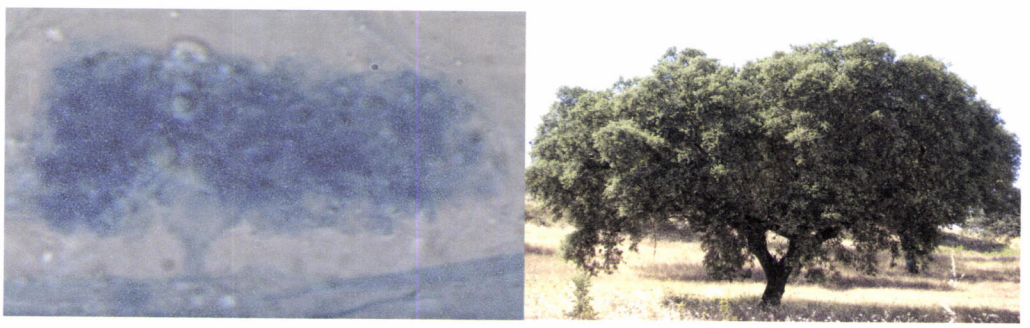
UNIVERSIDADE DE ÉVORA

Potential Value of Arbuscular Mycorrhiza in the Agricultural Systems of Alentejo Region – Portugal



Thesis submitted for the degree of Doctor in Biology by:

Isabel Maria de Oliveira Brito

Supervisor: Professor Michael J. Goss
Co-Supervisor: Professor Mário de Carvalho

Esta tese não inclui as críticas e sugestões feitas pelo júri.

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To Pedro, Gonçalo and Marta with love.

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Abstract

The Alentejo region, located in the south of Portugal and occupying a third of the continental part of the country, is the region with the greatest potential for small grain production. It produces more than 80% of the total national wheat harvest, the main small grain cereal crop, despite small average yields per unit land area. The restrictions to yield are largely imposed by its Mediterranean climate, with dry and hot summers and cold and wet winters. Soil limitations can also constrain yield. Development of biomass up to the flowering stage is critical for achieving good grain yields in wheat. Consequently, agronomic management practices, including appropriately designed crop rotations, focused on achieving good early crop development, together with low input systems based on no-till, and a rational use of nutrients, can contribute to increased productivity in the region. A possible mean of improving the use of nutrient resources is through the improved management of naturally occurring symbioses like arbuscular mycorrhiza.

Arbuscular mycorrhiza (AM), a symbiosis between the roots of a wide range of plants and obligatory symbiotic fungi, is commonly mutualistic, the long-term compatible interactions being based on a bidirectional nutrient transfer between symbionts. Fungal symbionts benefit from the photosynthates produced by the plants, while the latter gain because of the increased volume of soil that can be explored by the extraradical fungal mycelium. This enhanced soil exploration is one of the most obvious advantages of mycorrhizal formation and results in the ability to take up more nutrients, particularly those that have limited mobility in soil, such as phosphorus. In addition other benefits associated with AM plants are: alleviation of water stress, protection from root pathogens, tolerance to toxic metals and the stabilization of soil aggregates promoted by extraradical hyphae, globally conferring to AM a great potential to contribute to sustainable crop production systems.

Considering the impracticality of inoculating soil on a large scale, the objectives were focused on the potential value of AM under Mediterranean conditions and how they could be managed within the agricultural system of

Alentejo region, assuming that if there is adequate AM colonisation, benefits would directly or indirectly, sooner or later, accrue to the crop.

In a field experiment preformed to study the effect of soil disturbance (no-till and conventional tillage systems) on AM colonisation of wheat over the vegetation cycle the results indicated that mycorrhizal formation in wheat and AM fugal spore numbers were greater in no-till than under a conventional system, despite the high level of soil available phosphorus. Results also showed that under field conditions, AM colonisation of winter wheat increased gradually until late spring and then declined, following root development.

Once AM colonisation of wheat under Mediterranean conditions was confirmed at the field and that there was better colonisation under no-till systems, where the extraradical mycelium was kept intact, the next objective was to determine the ability of this mycelium to remain infective for the next crop. The maintenance of a high colonisation potential under the Mediterranean summer conditions is essential because it likely determines the time frame (from one cropping year to the other) and the possibilities (faster or slower colonisation) for AM to influence early crop development. Extraradical mycelium of native AM fungi (AMF) survived the dry and hot summer season and started new colonisations at the onset of the growing season, especially when it was not adversely affected by soil disturbance.

The proper management of weed populations that emerge after the first autumn rains might provide a valuable tool, functioning as a bridge, to ensure a quick and efficient AM colonisation of wheat young seedlings and help to assure a good early development known to be critical for successful wheat crop in Alentejo region. In a series of pot experiments using weed species chosen according to their relevance in regional agronomy, it was confirmed that allowing weeds to growth until extraradical mycelium is well established (one month) and then using herbicide to control them, preserves the extraradical mycelium network and greatly benefits initial colonisation of the crop and permits early nutrient acquisition.

Considering there are pronounced plant x fungus interactions, a more diverse AM fungal community offers more possibilities for functional AM symbioses to establish. A study comparing AM fungal diversity in the soil under

no-till and conventional tillage system, using nested PCR, indicated that conventional tillage reduces the diversity (number of ribotypes) and influence the community structure (frequency of each ribotype) of indigenous AM fungi.

In the context of Mediterranean agriculture there is potential value for AM, and it could and should be considered in the management of the agro-ecosystem given that the transfer of inoculum from one crop to the following one in the next cropping season is now established. The transfer can occur through the summer survival of extraradical mycelium, and from crop to weeds to a following crop through mycelia facilitative effects.

The temperature during the period of sowing the wheat crop (November) is too cool for the process of spore germination to be fast. This makes colonisation from spores too slow for effective AM colonisation of wheat after seeding, which is predominantly dependent on the presence of active extraradical mycelium.

Knowing that extraradical mycelium can survive over the Mediterranean summer means that the use of no-till or direct drilling systems that support a greater AMF diversity and maintain the extraradical mycelium intact, together with the choice of the crops to include in rotations, provides opportunities for agro-ecosystem management to benefit from mycorrhiza. Additionally, given that weed-mediated AM transfer is more efficient than the transfer from one crop to the following cropping season, an appropriate approach to weed management can strongly benefit the initial colonisation of the crop.

As wheat can actually take advantages of AM colonisation and knowing that under Mediterranean conditions the initial development of the crop is fundamental to ensuring sufficient biomass of the crop at flowering, there is unquestionable potential value in exploiting AM under properly managed agricultural systems in Alentejo region. Management practices like no-till, crop rotation and intelligent weed control clearly have the possibility to encourage arbuscular mycorrhiza.

Resumo alargado

A região do Alentejo situa-se no Sul de Portugal e corresponde a cerca de um terço do território continental. Por uma série de razões como sejam a situação geográfica o relevo e a dimensão da propriedade, é a região do país que apresenta maior potencial para a produção cerealífera, contribuindo com cerca de 80% da produção nacional de trigo, até ao momento o principal cereal produzido em Portugal. No entanto, devido em grande medida a limitações edafoclimáticas, as produtividades obtidas são relativamente baixas quando comparadas com outros países europeus.

O clima Mediterrânico, caracterizado por Verões quentes e secos e Invernos frios e chuvosos, obriga à utilização de variedades de ciclo relativamente curtos, para além disso a distribuição de precipitação é extremamente irregular provocando grandes oscilações de produção de ano para ano. Nestas condições, e para os cereais de Inverno no geral, a biomassa acumulada até à floração é determinante para a garantia de um bom enchimento do grão, por isso o crescimento inicial da cultura pode ser decisivo para alcançar uma boa produtividade.

Os solos são na sua maioria relativamente pobres. Derivados de xistos ou granitos, apresentando baixos teores de matéria orgânica, pH ligeiramente ácido e média capacidade de troca catiónica.

A adesão de Portugal à Comunidade Europeia em 1986, e a obrigatoriedade em seguir as directivas da Politica Agrícola Comum (PAC) cada vez mais baseadas na redução de subvenções aos agricultores, exigiu o repensar do sistema agrícola da região. A procura de sistemas de produção mais sustentáveis do ponto de vista ambiental e financeiro tornou-se obrigatória para a sobrevivência do sector agrícola. Assim, a aplicação de fundos estruturais no desenvolvimento e aquisição de novos meios de produção, o recurso a sistemas mais extensivos, o aumento da mecanização e o uso mais racional de factores de produção, têm vindo a operar uma mudança significativa no sistema agrícola do Alentejo. Exemplo disso mesmo é o recurso

cada vez mais generalizado à técnica de sementeira directa, actualmente praticada em várias culturas (trigo, milho, girassol) em mais de 50 000 ha.

Sistemas de baixo input baseados na sementeira directa, associados a um correcto delineamento da rotação de culturas e ao uso racional dos nutrientes são, em termos práticos, potenciais geradores de aumentos da produtividade. Neste contexto o recurso a simbioses que ocorrem de forma natural, como as micorrizas arbusculares, pode constituir uma importante via para a exploração mais adequada dos nutrientes do solo.

As micorrizas arbusculares (AM) são simbioses entre a raiz de uma grande diversidade de plantas e fungos do solo que são simbiontes obrigatórios, os fungos micorrízicos arbusculares (AMF). Esta associação simbiótica é normalmente mutualista, baseando-se na troca bidireccional de nutrientes. O fungo recebe da planta os produtos resultantes da actividade fotossintética e a planta, por sua vez, beneficia de uma série de vantagens decorrentes da exploração de um maior volume de solo proporcionado pelo micélio extraradical que se desenvolve, sendo a absorção de nutrientes, nomeadamente os menos móveis como é o caso do fósforo, a mais óbvia. Contudo, para além desta, outras vantagens são atribuídas às AM: alívio do stress hídrico, protecção contra agentes patogénicos, tolerância a metais pesados e estabilização dos agregados do solo, mostrando assim um enorme potencial no contributo para a produção agrícola sustentada.

Pretendeu-se com o presente trabalho compreender o potencial associado às AM, em situação de clima Mediterrânico, no contexto do sistema agrícola Alentejano, no sentido de através de práticas agrícolas adequadas valorizar os benefícios proporcionados pela micorrização, numa óptica de sustentabilidade do sistema agrícola. Assumiu-se que uma vez estabelecida a micorrização, os benefícios daí decorrentes, directa ou indirectamente, mais cedo ou mais tarde, recairiam sobre a cultura. Assim, no sentido de desenvolver conhecimentos que elucidassem sobre a ecologia funcional das micorrizas arbusculares no sistema agrícola em apreço, adoptou-se uma abordagem multidisciplinar de modo a que a integração e análise da informação a diferentes escalas pudesse ser feita.

Os ensaios de campo decorreram nos anos agrícolas de 1999/2000 e 2000/2001 na Herdade da Revilheira, próximo de Reguengos, pertencente à Direcção Regional da Agricultura do Alentejo. Estes ensaios integraram-se num programa de investigação agrícola mais abrangente que vem a decorrer na Herdade desde 1995, envolvendo estudos de mobilização de solo e rotação de culturas, entre outros. Com estes ensaios pretendeu-se avaliar o efeito da mobilização do solo, mais especificamente a sementeira directa e mobilização tradicional (lavoura com charrua e grade de discos) na colonização micorrízica do trigo, a principal cultura da rotação, ao longo do ciclo vegetativo da planta assim como o número de esporos de AMF. Os resultados obtidos mostraram que, apesar do elevado teor do solo em fósforo, no sistema de sementeira directa a taxa de colonização micorrízica do trigo assim como o número de esporos isolados foram substancialmente maiores do que os observados no sistema de mobilização tradicional do solo. A colonização micorrízica do trigo aumentou gradualmente até à Primavera e a partir daí decresceu, acompanhando o padrão do desenvolvimento radicular da cultura. Esta tendência foi observada em ambos os sistemas de mobilização do solo.

Uma vez confirmada a colonização micorrízica do trigo em situação de campo e atendendo a que no sistema de sementeira directa, onde o micélio extraradical do fungo é mantido intacto, se observaram melhores taxas de colonização micorrízica, importava saber a capacidade de sobrevivência deste micélio ao Verão quente e seco tipicamente Mediterrânico, mantendo a sua capacidade de gerar novas e mais rápidas colonizações no ano agrícola seguinte, influenciando de forma positiva o desejado crescimento inicial da cultura e assim constituir um factor a ter em conta na definição das operações culturais de uma exploração agrícola.

Para o efeito adaptou-se para o trigo uma técnica de estudo em ensaios em vasos previamente desenvolvido para milho como planta hospedeira. Resumidamente a técnica consiste em fazer crescer durante 3 semanas a cultura pretendida, após este período a parte aérea da planta é cortada e, apenas em metade dos vasos, o solo é retirado, crivado (4mm) e colocado novamente no vaso. Todos os vasos foram novamente semeados, dando-se início a um novo ciclo de 3 semanas de cultura, findas as quais, a operação se

repete nos mesmo vasos. Ao longo dos ciclos sucessivos acaba por se induzir uma diferença estrutural e de potencial de inóculo micorrízico entre os dois tratamentos do solo. Esta diferença é, em muitos aspectos, semelhante à operada em condições de campo pela sementeira directa e pela mobilização tradicional, embora se processe de forma mais acelerada. O uso da técnica dos ciclos de perturbação do solo depois de aferida para o trigo, associada a vasos de barro enterrados no campo permitiu o desenvolvimento do estudo da sobrevivência do micélio extradicular durante o período estival.

Verificou-se que o micélio extraradical e AMF sobreviveu ao Verão quente e seco da região, tendo mostrado capacidade de iniciar novas colonizações de AM no início do ano agrícola, especialmente se não for afectado pela perturbação do solo.

As infestantes germinadas após as primeiras chuvas outonais podem constituir um importante veículo para o rápido estabelecimento da colonização micorrízica na fase inicial da cultura do trigo uma vez que o micélio extraradical que lhes está associado funciona como uma interessante fonte de inóculo, estabelecendo-se uma ponte de micélio entre as infestantes e o trigo, particularmente no contexto da sementeira directa.

Esta hipótese foi investigada através de um ensaio em vasos, tendo-se seleccionado para o estudo algumas das infestantes comuns da região e várias formas (químicas e mecânica) para o seu controlo. Os resultados obtidos permitiram confirmar que de facto o crescimento prévio de infestantes, durante um mês, foi facilitador da colonização AM do trigo, para além de que o método utilizado para o seu controle foi determinante nesse efeito. Assim, quando as infestantes foram controladas com herbicida, nomeadamente glifosato, e portanto o micélio extraradical mantido intacto, a colonização micorrízica inicial do trigo foi maior e com efeitos positivos na aquisição de nutrientes, ao passo que quando as infestantes foram controladas através da perturbação do solo e a rede de micélio extraradical destruída, a colonização micorrízica observada foi menor.

A diversidade funcional dos AMF é bastante considerável, mesmo a nível intraespecífico. Para além de se verificar um marcado efeito planta X fungo, estão descritos efeitos sinérgicos e de cooperação entre espécies de

AMF que ocorrem simultaneamente no mesmo sistema radicular. Desta forma, em termos práticos, a diversidade dos AMF pode ter implicações importantes uma vez que a combinação planta x fungo pode ser decisiva para o efeito da simbiose num dado momento. De acordo com as condições ambientais prevalentes, as possibilidades de combinações fungo x hospedeiro bem sucedidas são seguramente maiores numa comunidade de AMF mais diversificada.

A mobilização do solo, ao provocar a ruptura do micélio extraradical e a inversão de horizontes do solo vai interferir de forma selectiva com os diferentes AMF em função as suas estratégias de vida e colonização. No sentido de avaliar os efeitos da sementeira directa e mobilização tradicional na diversidade de AMF no local de estudo, realizaram-se algumas experiências. As ferramentas moleculares actualmente existentes permitem que este tipo de estudo seja feito directamente a partir de DNA do solo o que constitui uma vantagem importante pois assim todas as formas de inóculo (esporos, micélio e fragmentos de raiz colonizados) podem estar representadas na amostra e ser avaliadas simultaneamente. Optou-se pelo uso da técnica de *nested* PCR (reacção em cadeia da polimerase) seguida de sequenciação e identificação de tipos ribosomais. Os resultados indicam que a mobilização tradicional do solo provoca a redução da diversidade de AMF do local (número de tipos ribosomais), para além de influenciar a estrutura da comunidade (frequência de cada tipo ribosomal).

O potencial dos efeitos associados às micorrizas arbusculares pode e deve ser considerado no contexto da prática agrícola em situação de clima Mediterrânico atendendo a que se observou transferência de inóculo de uma cultura para a seguinte na época de sementeira subsequente. Esta transferência processou-se quer através da sobrevivência de Verão do micélio extraradical, quer a partir das infestantes germinadas antes da sementeira da cultura, pelo estabelecimento de pontes de micélio.

A perturbação do solo associada aos sistemas de mobilização provoca a ruptura da rede de micélio extraradical fazendo com que a colonização micorrízica de novas culturas dependa de forma mais acentuada da germinação de esporos. Nestas circunstâncias a capacidade da cultura

precedente de uma rotação promover a produção de esporos é a única forma de aumentar o potencial micorrízico de um solo. Contudo, essa será sempre uma forma de inóculo menos eficiente para o estabelecimento de novas colonizações micorrízicas atendendo a que a colonização a partir da germinação de esporos é um processo mais lento do que a partir de uma rede de micélio pré estabelecida. Para além disso, na altura da sementeira do trigo (Novembro) a temperatura é já relativamente baixa fazendo com que o processo de germinação dos esporos seja ainda mais lento ou mesmo inibido, e portanto a colonização micorrízica do trigo na sua fase inicial esteja fortemente dependente da rede de micélio extraradical.

