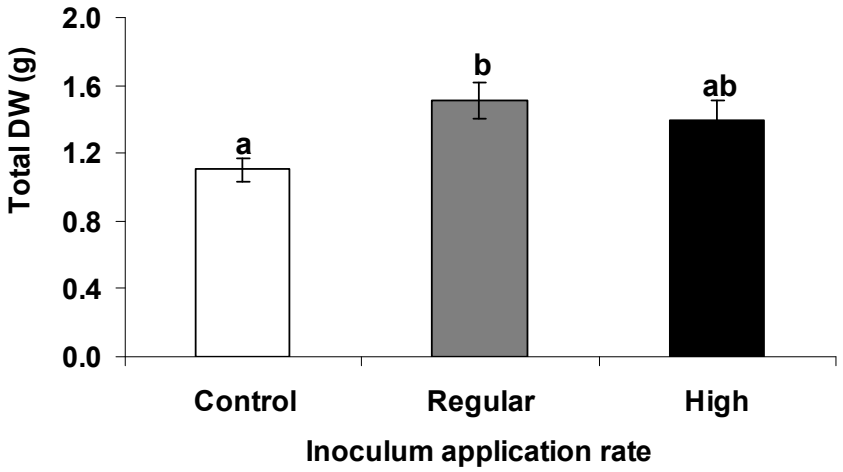

Figure A3. Total plant dry weight (DW, g) of *M. truncatula* and *O. basilicum* (pooled) grown with three different application rates of Myco-Gro. The plants were NM (Control; white bars) or received either the recommended application rate (Regular; dark grey bars) or ten times that rate (High; black bars). Means (n=3) and SE bars are shown. Different letters indicate significant differences according to Tukey's test (p=0.05).

Discussion

Despite the fact that the roots of basil and medic eventually got colonised when using Myco-Gro, the inoculum did not have much effect on the growth response of the plants. The levels of colonisation were quite variable, especially at 5 wks. Surprisingly, even when using ten times the recommended rate of application of Myco-Gro, there were no significant growth responses in the host plants, despite their much higher rate of colonisation compared to the ones treated with a

recommended rate. As a result of the poor mycorrhizal response, low colonisation levels at high rates of inoculum application, and considering the poor colonisation response obtained in a previous experiment using Myco-Gro (see Chapter 2), it was decided not to use this inoculum for the purpose of this project.

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