Sabendo que o micélio extraradical pode sobreviver ao Verão Mediterrânico, o uso da mobilização mínima ou sementeira directa ao permitir mater intacto este micélio, para além de permitir maior diversidade de AMF, associado à escolha de culturas da rotação, assume particular importância no favorecimento da micorrização de uma época de sementeira para a seguinte, possibilitando uma gestão do agro-ecosistema claramente propícia à micorrização. Adicionalmente, e considerando que a colonização micorrízica mediada pelas infestantes é mais eficiente do que a que ocorre de um época de sementeira a outra, um maneio adequado das infestantes (permitir que cresçam o tempo suficiente para que o micélio extraradical se estabeleça seguindo-se o uso de herbicida para o seu controle de modo a preservar intacto o referido micélio) pode ser extremamente favorável à colonização micorrízica inicial do trigo.

Dado que o trigo pode de facto beneficiar da colonização micorrízica e sabendo que em condições mediterrânicas o seu desenvolvimento inicial é fundamental para a estabilização da biomassa à floração, é evidente o potencial decorrente da micorrização no sistema agrícola do Alentejo. Práticas culturais como a sementeira directa, a escolha criteriosa de culturas a incluir numa rotação e um maneio cuidadoso das infestantes, são claramente facilitadoras do estabelecimento das micorrizas arbusculares.

Importa ainda salientar o importante papel que as micorrizas arbusculares podem assumir em condições bióticas ou abióticas marginas, funcionando como uma espécie de "seguro de vida" no sentido em que em

situações tidas como normais os benefícios conferidos pela micorrização podem até não ser óbvios, mas perante uma situação de desequilíbrio, as vantagens comparativas associadas às plantas micorrizadas tornam-se um factor importante, ou mesmo decisivo, para o ultrapassar. Este aspecto pode assumir particular relevância no contexto agronómico do Alentejo, atendendo à enorme variabilidade do clima Mediterrânico e à fraca qualidade dos solos cultivados.

O crescente interesse em sistemas agrícolas extensivos, com maior sustentabilidade ambiental e economicamente viáveis em termos de produção, obriga à identificação de formas de gestão das práticas agrícolas que permitam a manipulação controlada da comunidade de fungos micorrízicos arbusculares e a capitalização dos efeitos mutualistas da micorrização. O inóculo nativo deve ser favorecido de forma a promover a micorrização contribuindo para o desenvolvimento da produção agrícola sustentada.

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1. Introduction

1.1 Alentejo region - land use and climate

The Alentejo region is located in the south of Portugal. It has an area of 26,766 km^2 and corresponds to a third of the continental part of the country. The landscape of the region is characteristically large and slightly undulating area with rocky outcrops. The climate is Mediterranean (Csa, according to Köppen climate classification), characterized by hot, dry summers and cool, wet winters. Rainfall (500-800 mm/year) is concentrated mainly in the winter months and there is a relatively long dry summer period with temperatures that can go up to 40°C, associated with sinking air from subtropical high pressure cells, and may last for up to five months. The characteristic features of relief and climate, together with social and political forces have determined the land use in this region.

Until recently agriculture was characterised by the large expanse of *montado* (1.3 million hectares) characterized by species-rich grassland mixed with cork and helm oak trees. Traditionally the grassland was utilized by free-range pigs but this system has been replaced by sheep and cattle grazing perennial pastures with scattered tree cover and an extensive system of cereal production based on rotation with forage crops and fallow.

The utilised agricultural area (UAA) of the region is 1,792,285 ha (INE, 2007a) and the farming system is based in large estates. Holdings greater than 500 ha represent 1.6% of the total number of farms in the region and occupy 30.5% of the UAA (Table 1.1).

The dominant soil types are luvisols, lithosols and vertisols (FAO, 1990), that have mainly developed in a parent material of schist and granite and in some circumstances have toxicity problems (Carvalho, 1987; Alves, 1989). Soils are rather shallow and in general have very small organic matter content (less than 10 mg g^{-1}) and low pH (Table 1.2).

Wheat was traditionally the main crop of the rotation followed by 2 years of a less important cereal like oats or barley and proceeded by one or two years of fallow, depending on the soil, with sunflower as the spring crop. In 1980 the area of Portugal under wheat was 341,676 ha, with 77.3% of the production being in the Alentejo region. In the same year the average production was 1,259 kg ha^{-1}. Wheat stubble, straw, dry pasture and hay are the summer diet for livestock. Traditionally managed permanent orchards of fruit trees, olive and grapevines also occupy a significant area of the region. The Alentejo was responsible for 3.7% of the wine and 35.6% of the olive oil produced in Portugal during 1980, (INE, 2007b).

Table 1.1 – Size of holdings in Alentejo and % of total utilised agricultural area (UAA) they occupy.

Size of holdings by classes of UAA (ha)	% of total UAA
10 - 20 ha	2.4
20 – 50 ha	6.0
50 - 100 ha	8.2
100 - 500 ha	**45.9**
> 500 ha	**30.5**

(Source: Anuário de Campanha 2004/2005 and INE, 2007a)

Table 1.2 – Some characteristics of Portuguese soils. Values are a percentage of total cropped area (5,400,000 ha).

	Percentage of total cropped area		
	C.E.C.	O.M.	pH
High	4.2	27.5	11.8
Medium	**70.2**	2.2	5.3
Low	25.2	**70.4**	**82.9**

C.E.C. - cationic exchange capacity (meq./100 g of soil) (high >20, medium 10-20, low <10) O.M. – Organic matter (mg g^{-1}) (high >20, medium 10-20, low <10) pH (high >6.5 , medium 5.5 - 6.5, low<5.5). (Adapted from Alves, 1989 in Carvalho, 2003)

The greatest limitation for this current agricultural system in the Alentejo has been the small yields, rarely exceeding the 2,000 kg ha^{-1} for cereals, together with poor quality (e.g. wine). Beyond the variability and limitations

imposed by climatic conditions, which will be addressed in more detail below, many aspects of the sustainability of this system of agriculture are uncertain, as the level of inputs is quite high relative to the yield of cereals. Mechanisation, fertilisers and other agrochemicals have not been able to counterbalance poor soils (shallow and with very small organic matter content). Tillage practices are commonly both energy consuming and structurally destructive. In addition, there are examples of the use of unsuitable varieties and the untimely use of some cultural practices. Despite all these limitations this farming system has managed to survive because of central government financial intervention in supporting the price paid for small grain cereals.

Portugal accession to the European Union (EU) in 1986, along with Spain, stimulated deep structural changes in the agronomic system. Both countries were allowed to benefit from a relatively long transition period (between 7 and 10 years) designed to secure the harmonisation of prices for basic agricultural products in each country with the prices set by the Community's Common Agriculture Policy (CAP). EU rules required that they either adapted to the new reality by opening up to investments, and the phasing out of tariff barriers, or existing forms of agriculture would disappear. By the end of the transition period it had became evident that the modernisation model for agriculture followed in western Europe through the CAP had been wasteful of both financial and natural resources (Freire and Parkhurst, 2002). New models of rural development gained increasing numbers of supporters. These differed from previous ones in emphasising that the use of natural and social resources particular to each region should be the basis for increases in income and living standards. The emphasis on importing production methods and input, which entailed heavy capital investments and profound social and structural transformations, was abandoned. Integrated development within each region was emphasised over the rigid cross-regional criteria for modernisation. The reasoning behind the new approach was that agriculture represented only one of a variety of activities that kept populations in rural areas, and that these activities would stimulate other sustainable economic activities (Baptista, 1993 and 2001).

CAP was gradually implemented and the improvement of crop production means took place thanks to investments that allowed new production techniques and increasing of mechanization. Farmers chose more extensive systems that required less labour and were associated with less demanding production factors. Another important development has been the fact that the level of education among farmers has increased considerably, whether by a natural process of attrition or by producers acquiring new skills. In 2005 almost 18% of the farmers had attended more than primary school and of these almost half had completed a higher level of education (INE, 2007b).

The key trend in Portuguese agriculture, which is slowly assimilating all these changes, has been the major shift from arable crop to animal production, which has grown in proportion from 29% to 38% in the last 20 years, with the consequence that the area under forage and natural pasture has also risen from 34% to 48% over the same period (INE, 2007b). An important feature of Portuguese agricultural development in the region over the last decade has been the shift to extensive beef cattle, based mainly on expansion of the suckler cow herd. Portugal has supported this development by specific policy measures, which appear to be well-suited to the conditions in many of the less favoured areas like the Alentejo (COM, 2003). However, small grain cereals continue to occupy a significant role in the Nation's agriculture production for both human and animal consumption, despite the considerable reduction in the area of land under cultivation, resulting from trade liberalization and pricing policies.

Even after all these changes, the Alentejo region remains the most important region of the country for small grain cereal production, representing 55% of the cropped area and contributing 39% of the national production. As a result of the increase in land under irrigation, mostly promoted by private farmers taking advantage of EU funding, wheat has been replaced by maize, leading to a significant decrease in the area of land under wheat. In 2006 only 104,684 ha of wheat was grown in Portugal, with 85.5% of this area being in the Alentejo region. In 2006 wheat production averaged 2,380 kg ha^{-1} (INE, 2007b), although this was an exceptionally good year for wheat (Fig. 1.1).

One of the main constrains of wheat cropping in Alentejo is the great instability of production (Fig. 1.1), which is intimately related to the great variability of climatic conditions. In a Mediterranean climate under rain-fed agriculture, the development of biomass before the flowering stage is critical for grain yield in wheat (Carvalho,1987), with the major increases in biomass happening in late autumn and begin of spring. If the weather is too dry or too cold during periods of intense vegetative growth, wheat production can be severely impaired or greatly reduced. Conversely, if rainfall is sufficient during these critical periods, yield can be assured or even enhanced. The unpredictability of weather is a serious problem, having a direct impact on farmer's income and financial capacity. Agronomic management focused on achieving good early crop development can minimize the unavoidable variability due to weather.

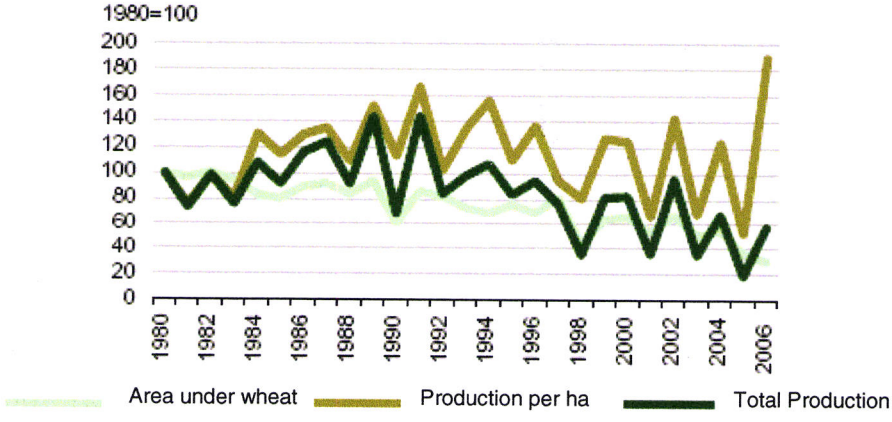

Figure 1.1 – Wheat cropped area (ha), productivity (kg ha^{-1}) and production (t) in Portugal, between 1980 and 2006. (Adapted from: National Statistics Institute (INE), 2007a)

In 2002 construction of the Alqueva dam was finished, giving rise to the largest artificial lake in Europe (250 km^2), it has greatly added to the potential for irrigated agricultural production in the Alentejo region. Considerable opportunities exist for irrigated spring crops, mainly maize, as well as using irrigation to offset the irregularity of winter rainfall. The increased area of olive tree and grape vines is also due to recent irrigation resources.

Large reforestation programs have been implemented on land abandoned for arable crop production because of its very low productivity. Well

adapted and, in the long term, highly profitable species like cork oak (*Quercus suber*) have been chosen together with other species such as *Pinus pinea*, that guarantee incomes over a shorter term.

Following the general trend, development in the Alentejo region has been directed to establish and implement new and more sustainable agricultural practices. The use of no-till (or direct-drilling) and reduced tillage systems is a good example of this development. The area under no-till has increased spectacularly over the last 10 years and has been used in the cultivation of almost all the key arable crops in the region, including wheat, corn, oats, barley and sunflower. Initially it was not easy to overcome farmers doubts and concerns about the reliability of the technique, but its inclusion in Agenda 2000, a financially-backed agro-environmental measure, provided support and encouragement. As benefits to crop yields coupled with reduced mechanical requirements (power and time) became evident, together with increased soil organic matter and other improvements, farmers got progressively more enthusiastic about the technique, such that their association currently reports more than 50,000 ha are cultivated under no-till.

In summary, the Alentejo is a region in Portugal with the greatest potential for small grain production. Despite some structural changes (like the increase of irrigated area) the future of small grain production lies on low input production systems based on no-till, appropriately designed crop rotations and a rational use of nutrients. A possible means of improving the use of nutrient resources is through the improved use of naturally occurring symbiosis like arbuscular mycorrhiza.

1.2 Arbuscular mycorrhiza - general considerations

Arbuscular mycorrhiza (AM) are the most common underground symbioses. They are formed between the roots of a extremely wide variety of host plants (angiosperms, gymnosperms and pteridophytes) and aseptate, obligatory symbiotic fungi, arbuscular mycorrhiza fungi (AMF), which belong to the recently created phylum Glomeromycota (Schüßler *et al.*, 2001). The

current classification is based on a consensus of morphological and molecular characteristics such that 7 genera with approximately 170 species are recognized. According to Koide and Mosse (2004) the term "mycorrhiza" can be used as both the singular and the plural, and this is the terminology adopted in this thesis.

Colonisation of land by plants some 4,000 million years ago was associated with the colonisation of the primitive roots by soil-born filamentous fungi (Simon *et al.*, 1993; Redecker *et al.*, 2000). More than 70% of all plant species in both agricultural and natural environments form AM associations and they can be found in a wide range of habitats (Smith and Read, 1997), including deserts, lowland tropical rainforests, high latitudes and altitudes and aquatic ecosystems.

Arbuscular mycorrhiza develops an intimate contact between the fungus and host plant root and intraradical and extraradical mycelia are established, with physiological and functional differences. The intraradical mycelium develops extensively between and inside the walls of exodermal and cortical cells of the root, where it forms intraradical structures - arbuscules and vesicles. Arbuscules (Fig. 1.2 A) are hyphae that branch dichotomously and profusely within the cell wall but outside the plasmalemma of root cells, remaining separated from the plant cell cytoplasm by an extension of the plant plasma membrane that surrounds the fungus and follows the contours of the hyphal branches. They can occupy up to 90% of the cell volume through the reduction of vacuolar space. The arbuscular structure results in a great increase in plant-fungal contact surface area, which contributes to the assumption that the bidirectional transfer of nutrients in AM probably occurs at the periarbuscular interfaces. However, whether arbuscules are the site of root-to-fungus carbon transfer is a matter of debate (Bago, 2000). The life span of an arbuscule is relatively short. It can exist for 2 to 3 weeks and after that period it collapses. In Figure 1.2 A, where fungal structures are stained in blue, different ages of arbuscules are reflected in the degree of branching. Once an arbuscule progressively degenerates to form remnant clumps, the plant cell remains alive. Vesicles are terminal or intercalary hyphal swellings (Fig.1.2 B) believed to function as storage organs. They contain abundant lipids and numerous nuclei.

Whether or not vesicles occur depends in the first place on the identity of the fungus, as neither *Scutellospora* nor *Gigaspora* genera develop vesicles. Members of all the other genera may develop vesicles to varying degrees and in either intercellular or intracellular positions in the cortex (Smith and Read, 1997).

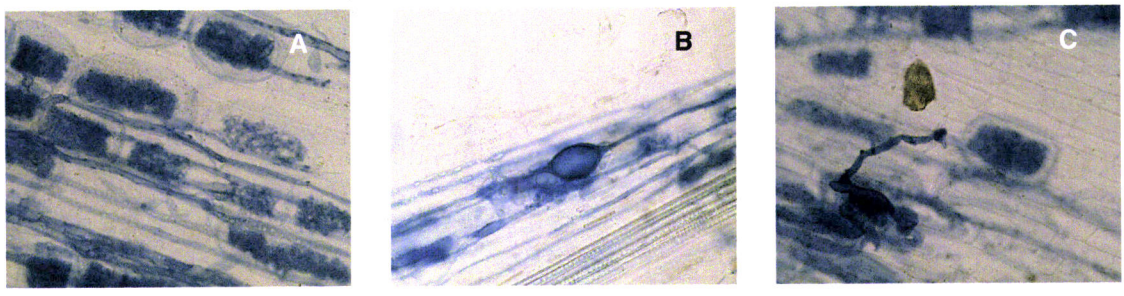

Figure 1.2 – Plant root colonised with arbuscular mycorrhiza. A - Arbuscules B – Vesicle C - Appressorium.

Colonisation of roots by AM can arise from three sources of inoculum: spores, infected root fragments and hyphae – collectively termed propagules (Smith and Read, 1997). Spores are long term survival structures and for long time AMF taxonomy was exclusively based in their morphology.

For the symbiosis to be established, molecular signalling events elicited by root exudates must take place, leading to various physiological and anatomical changes in both symbionts. There are likely several necessary steps leading to the formation of a functioning mycorrhiza, each of which require the sending out of appropriate signals by one member of the symbiosis followed by their recognition by the other member. Recognition and signalling events may serve to truncate a full-blown defence mechanism, allowing the symbiosis to become established (Koid and Schreiner, 1992; Giovannetti *et al.*, 1994). Formation of appressoria (Fig. 1.2 C) is one of the first morphological signs that recognition between the plant and the fungus has occurred and AMF hyphal penetration takes place by means of a combination of mechanical and enzymatic processes (Bonfante and Perotto, 1995). Great advances have been made over the last decade with the availability of new molecular and biochemical techniques permitting the understanding of the nature of plant signals and their perception by the fungal partner during early stages of the symbiosis (Paszkowski, 2006; Requena *et al.*, 2007).

The symbiosis is commonly mutualistic, the long-term compatible interactions being based on a bidirectional nutrient transfer between symbionts. The fungus gains all of its carbon from the plant, equivalent to between 10% and 20% of the host plant photosynthates (Graham, 2000). Thus AM symbiosis plays a significant role in carbon cycling between the atmosphere and biosphere.

The fungal hyphae within the root are connected to the extraradical mycelium and form a single continuum. This soil mycelium has a variety of functions (Friese and Allen, 1991), including formation of spores (propagules for dispersal in time and space), formation of runner hyphae (exploration of soil and new roots to be colonized), and nutrient uptake. It also constitutes a link between the plant roots and the soil environment and many benefits that accrue to plants from their association with arbuscular mycorrhizal fungi are a function of the increased volume of soil that can be explored by the extraradical mycelium. Sieverding (1991) estimates that for each centimetre of colonized root there is an increase of 15 cm^3 in the volume of soil explored, this value can increase to 200 cm^3 depending on environmental conditions. The enhanced volume of soil explored, together with the ability of the extraradical mycelium to absorb and translocate nutrients to the plant, results in one of the most obvious and important advantages of mycorrhizal formation: the ability to take up more nutrients. The more important nutrients in this respect are those that have limited mobility in soil, such as phosphorus (P). In addition to nutrient acquisition many other benefits are associated with AM plants (Gupta *et al.*, 2000): alleviation of water stress (Augé, 2004; Cho *et al.*, 2006), protection from root pathogens (Graham, 2001; Khaosaad *et al.*, 2007), tolerance to toxic heavy metals (Audet and Charest, 2006), tolerance to adverse temperature, salinity and pH (Sannazzaro *et al.*, 2006; Yano and Takaki, 2005) and better performance following transplantation shock (Subhan *et al.*, 1998). The enhanced tolerance to toxic metals afforded by arbuscular mycorrhizas can be of benefit in phytoremediation (Göhre and Paszkowski, 2006). The extraradical hyphae also stabilize soil aggregates by both enmeshing soil particles (Miller and Jastrow, 1990; Rillig and Mummey, 2006) and as a result of the production of substances that adhere soil particles together (Wright and Upadhyaya, 1998;

Goss and Kay, 2005). In addition, the extraradical hyphae may interact with other soil organisms either indirectly by changing host plant physiology, including root physiology and patterns of exudation into the mycorrhizosphere (i.e. in the small volume of soil immediately surrounding a mycorrhizal root), or directly by physically and/or metabolically interacting with other organisms in the mycorrhizosphere (Johanson *et al.*, 2004).

Overall arbuscular mycorrhiza are directly coupled with many aspects essential for the agronomic production enclosing a great potential to contribute to sustainable crop production systems.

1.3 General objectives and structure of this thesis

The changes in the chemical, physical and biological variables in the soil induced by agricultural practices interfere with the environment of AM establishment and function. The potential to make effective use of mycorrhizal symbiosis in Mediterranean agriculture depends on developing further understanding of the functional ecology of AMF in these singular agro-ecosystems.

The effectiveness of AM will depends on how agricultural practices (tillage system, use of fertilisers, crop rotations and pesticide use), together with the climatic conditions, affect the properties of the soil and on the responses of the fungi present to the changes induced by these practices. As there is a number of plant, soil, fungal and environmental attributes involved in the functioning of mycorrhizas, it is unlikely that there will be a unique relationship between mycorrhizal colonisation and mycorrhizal benefit. Furthermore, both colonisation of roots by mycorrhizal fungi and the functioning of the symbiosis are dynamic processes that need to be considered throughout time (i.e. the cycle of a particular crop or rotation). As a result quantification of AM impact on yields in agricultural systems has been largely unsuccessful, and the outcome of different field studies have often been contradictory (Ryan and Graham, 2002). Although the primary benefit to an AM host is usually nutritional and direct crop yield increases are often expected when AM are well established,

approach was undertaken in this study. Guidelines for the experimental program were defined to encourage an understanding of the potential value of AM in the agricultural systems of the Alentejo region, specifically in the context of low input cropping systems based on no-till. The objective was to evaluate how some common agronomic practices affect AM colonisation by native AM fungi, and how these practices might be adapted to increase AM inoculum and promote the early AM colonisation of wheat.

Following a general literature review on AM in the context of the agronomy of arable production systems (Chapter 2), a sequence of related sets of experiments was preformed and each one presented as a separate chapter. The primary step was to verify under field conditions whether wheat, one of the most important crops of the region, was colonized by indigenous AM inoculum and over what time period, and how the common soil tillage systems (no-till and conventional tillage) interfere with AM colonisation (Chapter 3). In view of the results obtained it was important to find out if the AM extraradical mycelium was able to survive over summer and start new AM colonisations in the growing season because these features would determine the time frame (from one cropping year to the other) and the way (faster or slower colonisation) in which AM can express their influence (Chapter 4 and Annexe 1). Additionally, knowing about the importance of an early good vegetative development of wheat under Alentejo's cropping conditions, the use of weeds to act as possible AM bridges to promote a better initial AM colonisation of the crop was investigated (Chapter 5). The effect of soil tillage practices on AM fungal diversity was also studied, given that in a poorly diverse or unbalanced AMF community the possible combinations of relationships between host plant and AM fungus are reduced and the expression of AM potential decreased (Chapter 6). The assembly and integration of the main findings, their implications and future work perspectives are then discussed (Chapter 7).

the interactions between host and fungus are far more complex. Thought AM associations can offer multiple benefits to the host plant it may not be obviously mutualistic at all points in time, and it is possible under some conditions that AMF may cheat their host plant into supplying resources with no apparent benefit to the plant (Gosling *et al.*, 2006). In some cases, this can cause a decline in growth (Ryan *et al.*, 2005). However, proving that AMF are actually "cheating" is difficult (Fitter, 2001), not least because of the wide range of benefits to the host, which may only become obvious at specific times or under certain environmental conditions or stresses. Therefore AM symbioses can function as an insurance against unfavourable conditions.

Trans-disciplinary research has an important role to play in understanding the complexities of the ecological approach to agriculture. On the other hand, strict disciplinary approaches are important for the understanding of the biology of organisms such as AM for management of these organisms in sustainable systems. In emphasizing the importance of integration and scale of the arbuscular mycorrhizal symbiosis, Miller and Kling (2000) support the view that insights into the contributions of AM fungi to belowground processes increasingly reveal how research into these fungi is pivotal to understanding many global environmental issues. These authors conclude that, to succeed, the mycorrhizal research community must go beyond their work and integrate it with that of other researchers. Such integration at different scales and organizational levels is a prerequisite for understanding the complex system that constitutes the mycorrhizal symbiosis and its function within an ecosystem. Also Jakobsen *et al.* (2004) state that the emerging results based on integration of molecular, physiological and ecological approaches, make it increasingly evident that there is a need for strategies involving cross-disciplinary research.

Very little has been reported about the performance of AM under Mediterranean climatic conditions in the context of agricultural systems in Alentejo region, except for one paper mainly dealing with the problem of finding good negative controls for AM study (Kleikamp and Joergensen, 2006).

Searching for a direct crop yield increase due to AM is likely to depend so much on site specificities (e.g. weather, disease patterns) and can be further hampered by soil and cropping history. Consequently, a multidisciplinary

2. Arbuscular mycorrhiza in the agronomic context

2.1 The use of AM fungal inoculum

Despite the ubiquous distribution of mycorrhizal fungi (Smith and Read, 1997), with only relative specificity between host plants and fungal isolates (McGonigle and Fitter, 1990), the obligate nature of the symbiosis requires that a plant propagation system be established for inoculum production. This takes place either under greenhouse conditions or *in vitro* laboratory propagation. These techniques result in high costs for inoculum production, which continues to be a serious problem since it is not competitive with that of phosphate fertilizers. Even though farmers understand the significance of sustainable agricultural systems, the reduction of P inputs by using AM fungal inocula alone cannot be justified except, perhaps, in the case of some high value crops (Saito and Marumoto, 2002). Nurseries, large input horticultural enterprises and non-agricultural applications, such as restoration of degraded or devegetated landscapes, are examples of operations where the use of commercial inoculum is current.

A number of factors contribute to uncertainty in the minds of potential users. These include the quality of commercially available products, especially the guarantee that these are pathogen-free, the conditions required for storage before application, the most effective application methods and what is the appropriate inoculum for the application. Furthermore, information provided by suppliers about an inoculum can be deceiving, given that total counts of spores or propagules may be given, but only a fraction may be effective for a particular plant or under specific soil conditions. There is a clear need for registration procedures that can stimulate the development of the mycorrhizal industry (Gianinazzi and Vosátka, 2004).

Advances in ecology during the past decade have led to a much more detailed understanding of potentially adverse consequences of introducing species into a new habitat, but there is little information available on the

ecological consequences of inoculating with mycorrhizal fungi. Schwartz *et al.* (2006) recommend that a careful assessment be made prior to inoculation that documents the need for inoculation and the likelihood of success, because the introduction of mycorrhizal fungi is not universally beneficial. In addition, there is inadequate knowledge of the basic biology and diversity of AM fungi (Abbott *at al.*, 1995; Saito and Marumoto, 2002).

Some on-farm inoculum production and application methods have been developed to allow farmers to produce locally adapted isolates and generate a taxonomically diverse inoculum (Mohandas *et al.*, 2004; Douds *et al.*, 2006). However, inocula produced this way are not readily processed for mechanical application in the field, and this is an obstacle to their utilization in large scale agriculture, especially for row crops. Moreover, it requires an additional mechanical operation, with the corresponding economic and possible soil compaction costs.

Although inoculation with AM fungi has potential significance for sustainable crop production, including environmental conservation, current knowledge and technologies limit the application for widespread use in many agricultural contexts.

2.2 Host plants and crop rotation

Crop rotation is an agronomic practice with a long history that is still performed and retains its general beneficial aspects associated with maintenance or improvement of soil fertility, reduction in erosion potential and in the build-up of pests and weeds, spreading of workload, lowering the risks from inclement weather damage, less reliance on agricultural chemicals, and a need for increased net profits.

Although most plants support the formation of arbuscular mycorrhiza, roots of some crops, such as those belonging to the *Chenopodiaceae* or *Brassicaceae*, do not form this symbiotic relationship. Furthermore, the use of these crops in rotations tends to lead to a reduction in mycorrhizal propagules. In contrast, the cultivation of mycorrhizal host crops increases AM fungal

populations and maintains mycorrhizal activity in soil (Vestberg *et al.*, 2005). As a result there tends to be much greater spore densities present (Black and Tinker, 1977; Karasawa *et al.*, 2000a) leading to improved colonisation of the succeeding crop, which may only take place in the following season (Gavito and Miller, 1998a; Miller, 2000).

Arihara and Karasawa (2000) studied the effects of fallow and prior cultivation of sunflower, maize, soybean, potato, sugar beet and canola (rapeseed) on the AM colonisation of a subsequent maize crop. They found that shoot weight and grain yield of maize were much greater in the plots following sunflower, maize, soybean and potato than those after canola, sugar beet or a fallow. The cultivation of a non-AM host such as sugar beet or canola, reduced the mycorrhizal propagules and consequent AM colonisation, P uptake and shoot dry weight (Arihara and Karasawa, 2000; Gollner *et al.*, 2004), even with no alteration of the availability of P in the soil induced by the previous crop (Karasawa *et al.*, 2001). These results established that cultivation of a mycorrhizal crop in the previous season promoted AM formation on roots of the following maize crop, which in turn enhanced its P uptake and growth, and finally increased the grain yield. The positive effect of having an AM host as the preceding crop for the maize was partly due to differences in AM fungal inoculum density (Karasawa *et al.*, 2002).

Reduction of AM fungal propagules can also be a significant consequence of bare-fallowing. Because AM fungi are strictly biotrophic, their survival depends on the presence of host plants. During a bare-fallow, the absence of host plants may cause the viability of AM fungi to decline and this decline may be further exacerbated by freezing conditions (Kabir *et al.*, 1997b). Harinikumar and Bagyraj (1988) reported a 40% and 13% decrease in AM fungi propagules after fallow and a non-mycorrhizal crop, respectively. The extent to which AM fungi communities can be restored is inversely proportional to the duration of a fallow and the extent to which the land is grazed at the time (Duponnois *et al.*, 2001).

Despite the ability of the extraradical mycelia to remain infective, even if the soil is frozen over winter (Addy *et al.*, 1997), it is important to maintain the level of AM fungal inoculum to maximize the benefits of AM fungi on the

following crop. Maintaining plant cover is very important, whether with cover crops or with cold tolerant crops, such as winter wheat. The choice of cover crops should be guided by the same principles as used to select the crop species to be adopted for a rotation. Preference should be given to mycotrophic cover crops capable of surviving in less favourable conditions while maintaining the AM inoculum potential in soil. Kabir and Koid (2000) demonstrated that using wheat or dandelion as mycotrophic winter cover crops increased subsequent sweet corn yield. Kabir and Koide (2002) reported that, relative to fallow, oats and rye were equally effective as cover crops in increasing mycorrhizal colonisation in a succeeding sweet corn crop, as determined by the density of mycorrhizal hyphae and soil aggregate stability. Their results also underlined the importance of host crop diversity as they found that the combination of two cover crops (rye and oats) was significantly better than sowing a single species for the colonisation of AM fungi, P uptake and yield of the following sweet corn crop.

Even though there is no obvious specificity between a host plant and colonizing AM fungal species, there are preferential associations (McGonigle and Fitter, 1990; Vandenkoornhuyse *et al.*, 2002; Gollotte *et al.*, 2004). Furthermore, the cultivated host species can influence the abundance of the different AM fungi species present (Troech and Loynachan, 2003). This explains how crop rotations can cause changes in a mycorrhizal fungal community (Johnson *et al.*, 1991; Hendrix *et al.*, 1995), the stability of complex soil biotic communities (Cavagnaro *et al.*, 2006) and increase biodiversity (Miranda *et al.*, 2005). In contrast, monocultures tend to select specific AM fungi, which tend to be inferior mutualists (Johnson *et al.*, 1992), making AM fungi a possible cause for the yield decline often observed when such cultures are grown over long periods of time.

Different host species and cultivars of the same species vary in the degree to which they form mycorrhizas (Azcón and Ocampo, 1981). The level of AM colonisation of a cultivar and the associated benefits, are heritable traits that can be selected through plant breeding (Kesava *et al.*, 1990). Breeding programs are commonly conducted on experimental stations, where mineral nutrients are not limiting factors. However, as increasing soil fertility can impair

mycorrhizal development, this can result in the selection of host-crop varieties that form mycorrhizas less readily. For example, Hetrick *et al.* (1993) reported that the wheat cultivars released prior to 1950 were consistently greater in their level of dependence on mycorrhizal formation for production relative to more recent releases. Zhu *et al.* (2001) showed that modern wheat cultivars were less responsive to mycorrhizal colonisation than were historical lines. The breeding of maize for resistance to fungal pathogens has produced lines that are less mycotrophic than previous varieties (Toth *et al.*, 1990).

2.3 Soil aggregation and tillage regimes

Arbuscular mycorrhizas make direct contributions to soil aggregation and aggregate stability (Tisdall and Oades, 1982), especially in no-till systems where hyphal networks remain intact. The direct effect of AM hyphae on soil aggregate formation was shown by Thomas *et al.* (1993), to be significant and at least equivalent to that of roots alone.

Important aggregate cementing agents produced by soil fungi and bacteria have been characterized as extracelular polysaccharides (Tisdall, 1991), although Wright and Upadhyaya (1996) reported the presence of copious amounts of glomalin, a glycoprotein, associated with AM fungi, later found to be located on the surface of active AM hyphae (Rillig *et al.*, 2001), and which could be important for soil aggregate stability (Miller and Jastrow, 1990). In a three-year study, Wright *et al.* (1999) measured an annual increase in both aggregate stability and weight of glomalin in the top 5 cm of the soil in no-till plots compared with ploughed plots. They also found that when soil was collected from the grassland adjacent to the tillage experiment, the structure of the top 0-10 cm of the grassland soil was more stable than of the cultivated soil after several years under no-till and 4 years under conventional tillage. Although there was not a full biochemical characterization of glomalin, in their study, Driver *et al.* (2005) showed that the material was tightly bound within the hyphal wall of AM fungi rather then being a primary release or secretion into the growth medium. They argued that glomalin has a role in the living fungus and any

functionality in the soil was only secondary arising, possibly due to its relative slow turnover rate in the environment (Steinberg and Rillig, 2003; Driver *et al.*, 2005; Goss and Kay, 2005; Rillig and Mummey, 2006).

Soil tillage serves many purposes, including weed control, preparation of the seed bed, and improved water capture and storage in the soil profile (Cook, 1992). It is also used to incorporate fertilizer, manure and pesticides and to reduce the incidence of disease and pests. Unfortunately, through the gradual loss of organic matter from near the soil surface, tillage can reduce aggregate stability and make soil more vulnerable to wind and water erosion. The environmental impacts of soil erosion became very evident in the 1930s in the USA, and since the 1950s, throughout the world there has been a gradual transition from the mouldboard plough to various forms of conservation tillage, including no-till or direct drilling, with minimum soil disturbance (Lal *et al.*, 2007). The basic principles of no-till agriculture include sowing directly into soil using the special planting equipment that cuts through or displaces the covering of crop residues. Retaining residues on the soil surface reduces erosion, evaporation and limits weed growth. It also improves water infiltration through the enhanced activity of the soil biota and the maintenance of macro-porosity, even if there is some increase in bulk density (Lal *et al.*, 2007). The transition to no-till has implications for environmental quality, particularly because of its effectiveness in reducing soil erosion and surface runoff, enhancing soil organic matter concentration near the soil surface, increasing soil biological activity and reducing the energy required for crop production.

The direct effects of the various tillage practices on fungi, particularly conventional tillage (CT) and no-till (NT) systems, are related to physical disruption of the hyphal network and to the mixing of surface residues within the soil profile, affecting the effectiveness of AM symbiosis in many ways (Kabir, 2005).

When host plants are present and the soil is not disturbed, hyphae from colonized roots and mycelia network are the main source of inoculum, they colonize roots more rapidly and efficiently compared with spores (Read *et al.*, 1976; Martins and Read, 1997). The latter are considered as "long-term" propagules (Kabir, 2005), mainly because it would take longer for spores to

germinate and make contact with roots compared with colonisation by runner hyphae from a well developed extraradical mycelium (Klironomos and Hart, 2002).

Since the 1980's many studies, under field conditions or in pots have been developed, most of them using maize as host plant, to evaluate the effect of soil disturbance on AM colonisation and its consequences, particularly for P uptake. In one of the first reports on the subject, Evans and Miller (1988) described a significant adverse effect of soil disturbance on AM colonisation of both maize and wheat roots (both mycorrhizal), and also upon the P absorption by these species but no effects were found with respect to spinach and canola comparisons (non-mycorrhizal). Moreover, the injection of benomyl, a potent inhibitor of mycorrhizal fungi, into the soil surface significantly reduced the influence of soil disturbance on P absorption. These results indicate that the negative effect of disturbance on P uptake is likely due to impaired AM associations. Later, Evans and Miller (1990) demonstrated that disruption of the hyphal network was directly responsible for much of the effect of soil disturbance on mycorrhizal colonisation. Besides, deep ploughing (to more than 15 cm) hinders subsequent mycorrhiza formation by reducing propagule density in the rooting zone (Kabir *et al.*, 1998b). Abbott and Robson (1991) found that under no-till there were more spores in the top 8 cm of soil whereas tilled soils had more spores in the 8-15 cm depth. Goss and de Varennes (2002), Antunes *et al.* (2006b) and de Varennes and Goss (2007) used the same technique for disturbing the soil in pot experiments, and, consistently found greater AM colonisation of soybean or annual medics, growing in pots of undisturbed soil.

The faster AM colonisation observed in undisturbed or no-till systems supports earlier uptake of P (Vivekanandan and Fixen, 1991; McGonigle and Miller, 1996b; McGonigle *et al.*, 1999), conferring a comparative advantage to the crop in the initial growth period, independently of soil P content (Fairchild and Miller, 1990; McGonigle and Miller, 1996b) although not always (Vivekanandan and Fixen, 1991).

The number of spores, length of extraradical mycelium and hyphal density, particularly in the row zone (Kabir *et al.*, 1998a), have been found to be enhanced when soil disturbance is reduced (McGonigle and Miller, 1996a;

Boddington and Dodd, 2000a; Galvez *et al.*, 2001; Borie *et al.*, 2006). In addition to the enhanced concentration of P in the plant, there are similar effects on zinc (Zn) and copper (Cu) concentrations (Kabir *et al.* 1998a), which is not surprising considering the metabolic activity of hyphae in this system is greater (Kabir *et al.*, 1997a). However, plant concentrations of other more mobile elements, such as potassium (K), calcium (Ca) or magnesium (Mg) did not change with tillage regime (Kabir *et al.*, 1998a).

The ability of AM fungi to promote growth in undisturbed soil is related to the spread of mycelium in the soil and the capacity of nutrient transfer to the roots and, in addition, a capacity for persistence and retention of functional ability of the extraradical mycelium from one plant generation to the next (McGonigle *et al.*, 2003) and this is why the survival of AM extra-radical mycelium survival is particularly important, over winter in cold climates or over summer in Mediterranean climates, where field crop production is restricted to a few months. In Canada, Kabir *et al.* (1997b) evaluated the timing of tillage on the survival of AM fungal hyphae, either connected to or detached from corn roots and whether the extraradical mycelium was intact or disrupted. They confirmed that fall tillage severely reduced AM hyphae viability, whereas spring tillage had little effect. They also found that attachment or proximity to roots favoured over winter survival, but disruption of the extraradical hyphae far outweighed the benefits of host root presence on survival.

Given that there is no such thing as a fungal effect or a plant effect, but a cross effect of both symbionts, the extent of colonisation in soil under different levels of soil disturbance is also influenced by the host plant. Under the same experimental conditions Mozafar *et al.* (2000) found an increase in AM colonisation of maize under no-till, although there were no differences in colonisation between tillage treatments in AM wheat. It should also be underlined that colonizing strategies of AMF differ considerably and the variation is taxonomically based at the family level (Hart and Reader, 2002). Consequently different survival strategies for soil disturbance or tillage regime can affect the population of AM fungi of a particular site.

Some exceptions to the promoting effects of no-till systems on AM colonisation have also been reported. On one hand, Gavito and Miller (1998a)

did not find any effect of tillage practices or fertilizer application on the AM fungal colonisation of maize under field conditions. On the other hand even though Mozafar *et al.* (2000) observed greater AM colonisation in maize under NT system than under CT, they did not see differences between tillage systems when wheat was the host crop. In other cases, despite the greater AM colonisation and P content of plants cultivated under no-till than in conventional cultivation systems, it did not translate into enhanced growth or yield (McGonigle and Miller, 1996b; Galvez *et al.*, 2001) suggesting an interaction of soil P and a yield depressing factor, possibly soil temperature, in no-till soils. In contrast to much previous research in mesic temperate climates, Lekberg *et al.* (2008) found that in the semi-arid tropics, P fertilizer, long fallow periods, and tillage did not significantly decrease the AM fungi inoculum potential. McGonigle and Miller (2000) confirmed that the high inoculum density in particular ecosystems, such as the pastures studied in Australia, likely overrides any soil disturbance effect and ensures that roots of all plants became colonized by AM fungi.

An increased presence of AM fungi in roots from less disturbed systems has been reported to be accompanied by greater colonisation by pathogenic fungi (McGonigle and Miller, 1996a; Mozafar *et al.*, 2000). The latter probably take advantage of the same mechanisms as AM fungi in terms of preserved integrity of the mycelium under these conditions. Mozafar *et al.* (2000) suggested that changes in nutrient concentration in the leaves of the plants tested in their study (wheat and maize) were likely due to the combined effects of colonisation of the roots by various mycorrhizal and non-mycorrhizal fungi and not to changes in the chemical or physical properties of the soil. The authors stress the need to take into account non-mycorrhizal roots parasites and especially non-filamentous obligate fungi in studies conducted under field conditions.

2.4 Weed management

AMF colonize the roots of most agricultural crops and of the weeds present (Yamato, 2004). In most of the cases AM fungi and weeds have co-evolved for longer time than AM fungi and crops.

Agro-ecological functioning of weed communities may be affected by AM fungi through facilitative effects mediated by the mycelial network. Mycelial interconnections among host species in a weed-crop mixture may cause patterns of resource uptake and distribution among host species that differ qualitatively from those occurring in plant communities where AM fungi are absent (Moyer-Henry *et al.*, 2006). For example, dying host species may release nutrients into the AM fungal mycelium (Smith and Read, 1997) which may then be redistributed among other host species, enabling facilitative effects in crop-weed mixtures. It is possible, therefore, that after selective weed control, nutrients acquired by host weeds may be transferred to host crop or cover crop via the mycelium. Such processes may result in greater nutrient cycling and reduce competitive effects from non-host weeds. If such phenomena are qualitatively important, then AM fungi may be capable of significantly altering the agro-ecological functioning of weeds. Properly timed control operations, such as sub-lethal post-emergence herbicide applications might be used to transfer nutrients from weeds to crops. In this scenario, the weeds might function as a temporary nutrient sink, restricting the competition for nutrients from non-host weeds and reducing leaching and other mechanisms of nutrient loss.

Facilitative functions may also occur in which one host species supports populations of mycorrhizal fungi that are beneficial to another plant species. Host species may provide carbon to the mycelium which may support formation of arbuscular mycorrhizas with other species. In effect the first host plants provide energy that serves, directly or indirectly, to support formation of AM colonisation on a second newly germinating host. This allows the seedlings of the second host to receive nutrients and other mycorrhizal benefits while minimizing the energetic costs of mycorrhizal establishment to seedlings. For

example weed communities in several cropping systems have been shown to enhance mycorrhizal colonisation and growth of subsequent crops, providing an alternative host between AM dependent crops (Kabir and Koid, 2000) or to maintain infective propagules over winter (Schreiner *et al.*, 2001). The negative impact of fallow periods or the cultivation of non host crops on the AM inoculum may be mitigated by the presence of weeds in the field. Jansa *et al.* (2002) hypothesized that the higher spore counts in no-till soil relative to conventionally cultivated land after a non-mycorrhizal plant (canola), was due to the increased presence of mycorrhizal weed plants in the no-till plots. These weeds may have supported AM fungal development in their roots and also allowed some spore formation under the canola. In the tilled plots, ploughing eliminated the majority of weeds and, therefore, AM fungi development during the growing of canola would be negligible. In Dehérain plots Plenchette (1989) also reported that mycorrhizal infectivity was maintained, when weeds were not controlled. However this effect would be beneficial only if the species that received the additional resources was desirable, and would have economic importance under the prevailing conditions. For example, under a Mediterranean climate, where the long, very dry summer might compromise the ability of extraradical mycelium to survive between the harvesting of one crop, in early summer and the seeding of the next crop in the rotation, in autumn. However, no reports investigating this have been found in the literature.

Feldmann and Boyle (1999) studied the interaction of weed competition and AMF in maize monoculture. They found decreased richness of AM fungal species and effectiveness in weed–free versus plots with weeds. Maize grew better in the presence of weeds and the authors concluded that the effective AM fungi over-compensate for any weed-mediated decrease in crop biomass. However, the benefit of enhanced AMF colonisation of maize observed by Galvez *et al.* (2001) in the absence of effective weed control did not translate into enhanced growth or yield.

Although it is possible that AMF may have negative effects on agro-ecological functioning of weed communities, simply by increasing abundance of problematic weeds (Jordan *et al.*, 2000), management of the existing weed population might provide an important tool to guarantee a more rapid

colonisation of a winter crop with the consequent advantages of an early adequate P nutrition. Abbott and Robson (1981) suggested that the many factors associated with the differences in effectiveness of different AM fungi in stimulating plant growth are due to their influence on the rapidity of infection rather than the ability of infected roots to take up P, suggesting that the benefit of the symbiosis is beyond the enhancement of nutrition.

Given that colonisation from spores is relatively slow because it requires a successful biochemical dialogue between plant roots and the AM fungal spores, the existence of weeds roots may be important in that fast colonisation of the sown crop because it can take place from a well established mycelium network as soon as germination occurs. Under natural ecosystems or reduced-tillage agricultural systems, young seedlings can germinate and "plug" into an already established AM fungi hyphal network which permeates the soil and links different plant species. The lack of specificity confers a great advantage for the success of AM fungi in mixed plant communities. Another benefit to the plant of interest is that photosynthates are needed only for maintenance of the pre-established AM fungi following colonisation and not for the initial development of the extraradicle mycelium (Dodd *et al.*, 2000).

Excessive tillage to control weeds, associated with frequent cultivation of non-mycorrhizal crops, could also hamper development of a diverse AM community (Gosling *et al.*, 2006). This is consistent with the findings of Abbott and Robson (1991), who reported a positive impact of weeds as host plants on the increase of AM fungal diversity.

Innovative forms of weed control with bio-herbicides make use of plant, bacteria or fungal secondary metabolites with allelopathic or semiochemist effect (Dias and Dias, 2007). In general allelochemicals seem to have an adverse effect on AM colonisation although AMF can alleviate allelopathic stress induced by some plants and improve crop growth and yield (Javaid, 2007). Still, bio-herbicides are not, or at least not yet, common practices for weed control and the most used mechanisms for that purpose are soil disturbance, herbicide application and, to some extent, crop rotation. Also to differing degrees, they all interfere with mycorrhization.

Soil disturbance affects AM through disruption of the hyphal network and the ability to start new AM colonisations. In addition deep ploughing (to more than 15 cm) hinders subsequent mycorrhiza formation by reducing propagule density in the rooting zone (Evans and Miller, 1990; Kabir *et al.*, 1998b).

The effects of herbicides on AM can be direct or indirect through the weakening of the host plant. However, mycorrhiza formation can alleviate their adverse effect on the plant (Garcia–Romero and Ocampo, 1998). Often, although not always (Santos *et al.*, 2006), herbicides negatively affect AM colonisation and sporulation depending on factors like the host plant, the active substance, rates of application and time lapse since application of the herbicide (Abd-Alla *et al.*, 2000; ChangJin and Bin, 2004; Garcia–Romera and Ocampo, 1998).

The use of non-mycorrhizal crops in the rotations, for example plants belonging to the *Chenopodiaceae* or *Brassicaceae* families, leads to a reduction in mycorrhizal propagules. In contrast, the cultivation of mycorrhizal host crops increases AM fungi populations and maintains mycorrhizal activity in soil (Vestberg *et al.*, 2005). As a result there tends to be much greater spore densities present (Black and Tinker, 1977; Karasawa *et al.*, 2000a) leading to improved colonisation of the succeeding crop, which might only take place in the following season (Gavito and Miller, 1998a; Miller, 2000). The elimination of weeds which can act as AMF hosts is a factor that may be especially important in rotations involving non-mycorrhizal plants (Gosling *et al.*, 2006).

The multiplicity of roles that could be played by weeds of an agro-ecosystem in relation to arbuscular mycorrhiza formation requires careful planning of the timing and method for their control if the benefits are to be captured in terms of crop production.

2.5 Nutrient management

The appropriate provision of macro and micro nutrients is a critical part of crop husbandry, to ensure both production potential and environmental safeguards are achieved. Investigations over the last two decades have

indicated significant potential for the use of arbuscular mycorrhizas in enhanced nutrient management practices.

2.5.1 Phosphorus

In most agricultural systems the application of P to the soil is necessary to ensure plant productivity. Phosphorus is largely taken up by plants from soil solution as inorganic PO_4^- ions. The recover of P applied as fertilizer or organic amendment by crop plants in the growing season in which it is applied is very small, as in the soil more then 80% of the P becomes immobilized and unavailable for plant uptake because of adsorption of ions on soil surfaces, precipitation of mineral phosphates or conversion to the organic form (Holford, 1997). Between 20 and 80% of soil P is present in organic form (Richardson, 1994). The conversion of organic P into inorganic forms and the consequent availability to plants depends on hydrolysis either by microorganisms or by enzymes originated in the organisms themselves (autolysis). The remaining P is found in the inorganic (Pi) fraction, which may contain up to 170 mineral forms (Holford, 1997). Pi may be held very firmly in crystal lattices of sparingly soluble forms, such as various calcium (Ca), iron (Fe) and aluminium (Al) phosphates, and may also be bound to the surface of clay minerals. This Pi exchanges very slowly with ions in the soil solution and constitutes a non-labile pool, which is regarded as unavailable to plants. A small but less tightly bound P fraction exchanges relatively rapidly with the soil solution and constitutes a labile pool that is available to plants.

Under conventional management practices, the role of the soil biota, including both saprophytic and mycorrhizal fungi, in nutrient cycling has been largely marginalized by the use of agrochemicals, particularly fertilizers but including fungicides, herbicides, and pesticides that counter disease and pests but can adversely affect other biota. Accumulation of P in the soil from applications of animal manure or fertilizer in excess of that taken up by the crop can increase the risk of P movement to surface and groundwater, with serious consequences for the aquatic environments. However, with increased societal

pressures to reduce the use of agrochemicals and fertilizers, a greater reliance on processes influenced by soil biota, and specially AMF are assuming greater relevance. According to Grant *et al.* (2005) management of the cropping system to improve the availability of P to the crop early in the growing season may improve P nutrition while reducing the potential for excess accumulation of P in the soil and the risk of its transport into water systems. This requires a detailed understanding of the process governing soil P cycling and availability in which AM symbiosis may play a significant role.

Provision of adequate P early in crop development is usually directly related to an improved final grain yield (Gavito and Miller, 1998b). For example, in maize, early P nutrition increased the number of kernels per plant, and similar increases in number or biomass of reproductive structures are reported for other crops (Gavito and Miller, 1998b). The corollary is that deprivation of early P leads to an initial reduction in shoot growth accompanied by a temporary stimulation of root growth (Mollier and Pellerin, 1999).

An increase in the absorption of P by mycorrhizal plants can result by both the increased physical exploration of the soil and by increased transport into mycorrhizal fungus hyphae. Modifications of the rooting environment can enhance the transfer of P to plant roots and changes in the efficiency with which plants utilise P can all contribute to more effective crop nutrition (Bolan, 1991).

Arbuscular mycorrhizal and non-AM plants show markedly different kinetics for P absorption, indicating that AM fungi hyphae have greater affinity for phosphate ions and a smaller threshold concentration for absorption than do plant roots (Smith and Read, 1997). P is translocated in hyphae as polyphosphates and translocation rates can be affected by concentration gradients and cytoplasmic streaming (Jakobsen, 1992). Sanders and Tinker (1973) observed that the rate of inflow of P into AM roots was much greater than that of non-AM plants. By assuming that the difference in the rate of inflow was all due to the AM fungi, they calculated that transport via mycorrhizal hyphae was six times that through a root hair.

Diffusion, rather then mass flow is the most important delivery system of P to plants, and movement through the soil is much slower than the rate of uptake into the roots. This results in a depletion zone around the roots (Smith

and Read, 1997). The size of the soil P depletion zone is significantly larger in AM plants then in non-AM plants. Li *et al.* (1991) found differences over 10 mm in some cases but in others this difference was greater than 110 mm, depending on the plant host colonised and the fungal isolate involved. In addition they estimated that 80% of plant P could be supplied by AM fungi hyphae from as far as 100 mm distance beyond the zone of direct root exploitation. Bucher (2007) concluded from analysis of physiology, molecular and cell biology, and genetics of P uptake in vascular plants that soil P availability and the formation of P depletion zones around roots and their mycorrhizas are the major physical parameters determining plant P acquisition efficiency.

A wide range of biological events, and consequent environmental changes, occur in the rhizosphere and particularly in the mycorrhizosphere, leading to direct or indirect effects on the availability of sparingly soluble forms of P. The exudation of organic substances by roots and their formation and excretion by AM fungal hyphae might be important in facilitating host plant accessibility to available P or making P more available (Bolan, 1991; George *et al.*, 1995). Koide and Kabir (2000) showed that extraradical hyphae of *Glomus intraradices* can hydrolyse organic P and can transport the resultant inorganic P to host roots. However, direct evidence that organic acids such as oxalic and citric acid, which can make sparingly soluble P sources available, have only recently been reported for AM fungi. Tawaraya *et al.* (2006) developed a system that allowed them to collect exudates specifically from hyphae. Their results showed that hyphal exudates can actually contribute to increased P uptake into colonized roots. They found no oxalic acid production, and suggested that the type of organic acid produced might be related to the specific mycorrhiza and soil in which the fungi grew. They also stressed the importance of soil pH in relation to the ability of hyphal exudates to solubilize P.

Other examples of mycorrhizosphere environmental modifications include the presence of P solubilizing companion microorganisms and changes in pH following AM colonisation of roots. P is more readily available at pH 6.5, but for pH values above this the amount of sparingly soluble Ca-phosphates

tend to increase, and for pH values less than 6.5 the levels of Al and Fe-phosphates increase.

In the rhizosphere, alkaline phosphatase activity is commonly greater than in the bulk soil, which contributes to increased availability of sparingly soluble P compounds. However mycorrhizal hyphae do not appear to influence soil phosphatase activity, even where hyphal length density is considerable (Joner *et al.*, 1995), but they can influence alkaline phosphatase secretion by other microorganisms, probably through competition for nutrients (Joner and Jakobson, 1995). In fact the quantitative importance of extracellular phosphatases for P nutrition in AM plants, which has frequently been used to indicate promotion of P uptake by mycorrhizas, is considered to be insignificant relative to the total phosphatase activity in soil (Joner *et al.*, 2000).

Jayachandran *et al.* (1989) suggested that siderophores produced by AM fungi or other soil microbes could significantly increase P availability in low-pH soils, and that is a feasible mechanism by which AM plants could acquire P sources unavailable to non-AM plants.

Phosphorus acquisition by AM plants varies not only with plant species and cultivar but also with the AM fungus colonizing the roots (Munkvold *et al.*, 2004). The influence of the AM fungal species or isolate is effective at different levels: the size of the depletion zone, on the speed and rate of soil exploration by hyphae, phosphatase activity, the effectiveness of the symbiosis (as measured by the proportional AM benefit compared with the benefit from added P), P uptake by hyphae, shoot P content, growth performance at a given pH and accumulation of polyphosphates in the extraradical mycelium.

Fertilizer application, particularly P fertilizer and the associated increase in soil P level, decreases AM fungal infectivity and effectiveness (Dickson *et al.*, 1999; Kahiluoto *et al.*, 2000; Sorensen *et al.*, 2005) and spore density (Chandrashekara *et al.*, 1995). Irrespective of high levels of P in the soil, it is the concentration of P in the root system that determines whether AM fungi colonize roots (Menge *et al.*, 1978). This suggests that the effect of P level in the soil on AM formation appears to be indirect, through the influence on P concentration in plant tissue, rather than directly on the fungus in the soil. This may explain why fertilizer application does not always reduce mycorrhizal associations. Fairchild

and Miller (1988) observed extensive colonisation at very high rates of fertilizer addition. Paradoxically, if the available P in the soil is very small, AM and spore production may be restricted and AM may increase as a result of P application (Grant *et al.*, 2005). For example, sunflower grown in a sand medium that was almost P-free showed only poor mycorrhizal development and the infection increased as P was added (Koide and Li, 1990).

Less soluble forms of P fertilizer, such as rock phosphate, have a very slow effect on P availability and mycorrhizal colonisation of the host plant is favoured. The slow release of P likely prevents the P concentration in root tissue from reaching a level that can inhibit the formation of a mycorrhiza.

Also the moderate application of farmyard manure seems to be less detrimental to root colonisation by AM fungi than is the application of the same amount of nutrients in the form of inorganic NPK fertilizer (Joner *et al.*, 2000). Gryndler *et al.* (2006) reported reduced growth of AM fungi (assessed by hyphal length and the signature fatty acid 16:1ω5) following mineral fertilizer application, but a manure application increased the growth of AMF. Colonisation of roots followed the same pattern as the growth of AM fungal hyphae. In contrast, Allen *et al.* (2001) reported a greater AM colonisation of dry bean and sweet corn in unamended than in manured soils.

Although no changes in AM fungi species composition were observed, cumulative P in soil decreased the size of the AM fungi communities (Kahiluoto *et al.*, 2001 and 2004), but did not affected the hyphal P transport capacity of hyphae (Kahiluoto, 2004). Fertilizer application also seems to select for inferior AM mutualists (Johnson, 1993).

Each combination of AM fungus, plant species or cultivar, and soil environmental condition is unique with several possibilities in terms of outcome for beneficial effects on the plant and in terms of the uptake of P and other nutrients (Kahiluoto, 2004; Jansa *et al.*, 2005). However, the evidence for other nutrients is limited as they have been studied less. Pandey *et al.* (2005) found P uptake by wheat, rye and triticale was 10%, 64% and 35% greater respectively, in the presence of mycorrhizas than in their absence. The authors suggested that the differences in the enhancement of P uptake induced by mycorrhizal formation in triticale seem to be inherited from the wheat genome rather than

from the rye. Different species of the same genera of the AM fungal symbionts, *Glomus mosseae* and *Glomus intraradices*, also exhibit a degree of functional complementary in terms of spatial P acquisition (Drew *et al.*, 2003).

Burleigh *et al.* (2002) investigated whether functional diversity between AM fungi species is limited to the level of mycorrhizal formation, plant nutrient uptake and plant growth. Their study advanced current understanding of functional diversity and showed that plants can respond differently to AM fungi at the morphological and physiological levels and also at the level of gene expression. Functional diversity in mycorrhizal Pi uptake in different plant-AM fungi combinations and the control mechanisms involved are likely to be dependent on the molecular cross-talk between plant and fungal symbionts. Bucher (2007) suggested that to improve our knowledge of the role of fungal and plant metabolic status, elucidation of the chemical signals that orchestrate P transporter gene expression will possibly be the critical step towards a systems view of P uptake dynamics.

2.5.2 Nitrogen

In addition to the commonly reported enhanced P acquisition by AM plants, enhanced nitrogen (N) acquisition is also often reported (George *et al.*, 1995). Although it is thought that AM fungi have little ability to increase plant uptake of more mobile ions such as NO_3^-, as these move rapidly through the soil to roots, they do transport the less mobile NH_4^+ (Smith and Read, 1997).

Corkidi *et al.* (2002) observed that enrichment with N consistently decreased root colonisation by AM fungi in grasses grown in soils with high P availability, but not when they were grown under conditions of limited P availability. Such results point to the conclusion that when mycorrhizal costs exceed the benefits AM colonisation is severely reduced, and are consistent with the hypothesis that N fertilizer application alters the balance between costs and benefits in mycorrhizal symbiosis. The authors also observed that AM fungal communities from N fertilized soils are less mutualistic than those from unfertilized soils.

Whether AM can take up or transform organic forms of N has long been a subject of debate, but recently Hodge *et al.* (2001) found that AM can enhance decomposition and increase N capture from complex organic material (grass leaves) in soil, independently of the host plant.

Considerable research has been conducted on the benefits conferred by the simultaneous AM colonisation and nodulation of legumes with N_2 fixing bacteria. A synergistic effect between both microsymbionts and the host leguminous plant lead to the idea of a tripartite symbiosis (El-Hassanin and Lynd, 1985; Niemi and Eklund, 1988) and has been explained on the basis of a high P demand in the N_2 fixation process, which is offset by AM symbiosis (Stribley, 1987; Smith and Read, 1997). However, there is now evidence that P uptake is not the main driver for the development of the tripartite symbiosis. In fact both symbioses share transduction pathways and effects of the tripartite symbiosis (e.g. increased nodulation) can be observed within 10 days after plant emergence, when the seedling still relies on P contained in the cotyledons (Goss and de Varennes, 2002; Antunes *et al.*, 2006a).

Variation in the enhancement of N acquisition by AM plants may also occur with different AM fungal isolates (Azcón-Aguilar *et al.*, 1980). Antunes *et al.* (2006b) found no difference in N_2 fixation of soybean plants colonized with *Glomus clarum* or *Gigaspora Margarita*, however Chalk *et al.* (2006) highlighted the current paucity of quantitative data and lack of understanding of the interactions of legume genotype with AM fungal species in respect of the potential of AM to enhance legume yield and symbiotic dependence, particularly under field conditions.

Common mycorrhizal networks can interconnect component intercropped species or cultivars by extending AM mycelia from one plant roots to another (He at al., 2003). The direct transfer of N from soybean to maize mediated through AM fungi has been shown by van Kessel *et al.* (1985) and between berseem and maize by Frey and Schüepp (1992). He *et al.* (2003) reviewed evidence for one-way transfer of legume-N to a non N_2-fixing mycorrhizal plant and a few studies showing transfer from non-fixing mycorrhizal plants to N_2-fixing mycorrhizal plants as a kind of bidirectional transfer. Hauggaard-Nielsen and Jensen (2005) believe that a better understanding of the mechanisms

behind facilitative interactions may allow a greater benefit from these phenomena in agriculture and environmental management.

2.5.3 Other Nutrients

Clark and Zeto (2000) reviewed a number of studies where improved acquisition of sulphur (S) was observed in AM plants relative to uncolonized plants. Even though hyphae have not been shown to be highly active in S transport, the uptake of sulphur in AM plants appears to be very dependent on host plant, AM fungal symbiont, temperature and soil pH.

High concentrations of boron (B) in soil may have negative effects on root AM colonisation (Ortas and Akpinar, 2006) and B acquisition in AM plants seems to be relatively inconsistent. It has been reported as enhanced (Kothari *et al.*, 1990), reduced (Clark *et al.*, 1999) or not affected (Lu and Miller, 1989) in shoots of AM plants, and it is also reported that AM fungi vary extensively in their ability to acquire or restrict availability of B (Clark *et al.*, 1999). A similar variability in nutrient acquisition, including the response to biotic and edaphic factors, has been noted for potassium, calcium, magnesium and sodium (Clark and Zeto, 2000).

Uptake of both Zn (Ryan and Angus, 2003) and Cu are enhanced in AM plants but to a lesser extent than reported for P. These nutrients may not be as readily translocated from roots to shoots as is P, but the amount is probably adequate given that plants have a smaller requirement for these micronutrients. Its distribution in roots and shoots depends on soil P level. Zn, like P, is diffusion limited and its mobility in the soil is very small. AM involvement in Zn nutrition has been implicated from the negative effects of both tillage (Evans and Miller, 1988) and long fallow (Wellings *et al.*,1991) on crop growth. Beneficial responses to AM fungal colonisation vary according to the different environmental conditions impacting the plants, especially soil pH.

AM plants generally acquire less manganese (Mn) than non-AM plants (Arines and Vilariño, 1989; Bethlenfalvay and Franson, 1989) although in acid soils, where Mn is more soluble, enhancement of Mn has been reported (Habte

and Soedarjo, 1995). Alleviation of Mn toxicity has also been reported frequently, it seems to be due to a more favourable equilibrium of Mn oxidising and reducing microorganisms in the mycorrhizosphere of AM plants (Nogueira *et al.*, 2004).

Acquisition of Fe by AM plants is strongly affected by soil pH and the fungal symbiont. Amounts are reported to be enhanced or reduced depending on the conditions (Al-Karaki and Clark, 1998; Nogueira and Cardoso, 2002).

The presence of some elements also affects the way others are taken up in the presence of AM fungi. Liu *et al.* (2000) reported that, in the experimental conditions tested, the effect of AM fungi on Zn, Cu, Mn, and Fe uptake varied with micronutrient and P levels added to the soil.

For potentially toxic elements (e.g. cadmium, plumb, nickel, barium, arsenic) the ability of AM to growth in heavily contaminated sites has long been reported (Heggo *et al.*, 1990; Ahmed, 2006). Apparently the toxic elements are sequestered in the hyphae, in the polyphosphate granules, and not transferred to the plant (Smith and Read, 1997; Rivera-Becerril *et al.*, 2002). Even when transferred their negative influence on the plant metabolism is not critical because in AM plants the vegetative part is more developed allowing the dilution of trace elements. Different isolates have diverse tolerance to excess levels of many trace elements (Turnau *et al.*, 2001). The size and diversity of AM fungi populations may be modified in metal-polluted soils (Del Val *et al.*, 1999) and it appears that a prolonged exposure to this type of elements can result in the development of tolerance by the AM fungi (Oliveira *et al.*, 2001).

2.6 Water management

It is known that AM colonisation affects water relations of plants, particularly during drought periods (Kothari *et al*, 1990; Subramanian *et al.*, 1995; Sieverding, 1986). As with other aspects of physiology of AM plants, it is important to distinguish direct effects of AM colonisation on drought resistance from indirect effects resulting from changes in plant size and nutritional status induced by mycorrhization. Various mechanisms are pointed out as responsible

for the drought alleviation on AM plants. These include more effective scavenging of soil water (Sieverding, 1991), hormonal involvement (Krikun, 1991), improved P nutrition (Fitter, 1988), improvement in structure caused by extraradical mycelium (Smith and Read, 1997; Augé *et al.,* 2001) and also improved soil roots contact, stimulation of gas exchange through increase sink strength and possible effects on osmotic adjustment as reviewed by Augé (2001). Contribution of soil hyphae to water absorption is also pointed out although some authors consider direct water transport through hyphae to plant improbable or little significative (Kothari, 1990; George *et al.*, 1992).

AM symbiosis affects soil structure and this affects water retention properties. Therefore it seems likely that mycorrhizal symbioses will affect soil moisture retention properties. A path analysis modelling approach preformed by Augé (2004) revealed that soil hyphal colonisation had larger direct and total effects on dehydration tolerance of bean than did root hyphal colonisation or several other soil or plant variables.

Mycorrhizal effects on plant water relations are not as dramatic and consistent as those on P acquisition on host growth (Ryan and Ash, 1996). However, modest changes, if sustained, can have meaningful effects on plant fitness. Augé (2001) is critical about many studies that offer only momentary or short-term snapshots of leaf or root water relation behaviour but, viewed as a whole, the literature suggests a substantive if only occasional AM influence on host water relations and drought physiology.

Drought water stress may influence not only the AM plant behaviour, but previously to that, the way colonisation itself is established, leading to a low level of colonisation (Ryan and Ash, 1996). Experiments made by Karasawa *et al.* (2000a, 2000b) with maize under various soil moisture conditions revealed that colonisation improved with increasing soil moisture status, even when cultivated after a non-AM crop, thus promoting AM formation, P uptake and plant growth. The increase in AM colonisation with the increase in soil moisture status, despite the limited AM inoculum suggests that greater soil moisture improves the efficiency of AM colonisation, promoting AM formation and masking the influence of a less favourable AM fungal population in the soil, thereby reducing the influence of a negative cropping history.

Al-Karaki and Clark (1999) observed that AM colonisation increased as inoculum rate increased, in plants grown with and without water stress, although leaf area and shoot and root dry matter and also mineral acquisition traits increased as inoculum rate increased up to the second, out of 3 levels of inoculum tested (120, 240 and 360 spores per 100 g of dry soil). These results suggest that the soil moisture level along with the rate of AMF present influences the response to root colonisation.

The diversity of inoculum present can also make a difference in terms of AM induced drought resistance. After testing a commercial inoculum Quilambo *et al.* (2005), verified that it only had a positive effect on growth under well-watered conditions, under drought conditions the indigenous Mozambican inoculum preformed better as it is more adapted to the water limited circumstances of the region.

More recently Atkinson (2004), through the measurement of soil water potential, argued that relative to non-mycorrhizal plants, AM plants are better adapted to their environment in terms of the rational use of water. He found that AM plants used less water as the soil water potential decreased, so that rather than having access to more water they used the same quantity of water but over a longer period than did non-AM plants.

2.7 Pesticides and AMF

The use of pesticides, particularly fungicides, appears to impair mycorrhizal formation and development. In fact most of the time the effects of these chemicals are detrimental to AM fungi (Manjunath and Bagyaraj, 1984; Salem *et al.*, 2003) although the degree of toxicity varies with the active ingredient, the application rate (Habte and Manjunath, 1992) and the AM fungus (Schreiner and Bethlenfalvay, 1997). Systemic fungicides would be expected to be more detrimental to AM fungi then non-systemic ones. However, this distinction does not seem to be a key variable. For example, captan, a non-systemic fungicide, can significantly reduce mycorrhizal formation while conversely triadimefon and pyrazophos, both systemic fungicides, actually

promoted AM formation (von Alten *et al.*, 1993). Kjøller and Rosendal (2000) concluded that external hyphae are more sensitive to the application of systemic fungicides, than are the internal hyphae.

An extensive review made by Menge (1982), on the effects of many fumigants and fungicides specifically on AM fungi, is highly recommended despite the passage of time since it was published. A general analysis, confirmed by Vyas and Vyas (2000), indicates that for AM development, soil-applied products should be avoided.

2.8 Conclusion

There is a lot of available information in the literature about the diverse range of benefits granted by AM in the context of agronomic production. In general there are clearly identifiable trends, although always dependent on the specifics of the system: AM can play an important role in nutrients acquisition in unbalanced soils, whether by excess (Al, Mn) or deficiency (P) of some nutrients, enclosing a great potential to overcome some of the major production limitations of the Alentejo cropped soils. Arbuscular mycorrhiza are favoured by reduced or no-till systems, mainly because an important component of the symbiosis, the extraradical mycelium, is kept intact. In addition they may act together with other components of the systems, like weeds, in a cooperative way.

There are important gaps in knowledge that give rise to great uncertainty about the sustained presence of AM under Mediterranean climate conditions in agricultural production systems typical of those used in the Alentejo. Understanding the dynamics of AM in this system, may constitute an important step forward in terms of establishing more sustainable crop production strategies for this region.

3. Effect of conventional tillage and no-till systems on arbuscular mycorrhiza colonisation of winter wheat under Mediterranean conditions

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are a main component of the soil in most agroecossystems. The obligate symbiosis they form with the majority of crop plants (Smith and Read, 1997) is usually described as mutually beneficial because the fungus benefits from the carbohydrates produced by the plant and in return the plant benefits from several advantages most of then derived from the enlarge volume of soil exploited by the extended hyphal network of a colonised root system. As a direct effect the absorption of mineral nutrients is facilitated, mainly the less mobile nutrients, like phosphorus (P). AMF can also enhance the host plant resistance to root pathogens by a systemic bioprotectional effect (Khaosaad *et al.*, 2007) and the tolerance to abiotic stress, such as drought (Al-Karaki, 2004). The direct effect of AM hyphae on soil aggregate formation was shown by Thomas *et al.* (1993), to be significant and at least equivalent to that of roots alone, supporting Tisdall and Oades (1982) concept of AM contribution to aggregate formation.

Wheat is the main small grain cereal crop produced in Portugal. More than 80% of the total national production is from the Alentejo region (INE, 2007), in southern Portugal. It is sown from October to December (depending on variety) and harvested between June and July of the following year. This sequence includes a short vegetative cycle of only five or six months, which together with both climatic and edaphic conditions leads to very small average yields (1,500 kg ha^{-1}).

Mediterranean climates are characterized by the mild and wet winter and dry and hot summer. In these conditions, the crop does not perform very well due to the precipitation being concentrated during the winter season and its distribution is irregular. The hot summer causes the grain filling period to be short, leading to a lower yield potential than that found in temperate regions.

Yield potential under Mediterranean conditions is very much dependent on the grain number per unit of area, and this depends on the autumn and winter growth of the crop.

Wheat varieties differ greatly in degree of colonisation by AMF (Azcón and Ocampo, 1981) and in the ability of the symbiosis to improve biomass and yields, ranges from negative (a parasitic effect) to 5 times greater yields than non-mycorrhizal plants (Karagiannidis and Hadjisavva-Zinoviadi, 1998; Ryan *et al.*, 2005). Modern breeding programs seem to be one of the most important factors contributing to such a wide diversity. Current wheat cultivars are much less responsive to AMF compared with the consistent AM dependence (benefit) of wheat cultivars released before 1950 (Hetrick *et al.*, 1993; Zhu *et al.*, 2001). Mycorrhiza-inducible P transporter genes have been reported in wheat (Glassop *et al.*, 2005) and over 50% of P uptake by wheat plants may be absorbed via AM fungi (Li *et al.*, 2006) even though available soil P content may depress AM colonisation in wheat (Ryan *et al.*, 2005; Mohammad *et al.*, 2005). The threshold observed by Covacevich *et al.* (2007), under the conditions studied, was 27 mg P kg^{-1} soil. Above this concentration, indigenous AM colonisation stabilized at a minimum of 10%. Both the soil P threshold and the minimum AM colonisation values must be considered in planning the fertilizer regime to be applied for increasing the growth of wheat without depressing mycorrhiza formation.

Little is known about seasonal variation in mycorrhizal development of agricultural crops under field conditions and it is widely believed that AM percentage colonisation of wheat is small, but this is not necessarily the case. AM colonisation can be determined only in late spring (Hetrick *et al.*, 1984) and can range from 1 to 40% within the 2 months of seeding (Dodd and Jeffries, 1986). Saif and Khan (1975) reported that more than 50% of root segments of winter wheat were mycorrhizal a month after seeding. Soil temperature seems to have an important influence on AM development allowing only small AM fungi colonisation during winter months following seeding, when soil temperature is low, but increases gradually during spring (Mohammad *et al.*, 1998; Al-Karaki *et al.*, 2004).

The direct effects of the various tillage practices on AM, namely conventional tillage (CT) and no-till (NT) systems are related to physical disruption of the extra radical mycelia network (Evans and Miller, 1990) and the mixing of surface residues within the soil profile, thereby limiting the effectiveness of the AM symbiosis. The number of spores, length of extraradical mycelium and hyphal density, particularly in the row zone (Kabir *et al.*, 1998a), have been found to be enhanced when soil disturbance is reduced and the mycelia network is preserved and not detached from roots. This leads to a greater AM colonisation of plants cultivated under NT systems then CT systems (Evans and Miller,1988; McGonigle and Miller, 1996a; Kabir *et al.*, 1997a; Kabir *et al.*, 1998b; Boddington and Dodd, 2000a; Galvez *et al.*, 2001; Borie *et al.*, 2006; Castillo *et al.*, 2006).

When host plants are present and the soil is not disturbed, hyphae from colonised roots and soil mycelia network are the main source of inoculum. They are more rapid and efficient in initiating colonisation (Read *et al.*, 1976; Martins and Read, 1997) than spores, which can be considered as "long-term" propagules (Kabir, 2005), mainly because spores take longer to germinate and make contact with roots compared with infection by runner hyphae from well developed extraradical mycelium (Klironomos and Hart, 2002). Furthermore, deep ploughing (to more than 15cm) hinders subsequent mycorrhiza formation by reducing propagule density in the rooting zone (Kabir *et al.*, 1998b; Abbott and Robson, 1991).

The faster AM colonisation observed in undisturbed or no-till systems supports earlier uptake of P (Vivekanandan and Fixen, 1991; McGonigle and Miller, 1996b; McGonigle *et al.*, 1999).This can confer a comparative advantage to the crop in the initial growth period that commonly translates into improved final grain yield, irrespective of soil P content (Gavito and Miller, 1998b; Fairchild and Miller, 1990; McGonigle and Miller, 1996b), although not always (Vivekanandan and Fixen, 1991). Such an outcome is highly desirable for wheat production under the specificity of Mediterranean conditions, where the biomass build up to the flowering stage is decisive for grain yield (Carvalho, 1987).

Generally, colonisation of winter wheat is affected by site-specific environmental and agricultural conditions. Considering the impracticality of

inoculation on a large scale and the perspective of sustainable production, the potential to make effective use of mycorrhizal symbioses in Mediterranean agriculture depends on developing a further understanding of the functional ecology of AMF in agroecosystems as influenced by management strategies such as tillage.

This experiment was conducted in the field to tests the following hypotheses:

- Soil disturbance and mixing of surface residues induced by tillage will influence the ability of indigenous AM fungi to produce spores and to colonize autumn-sown wheat under Mediterranean conditions.
- There is a seasonal pattern of AM colonisation of dry land winter wheat.

3.2 Materials and methods

Wheat is not a common host plant for mycorrhizal colonisation studies due to the great variability in reported effects, which appear to be particularly dependant on site-specific environmental conditions. In contrast, maize or legumes, such as soybean, are frequently studied as host crops because of their commercial value and because they are commonly considered as being very mycotrophic. Their mycotrophic nature makes the studies easier to interpret since the effects are very obvious. However, given the specific climatic, agronomic and socioeconomic conditions in the Alantejo Region of Portugal, wheat was the appropriate crop to study despite the lack of information with which the results of this investigation could be compared.

3.2.1 Experimental site and soil

The experiment was conducted under field conditions at the Revilheira farm (Regional Agriculture Experimental Station) near Reguengos, Alentejo (38°28´N 7°28´W) in 1999-2000 and 2000-2001. This site has been used since 1995 as part of a large research program requiring soil tillage and crop rotation experiments. Two tillage systems were compared: conventional tillage

(mouldboard plough followed by a disk harrow)(CT) and no-till, direct seeding (NT). The rotation was wheat-triticale-sunflower, although the focus was directed at wheat as the main crop in the rotation. The experiment had a randomized block design with 3 replicas. To have the 3 elements of the crop rotation present in each year, 3 adjacent fields were used and crops rotated among them (Fig. 3.1). The soil was a Luvissol and the chemical characteristics of the 0-10 cm and 10-20 cm soil layers for the two tillage treatments are given in Table 3.1. The soil analyses were performed in the Soil Chemistry laboratory of Évora University. Soluble P and K were extracted by the Egner-Riehm method (Riehm, 1958) and P was quantified by molecular absorption in ascorbic acid and ammonium molybdate, and K analysed by flame photometry. Organic matter was quantified according to Anne (1945).

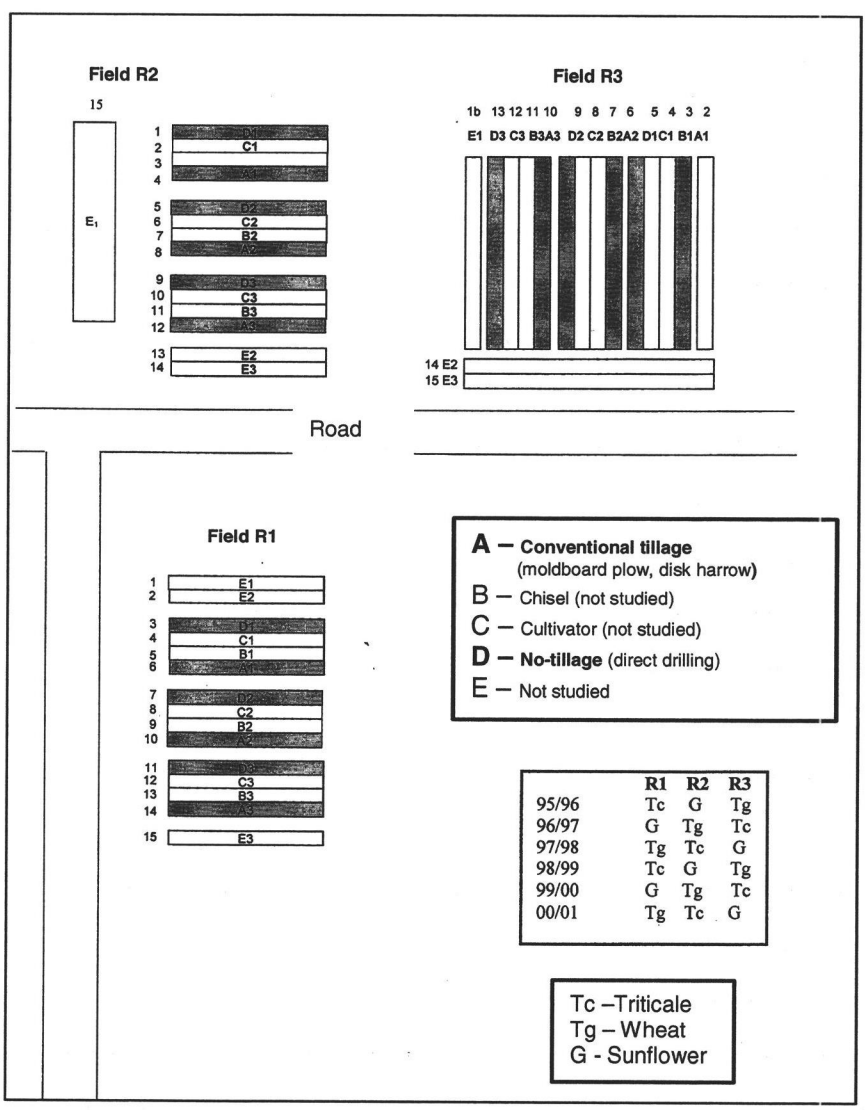

Figure 3.1 – Experimental layout of the Revilheira field experiment.

3.2.2 Field and laboratory procedures

In the second week of November 1999, the CT plots were mouldboard ploughed and then disk harrowed to control weeds and prepare the seed bed. Wheat (*Triticum aestivum* L., var. Coa - a regionally developed variety) was sown on November 19th, at a seed density of 190 kg ha^{-1}. Fertilizer (150 kg ha^{-1} of 18.46.0) was broadcasted at seeding. In January 2000 post-emergence chemical weed control was preformed with diclofop-methyl, (3 L ha^{-1} Iloxan by Bayer) and tribenuron-methyl (20 g ha^{-1} Granstar by Dupon). In February 2000, crops received a top dressing of 150 kg ha^{-1} of ammonium-nitrate 26 %. The crop was harvested on July 6th.

Plots of the NT treatment received pre-seeding weed control just before sowing, preformed with glyphosate (1.5 L ha^{-1} Roundup by Bayer), and then were sown using a John Deere direct seeder. All other procedures were the same as those described for CT plots.

Weather data was obtained from Centro Geofísica de Évora, Reguengos meteorological station.

3.2.2.1 Root sampling and AM colonisation rate

Root sampling for AM colonisation assessment started in March 2000, because that was when the colonisation became evident in previous experiments, and ended in June 2000, when the crop was close to maturity. At approximately 2 week intervals, roots from 3 plants of each plot were sampled with a hand-operated soil probe (5 cm diameter) to a 20 cm depth. The 3 soil cores collected were taken to the laboratory and stored at 4°C.

As soon as possible after collection, the root samples were carefully washed, and by visual discrimination fine white roots were selected. Thick, lignified roots were avoided as they did not stain uniformly. The roots were cut into approximately 2 cm long and stained. The staining procedure consisted of:

a) clearing in 10% (w/v) KOH in an autoclave for 15 minutes at a temperature of 121°C to eliminate cytoplasm contents. b) profuse washing with tap water to remove excess KOH. c) staining in a solution containing 0.1% Trypan Blue in lactoglycerol (1:1:1 glycerol, 80% lactic acid, water) for 10 minutes at 65°C. Trypan blue is a diazo dye that binds to chitinaceous structures of fungi but not to root material. d) storage of stained roots in 50% (v/v) glycerol-water. In addition to preserving stained roots for long periods (years) this solution dissolves Trypan blue that not attached to fungal structures, resulting in a better contrast between roots and fungal structures.

Assessment of AM colonisation was by the grid line intersection method of Giovannetti and Mosse (1980): 3 sub-samples were observed under a dissecting microscope at x 40 magnification. Roots were spread out evenly in a plastic Petri dish with a grid of lines marked on the bottom of the dish. When assessing the percentage of infected roots, the gridlines only act as a device for the systematic selection of observation points. Vertical and horizontal gridlines were scanned and the presence or absence of infection was recorded at each point where roots intersect a line. For each observation (sub-sample) 200 root/gridline intersects were recorded.

3.2.2.2 Spore extraction and counting

In two consecutive years 1999 and 2000, before sowing but after the seed bed preparation in CT plots, soil samples were collected from the top 20 cm of the profile to quantify AMF spores. From each plot a composite sample was obtained, which was sieved and air dried. Two sub-samples of dried soil from each plot were taken for spore extraction according to Gerdemann and Nicolson (1963) as follows: a) 100 g of dry soil was mixed with tap water in a 1L beaker and passed through sieves (710 µm and 45 µm mesh), b) the soil was washed through the sieves with running water using a shower head where necessary c) the contents of the 45 µm sieve were backwashed into a 50 mL centrifuge tube, until the latter was half full, d) an equal volume of 60% (v/v) commercial sugar (sucrose) solution was gently added to the pellet at the

bottom of each tube using a syringe with a plastic tube extension, to form an obvious interface between the water (above) and sugar phase (below) e) the capped tubes were centrifuged at approximately 2500 rpm for 3 minutes in a bench centrifuge, f) spores caught at the interface between the two layers were removed with the syringe and tube attachment, starting above the interface and working down into the sugar phase using a circular motion as some species produce spores which can sink in the sugar solution while others can float just above the interface, g) the contents of the syringe were dripped into a clean 45 µm sieve, and washed thoroughly to remove traces of sugar solution, h) material on the sieve was backwashed into a Petri dish and viewed under a stereomicroscope. A Petri dish with circular wells, and normally used to count nematodes, was adapted to facilitate the counting.

The spore counting was made according to two broad morphotypes: small hyaline spores and all the other. Only bright, apparently viable spore were counted.

3.2.3 Statistical analysis

The observations followed a normal distribution confirmed by Shapiro-Wilk's W test (Shapiro *et al.*, 1968) and homogeneity of variances was confirmed by Levene's test (Conover *et al.*, 1981).

The data were analyzed with MSTAT-C (version 1.42, Michigan State University) statistical package as one factor randomized complete block design, with 2 levels for the factor (NT and CT) and 3 repetitions. When the F-test of the treatment mean square indicated that there were significant variances (α= 0.05) due to treatment effects, means were highlighted "*".

3.3 Results

Compared to the 30 years average, the rainfall during the period under study was unpredictable and the distribution of precipitation was erratic (Fig 3.2). This is typical of a Mediterranean climate. Before flowering (April 2000),

there was only 26% of the 30 years average rainfall for that period. In contrast rainfall during April was more than three times the 30 years average for the month, and it was concentrated in very intense events (Fig. 3.2). May was also wetter; about double the 30 years average.

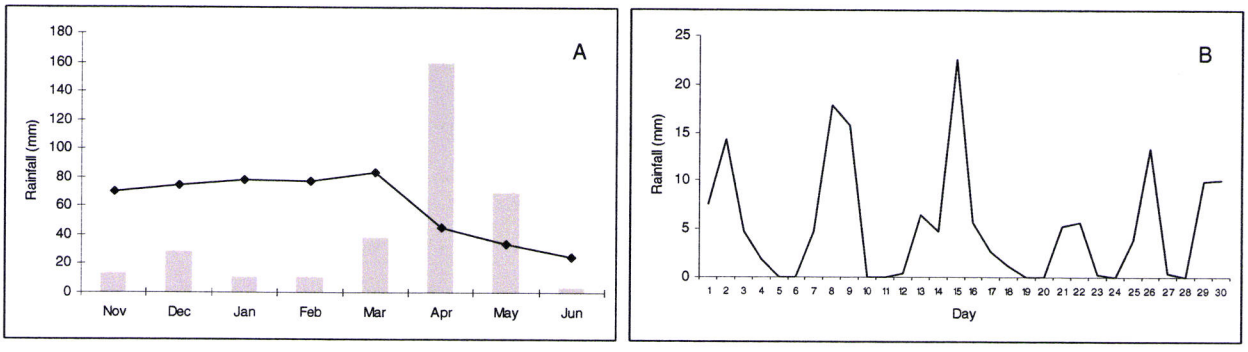

Figure 3.2 – A - Rainfall (mm) on the period under study (bars) and 30 years mean (line). B - Daily rainfall (mm) in April 2000. (Source: Centro Geofísica de Évora, Reguengos meteorological station)

During the winter months, particularly in November and January, the mean temperature was 2 to 3 °C less than the 30 years average. Furthermore, in the early spring months the temperature was as much as 2 °C cooler than the 30 years average. Overall, the temperature regime during the studied period did not diverge greatly from the 30 years average, although it can be considered as a slightly cooler growing season (Fig. 3.3).

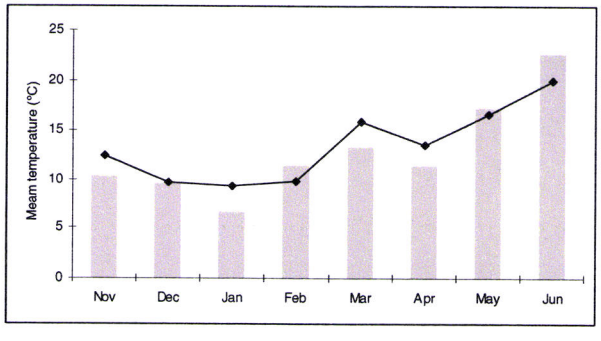

Figure 3.3 – Mean temperature (°C) on the period under study (bars) and 30 years mean (line). (Source: Centro Geofísica de Évora, Reguengos meteorological station)

There was more available P and K and a greater OM content in the top 10 cm of NT plots than in the conventionally tilled soil (Table 3.1). As expected, the values for the two soil layers in CT were not very different, likely because of the inversion and mixing of soil during cultivation. For K_2O, the concentration was greater in the top 10 cm under NT but in the 10-20 cm layer under CT.

Soil pH varies between 5.3 and 5.9 with no relevant differences across tillage systems or soil layers.

Table 3.1 – Essential chemical characteristics of the soil from the field experiment.

Field Plot	Soil layer (cm)	P_2O_5 (mg kg^{-1})	K_2O (mg kg^{-1})	pH (water)	OM (mg g^{-1})
No Till	0-10	59	83	5.3	16
	10-20	25	59	5.7	9
Conv. Tillage	0-10	19	59	5.6	10
	10-20	16	63	5.6	10

In 1999-2000 cropping season, the difference in the number of hyaline spores and other spores between NT and CT plots was not sufficient to be statistically significant at $p \leq 0.05$ (Table 3.2). In 2000-2001 cropping season the number of hyaline spores was also not significantly different at $p \leq 0.05$ between NT and CT treatments (Table 3.2). However the total number of spores was greater in NT plots, because significantly more spores counted as "other" were present in NT plots then in CT plots.

Table 3.2 - Effect of soil disturbance (no-till and conventional tillage) on the number of spores in 100g of the top 20cm of soil.

Soil Disturbance	Cropping Season	Hyaline spores	Other spores	Total number of spores
No-till	1999/2000	12.5	22.3	34.8
Conventional Tillage		6.8	16.3	23.2
SEM		2.78	5.81	6.37
CV (%)		40.78	42.54	31.07
No-till	2000/2001	57.3	45.1*	102.4
Conventional Tillage		53.2	26.1	79.3
SEM		22.74	6.51	16.39
CV (%)		58.17	25.83	25.51

For each sampling date means in the same column followed by * are significantly different (α=0.005). SEM - standard error of the mean, CV – Coefficient of variation

Tillage affected the AM colonisation of wheat. It was greater in NT plots from the beginning of sampling period and over the rest of the vegetative growth phase, being highly significant for the first 4 sampling dates (Table 3.3). The difference between tillage treatments was particularly evident in the second sampling date at the beginning of heading stage, when AM colonisation in NT was more than 9 times that in CT plots. It should be noted that a few days before that sampling occasion (Julian day 102) there was an intense rainfall event (Fig. 3.2). Thereafter, AM colonisation continued to increase in wheat cultivated under both tillage systems, although with a much slower progression in the CT plots. By the end of the flowering stage, although AM colonisation had increased in NT plots, any difference between tillage treatments was not significant at $p \leq 0.05$.

For both tillage systems, AM colonisation rate increased until late spring and declined after the plants reached the grain filling stage. The oscillations of AM colonisation rates recorded between the day 102 (12thApril) and 145 (25thMay) might have been influence by atypical weather events (Fig. 3.2).

Table 3.3 – Effect of soil disturbance (no-till and conventional tillage) on wheat arbuscular mycorrhiza colonisation (%) over the vegetation cycle.

		Wheat Arbuscular Mycorrhiza Colonisation (%)					
Sampling	Julian days	88	102	118	131	145	159
Growth stages	Feeks scale	10	10.2	10.5	10.5.1	10.5.3	10.5.4
	Description	stem elongation	heading	heading	flowering	flowering	grain filling
Tillage regime	No-till (NT)	15.7 *	16.9 *	23.1 *	15.2 *	21.1	14.9
	Conventional Tillage (CT)	6.8	1.8	5.4	5.3	10.8	8.6
	NT/CT	2.3	9.3	3.9	2.9	1.9	1.7
	SEM	1.28	1.49	2.37	1.75	3.03	2.25
	CV (%)	16.13	22.64	23.52	24.10	26.88	27.18

For each sampling date means in the same column followed by * are significantly different (α=0.05). SEM- Standard error of the mean, CV – Coefficient of variation

3.4 Discussion

The number of spores and other forms of inoculum present in soil at the beginning of the cropping season considered to be related to the previous crop in the rotation, given that the latter supported the development of the AMF which produced the majority of spores present. Consequently, the cultivation of a mycorrhizal host crop, such as sunflower, tends to increase AM fungi populations and maintain mycorrhizal activity in soil (Black and Tinker, 1977; Arihara and Karasawa, 2000; Karasawa *et al.*, 2002; Vestberg *et al.*, 2005).

It can be assumed that sunflower (harvested in late July 1999) was a good preceding crop since the presence of active roots during part of the dry summer season enabled the presence of AM inoculum for wheat, the main crop of the rotation.

The average number of spores counted per 100g of dry soil in 1999-2000 season was quite small in comparison to that found in the 2000-01 season, or in similar studies (Jansa *et al.*, 2002). It is possible that there was an unidentified problem in the spore isolation procedure in the first season. The decanting process, an opening in the sieves or an incorrect manipulation of the sucrose injection could have contributed to the small spore count.

The average number of spores counted per 100g of dry soil in the 2000-2001 season was in the same range as that reported Stahl *et al.* (1988) after a mycotrophic crop but, a bit less than the values presented by Troech and Loynachan (2003) under similar circumstances, and considerably smaller then the numbers presented by Saif and Khan (1975). However it should be noted that spore number is influenced by season (Schalamuk *et al.*, 2006), having the largest increase after the period of maximum root growth (Saik and Khan, 1975). The soil sampling for spore counts in the present study was preformed about 2 months after sunflower was harvested, as the aim was to establish how many were available as spore inoculum for wheat at the beginning of the cropping season.

The total number of AMF spores was greater under NT then under CT as reported by Abbott and Robson (1991), Boddington and Dodd (2000a), and

Castillo *et al.* (2006), but the number of hyaline spores was not affected by tillage intensity unlike the results of Mozafar *et al.* (1998). The relationship between soil organic matter content and number of spores found by Klironomos *et al.* (1993) is evident in this experiment, as soil organic matter content was greater in NT than CT plots.

The number of spores classified as "others" was significantly affected by the tillage system but not the number of hyaline spores. Consistent with reports from Douds *et al.* (1995), Jansa *et al.* (2003), Oehl *et al.* (2003) and Castillo *et al.* (2006), the results indicate that AMF species, or morphotypes, are differentially susceptible to soil disturbance. Considering the scale of the groups defined it is not reasonable to comment on the influence of tillage on particular taxonomic groups of AMF. However these results point the importance of an in depth study on the effects of tillage practices on AMF biodiversity.

Spores are only a part of the possible AMF inoculum and are not necessarily directly correlated with the rate of colonisation (Abbot and Robson, 1982) given that hyphae are considered the main source of inoculum in soil (Sylvia and Williams 1992). Moreover germination of AMF spores occurs more slowly or is entirely inhibited at soil temperatures below 18°C (Daniels and Trappe, 1980; Koske, 1981), making possible colonisation of wheat immediately after seeding predominantly dependent on extraradical mycelium. AM colonisation of wheat at the first sampling date (Julian day 88) was much greater under NT then under CT conditions and the same trend was consistent over the growing period. This is consistent with soil tillage disrupting extraradical mycelium and causing detachment from roots thereby adversely affecting AM colonisation, as previously reported for maize and wheat (Castillo *et al.*, 2006).

Winter wheat colonisation by AM fungi was detected only after the warming of surface soil until the temperature reached and remained above 13°C. Once established, the level of colonisation increased with time, peaking at flowering in both tillage treatments. A subsequent decline in colonisation was observed and at grain filling stage the difference between the tillage systems became less evident. The same AM colonisation pattern was described for

wheat by Mohammad *et al.* (1998) and Al-Karaki *et al.* (2004) where AM development seems to go along with root development.

Apparently C4 and C3 plants have different patterns to their rate of colonisation according to their metabolic strategy (Arias *et al.*, 2001). In maize, a C4 plant, AM colonisation rate decreases over time until flowering; whereas in C3 plants, like wheat, the AM colonisation rate increases until the flowering stage and then declines thereafter.

Sampling started quite late in the crop cycle because in previous investigations (data not shown) AM colonisation was not detected on wheat roots before March. The technique used for assessment of AM colonisation developed by Giovannetti and Mosse (1980), did not allow the detail obtained with the technique developed by McGonigle *et al.* (1990) and adopted in subsequent studies. The AM colonisation rates obtained in this study are possibly somewhat underestimated, and any colonisation that took place before the first sampling date might have been missed.

The unusually large rainfall occurring in April, but also in May, might have been the cause of variations in the progression of colonisation rate observed on the 2nd and 4th sampling dates. In NT, where soil drainage is more efficient, these variations are less evident. Poor drainage conditions can interfere with spore production, reducing it (Troech and Loynachan, 2003). It is also known that host plant can have some control over mycorrhizal fungi activity via a regulation of carbohydrate transfer, one way being the regulation of arbuscule numbers (Koide and Schreiner, 1992). Under water stress conditions these mechanisms can be active and eventually contribute to oscillations of AM colonisation rate during the period. These oscillations can also be attributed to the normal course in the development of new roots after an intense rainfall period, favoured in CT treatment where the resistance for the growth of new roots is less important.

The relatively high level of soil available P (59 mg kg^{-1}), especially in the top 10 cm layer of NT plots, may function as inhibitor of AM development on wheat (Covacevich *et al.*, 2007). However, AM colonisation did occur under NT, reaching 23%, indicating that indigenous and therefore more adapted isolates of AMF population can colonize wheat despite high levels of soil available P.

In agronomic systems AM colonisation and crop responsiveness is the final product of a complex chain of interactions between anthropogenic and environmental factors. To study the effect of soil disturbance on AM fungi under field conditions is subject to many constraints.

This was a first field approach to identify possible effects of tillage on the development of AM in winter wheat under Mediterranean conditions. The study was carried out within a long term field experiment that had been carried out for more then 10 years with very specific agronomic objectives. With only a few parameters investigated, the effects of soil disturbance on winter wheat AM colonisation were evident, and suggested several other questions that required answers: what is the ability of extraradical mycelium to survive over the Mediterranean dry summer and start new colonisations in the next cropping season, does the increased AM colonisation of winter wheat observed under no-till have any effect on plant P content, on crop biomass or grain production? However, irrespective of a direct effect on the crop yield, the use of no-till clearly supported AM development and biodiversity and naturally diverse and spatially heterogeneous agroecological systems are definitely desirable as their resilience may be decisive in dealing with a multiplicity of possible biotic and abiotic stresses.

3.5 Conclusions

Based on the test of the initial hypotheses, this field experiment supports the flowing statements:

- Sunflower was a good preceding crop for winter wheat under Mediterranean conditions.
- The total number of spores was greater in NT than CT plots, and AMF are differentially susceptible to soil disturbance.
- Wheat was colonised by indigenous AM inoculum, despite the high level of soil available P.

- AM colonisation rate of wheat was significantly greater in the NT treatment than the CT treatment during most of the vegetative growth of the crop.
- AM colonisation of winter wheat under Mediterranean conditions increased gradually until late spring and then declined. This pattern followed that of root development.

4. Infectivity of arbuscular mycorrhiza extraradical mycelium after Mediterranean summer and the effect of soil disturbance

4.1 Introduction

Arbuscular mycorrhizal (AM) colonisation can be started by three different types of propagules: spores, extraradical hyphae and colonised roots fragments. However the ability to produce new mycorrhiza (infectivity) is not the same for all of them depending on the AMF life-history strategy under specific environmental circumstances. The different strategies adopted by AMF to effect colonisation can be defined at the family level, showing that beside a morphological and developmental basis there is a functional basis to AMF taxonomy.

According to Hart and Reader (2002) members of the *Glomaceae* usually contact roots quickly and produce extensive mycelium in roots compared with in soil. Members of the *Gigasporaceae* typically contact roots more slowly and establish an extensive mycelium in soil rather than in roots. Members of the *Acaulosporaceae* also contact roots more slowly and establish a much less extensive mycelium in either roots or soil than members of the other two families. Overall, *Glomus* and *Acaulosposra* isolates colonise from all inoculum types, whereas *Gigaspora* and *Scutellospora* isolates colonise mainly from spores and to a limited degree from root fragments (Abbott *et al.*, 1992; Brundrett *et al.*, 1999; Klironomos and Hart, 2002).

The main hyphal types identified in the soil by Friese and Allen (1991) include the absorptive hyphae network, primarily involved in the acquisition of soil resources and runner hyphae that go along or among root segments and are the ones that form new infection units. Extraradical mycelium is quicker to start new colonisation than other sources of inoculum, mainly because it would take longer for spores to germinate and make contact with roots as opposed to runner hyphae colonisation from a well developed extraradical mycelium (Read *et al.*, 1976; Jasper *et al.*, 1989b). In addition if the number of spores is not very

great, mycorrhizal infectivity will depend more heavily on colonised mycorrhizal roots and on the hyphal network.

Disturbed soils are a consequence of tillage and therefore are frequently associated with agronomic practice. One consequence of soil disturbance is the disruption of extraradical mycelium network, and it is this process rather than detachment of hyphae from the host root (Jasper *et al.*, 1989a) that causes a loss of AMF infectivity. In addition, the mixing that is associated with tillage implements incorporates surface residues within the soil profile and reduces propagule density in the rooting zone (Abbott and Robson, 1991; Kabir *et al.*, 1998b). This is why no-till systems lead to a greater AM root colonisation compared with conventional cultivation (Evans and Miller, 1988; McGonigle and Miller 1996a; Kabir *et al.*, 1997a; Kabir *et al.*, 1998b; Boddington and Dodd, 2000a; Galvez *et al.*, 2001; Borie *et al.*, 2006; Castillo *et al.*, 2006). Soil disturbance will also differently influence the survival and persistence of propagules because of the variation in life strategies between AMF taxonomic groups. The relative importance of spores, colonised roots or hyphae as propagules for a given life strategy likely determines how severely infectivity is decreased by soil disturbance. In soils containing a large number of propagules overall infectivity is less affected by soil disturbance, especially if the inoculum is composed by a high number of spores or mycorrhizal rootlets, since the mycelia network is the form of propagule which is most sensitive to disturbance (Jasper *et al.*, 1991; Requena *et al.*, 1996).

Augé (2001) cites more then 150 studies about the effects of drought, aridity and soil moisture gradients on behaviour of AMF on several combinations of AM fungal and host species and duration of drought. In most of them the studied parameter is AM colonisation rate and the results are very variable depending on the blended factors. Within these references, only a few are concerned specifically with the survival of propagules and infectivity.

The survival and persistence of less resistant forms of inoculum, like extraradical mycelium, is also very much dependent on climatic conditions, namely soil temperature and moisture content. Under a Mediterranean climate, region Jasper at al. (1989b) produced the first direct demonstration of the ability of AMF external hyphae networks to maintain infectivity in dry soil. They verified that hyphal network of *Acaulospora leavis* could maintain its infectivity as soil

dries, matric potential reached – 21 MPa, for at least 36 days. Depending on whether sporulation had occurred at the time of the onset of drying, on the AMF taxonomic group, on the associated host plant and on any soil disturbance, the capacity of extraradical hyphae to remain infective under drought circumstances is variable (Jasper *et al.*, 1993; Klironomos *et al.*, 2001). In *Acaulospora leavis*, after sporulation had commenced, infectivity quickly declined with soil drying, making it unlikely to be adversely affected by soil disturbance once spores have formed. In contrast, infective hyphae of *Scutellospora calospora* can survive for at least 11 weeks, regardless of the timing of the onset of soil drying in relation to sporulation, and can even show increased colonisation following drought. Under these latter conditions, colonisation by *Glomus* species decreased or show no change (Jasper *et al.*, 1993). Apparently AMF that have invested heavily in an extensive external hyphal network might be less susceptible to drought as there would be greater access to the water in finer soil pores.

In Mediterranean climates autumn rains are sporadic and might wet the soil for a few days or weeks, but the rain is frequently followed by warm sunny weather and the soil and propagules dry. Depending on the predominance of different AMF and propagules, sporadic rainfall and associated drying can affect the occurrence of mycorrhiza. A false break is apparently more detrimental on the infectivity of spores than of hyphal network as it requires significantly more time and plant energy for spores to establish a functional mycelium under these circumstances (Braunberger *et al.*, 1996). Additionally in the absence of host plants, typical for dry season conditions in Mediterranean regions, hyphae assume an increasing importance as infective units (McGee *et al.*, 1997).

Provision of adequate P nutrition early in crop development is important for final grain yield (Gavito and Miller, 1998b), and faster AM colonisation often supports earlier uptake of P (Vivekanandan and Fixen 1991; McGonigle and Miller 1996b; McGonigle *et al.*, 1999), conferring a comparative advantage to the crop in the initial growth period, independent of soil P content (Fairchild and Miller, 1990; McGonigle and Miller 1996b) although not always (Vivekanandan and Fixen, 1991).

Phosphorus acquisition by AM plants varies not only with plant species and cultivar but also with the AM fungus colonizing the roots (Munkvold *et al.*, 2004). Within the same genera, AMF can exhibit a degree of functional

complementary in terms of spatial P acquisition (Drew *et al.*, 2003). Even intraspecific functional diversity has been detected in both *G. mosseae* and in *G. intraradices* (Avio *et al.*, 2006), although little variation in ^{32}P uptake by extraradical mycelium was detected among isolates of these two species originating from the same field site (Jansa *et al.*, 2005), suggesting some local adaptation. The influence of the AM fungi species or isolate is effective at different levels: the size of the depletion zone, on the speed and rate of soil exploration by hyphae, phosphatase activity, the effectiveness of the symbiosis (as measured by the proportional AM benefit compared with the benefit from added P), P uptake by hyphae, shoot P content, growth performance at a given pH and accumulation of polyphosphates in the extraradical mycelium. Burleigh *et al.* (2002) advanced current understanding of functional diversity and showed that plants can respond differently to AM fungi at the morphological and physiological levels and also at the level of gene expression. Functional diversity in mycorrhizal P uptake in different plant-AM fungi combinations and the control mechanisms involved are likely to be dependent on the molecular communication between plant and fungal symbionts. Elucidation of the chemical signals that orchestrate P transporter gene expression will possibly be the critical step towards a systems view of P uptake dynamics (Bucher, 2007). Given the complexity of P absorption in AM plants, calculations of AM contributions to P uptake from total plant P can often be highly inaccurate and the lack of plant responsiveness does not mean that an AM fungus makes no contribution to P uptake as perceived by many authors (Dekkers *et al.*, 2001; McGonigle *et al.*, 2003; Smith *et al.*, 2004; Li *et al.*, 2006).

Depending on distinct functional AMF groups, soil and environmental conditions, the colonisation ability and efficiency will determine the symbiotic performance of AM. This performance is not necessarily associated directly with plant growth promotion, but because of all the beneficial processes influenced by mycorrhiza, such as the enhanced resistance to several biotic and abiotic stresses and the promotion of soil aggregate formation, their formation should be encourage.

Mediterranean climates are noted for elevated temperatures and greatly restricted water availability in summer; features that are deleterious for crop growth. Knowledge of the ability of extraradical mycelium to remain infective for

the next crop under these conditions is important information because it will likely determine the time frame (from one cropping year to the other). The infectivity governs the potential impacts (faster or slower colonisation) through which AM influence early plant development. It also interferes with the establishment of agronomic management decision systems that recognize a role for mycorrhiza, since the need and timing of several field operations will be dependent on the potential for AMF colonisation.

This study was conducted to tests the following hypotheses:

- Extraradical mycelium of native AMF can survive the dry and hot summer season when plants are absent and start new colonisations on the onset of the growing season.
- Disturbance of the extraradical mycelium influences its ability to remain infective.
- Early AM colonisation is linked to early P nutrition in wheat .

4.2 Materials and Methods

Wheat (*Triticum aestivum* L., var. Coa) was chosen as the host plant because of its importance as a small grain crop in the region, and because of its use in my previous studies. To have better control of the factors involved, the experiment was conducted in clay pots and consisted of two steps.

The first step was performed according to the protocol developed in a preliminary experiment described in Annexe1. The objective was to promote indigenous mycorrhizal development associated with the studied host crop and to establish a differential AM potential through contrasting levels of soil disturbance (Fairchild and Miller, 1988). The greatest amount of disturbance was achieved by passing soil through a 4 mm sieve.

The second step took place once the difference in extraradical mycelium development between disturbed and undisturbed soil was established. In this step the effect of climatic Mediterranean summer conditions on AM infectivity of wheat and subsequent nutrients uptake was evaluated.

4.2.1 The cycles of disturbance technique

Fairchild and Miller (1988) developed a "Cycles of Disturbance Technique" to study the differences in AM colonisation and P absorption in disturbed and undisturbed soil. Air dried soil was sieved (5 or 4 mm mesh size), packed into the pots to a natural bulk density of approximate 1.2 g cm^{-3} and sown with wheat. Three weeks after emergence, plant shoots were excised and measured. Half of the pots were taken and the soil removed as two separate layers, which were each passed through a 4 mm sieve. All root material separated on the sieve was cut into 2 cm long segments and mixed into the soil of the appropriate layer. Soil was repacked in the pots to reconstitute the same layering. In the remaining pots the soil remained undisturbed. All pots were then reseeded and a new cycle initiated. The authors argued that the possible microbial flush of N (mineralization) in the soil caused by disturbance would be negligible compared with the relatively large concentrations of N (100 μg g^{-1}) added to the soil at the start of their experiment. In addition, they argued that this relatively small amount of N would mainly be released in the initial phase of the experiment. The effect of soil disturbance on AM infection could be mediated through changes in soil physical properties, however bulk density measurements were unable to discern any significant differences between the two soil treatments.

Sporulation by AM fungi mainly occurs when the plant is reaching the end of vegetation cycle because carbon flow from the plant to the fungus is reduced at that time, thereby inducing a stress situation and stimulating spore formation. Spores that develop during the plant vegetation cycle take in general some weeks to develop. Trap cultures used to study spores production take at least 2 months and some AMF, like many *Glomus* species (Brundrett *et al.*, 1999), only produced spores after a second pot culture generation. Hence the 3 weeks duration of each cycle mainly interferes with the non-spore forms of inoculum, i.e. extraradical mycelium and colonised root fragments.

Using this technique, and after 3 or 4 cycles of disturbance, greater colonisation rates have been observed consistently in maize and soybean

plants coming from undisturbed soil pots (Goss and de Varennes, 2002; McGonigle *et al.*, 2003; Antunes *et al.*, 2006a).

A number of advantages are associated with this technique, namely the fact that it requires no toxic compounds, causes little nutrient release, exploits the naturally occurring inoculum and allows a common history of inoculum and host plant throughout the successive cycles. To some extent, it also allows the simulation of a common agronomic practice like soil tillage and the study of its impact on AM. This approach was adopted here.

4.2.2 Experiment step 1

The soil, a Luvisol, was collected from the top 20 cm of arable field on the site used for the field experiment described in Chapter 3. At the start of the pot experiment it contained 28 mg P_2O_5 kg^{-1}, 94 mg K_2O kg^{-1}, 710 mg total N kg^{-1}, from which 20 mg NO_3 - N kg^{-1}, 15 mg OM g^{-1} and a pH (in water) 6.4. It was air dried and sieved (4 mm) before use. As about 300 kg soil was required, it was sieved in a cereal winnowing-machine. This soil was first used in the preliminary experiment described in Annexe 1. Then it was sieved (4 mm) again and mixed together to homogenize AM inoculum distribution. This sieving was carried out using net trays specially made for the purpose (Fig. 4.1 A). These were also used to sieve soil between each cycle of disturbance.

In February 2005 the soil was packed into 7 L clay pots to a bulk density of ~1.3 g cm^{-3}. The experiment was conducted in greenhouse from February to July. Day temperature inside the greenhouse ranged between 17 and 30°C and after March the cooling system was on. Photosynthetic active radiation (PAR) during last plant growth cycle was 350 µmol m^{-2} s^{-1}, with shadow protection to maintain temperatures at or below 30°C.

Six pre-germinated seeds were sown into each of the 50 pots and 5 days later thinned to 4 plants per pot, to give a similar plant density to that used in the field experiment. The pots were watered weight with distilled water, and after the 1st week this was applied from the base to avoid consolidation. Three weeks after planting the shoots were excised and their length measured before being dried at 70°C for 48 hours and weighed. In half of the pots the soil was removed

as two 10 cm layers and passed separately through a 4 mm sieve. All root material separated on the sieve was cut into 2 cm long segments and mixed into the soil of the appropriate layer. Soil was repacked in the pots and arranged in the same two layers and bulk density. In the remaining pots the soil was not disturbed. More pre-germinated seeds were then added to each of the 50 pots and a new cycle initiated. In total 5 cycles were preformed. In cycle 2, 4 and 5 one week after transplant, 50 mg N kg^{-1} (120 kg of N ha^{-1}) were applied (10 mL of 1M solution of NH_4NO_3 diluted on 100 mL of distilled water per pot). In cycle 4 plant laves showed Zn deficiency symptoms and on 3rd week of cycle 5, plants were supplemented with 3.4 mg Zn kg^{-1} (320 µL of 1M solution of $ZnSO_4.7H_2O$ per pot, corresponding to 8 kg of Zn ha^{-1} and 4 Kg of S ha^{-1}).

After the last cycle, in 28 July 2005, pots were buried in the field, with the top at surface level. This period is coincident with the normal dates for wheat harvest in the field.

4.2.3 Experiment step 2

In the first week of October pots were unburied, taken back to greenhouse and slowly watered to field capacity. Eight pre-germinated wheat seeds were sown into each of the 50 pots and 5 days later thinned to 4 plants per pot (Fig. 4.1 B and C). There was no disturbance of the soil at this stage because the objective was to evaluate the survival of the extraradical mycelium differentially developed by the soil treatments in Step 1 of the experiment. Pots were watered to weight from the top with distilled water and later from the base of the pots, according to plant needs. Application of N, Zn and S was made at the second week after planting and at the rates reported previously.

During step1 of this experiment the 5 days plants removed from the pots at thinning were stained for AM colonisation evaluation. In these roots it was minimal, indicating that attempts to make earlier detection of AM colonisation were not likely to be successful. Previous experiments have shown that in 21 days plants, differences in AM colonisation between disturbed and undisturbed soil were evident. The restricted soil volume in the pots limited the period of normal growth and development of the plants. Nevertheless, it was important to

follow, for as long as possible, the evolution of AM colonisation over time. Consequently, 3 sampling dates were defined: 10, 21 and 35 days after planting.

At each sampling date shoots were excised, their length measured, dried at 70°C for 48 hours and weighed. Plants were ground and analysed for nutrient content by colorimetry after ashing the samples at 500°C, dissolving the residue in 0.3 M HCl and submitting the material to mass spectrometry. These analyses were made in the Agricultural Chemistry and Environment Department, Institute of Agronomy, Lisbon. Nutrient acquisition was calculated multiplying nutrient concentration by the shoot dry weight.

Roots were carefully removed from seven pots per treatment and a random sample excised and brought to the laboratory. Thicker roots and the ones profusely developed in the base of the pot were avoided because these are usually very poorly colonised by AMF.

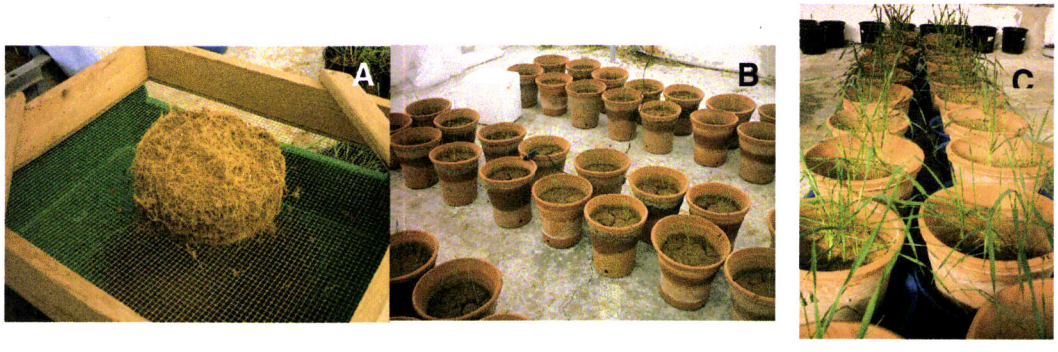

Figure 4.1 - A - Detail of the trays used to sieve soil with a 4mm mesh. B - Aspects of wheat growing in the pots a few days after planting. C – Wheat 21 days after planting.

In the laboratory roots were washed and stained with Trypan Blue according to the procedure described in Section 3.2.2.1 and kept in 50% (v/v) glycerol-water for observation of mycorrhizal colonisation under a compound light microscope. Assessment of AM colonisation was made by the magnified intersections method (McGonigle *et al.*, 1990). In brief, roots were mounted in glycerine on microscope slides aligned parallel to the long axis of the slides and covered with 40x22 mm cover slips. Three microscope slides were prepared for the root sample taken from each pot. Slides were observed at a 200x

magnification and the quantification was done by moving the field of view of the microscope across each slide perpendicular to its long axis. An approximately constant distance of a field of view was used between passes. Except when cortex was missing or for any reason the root visualization was not obvious, all intersections between roots and the vertical eyepiece crosshair were considered so that a minimum of 200 intersections per sample were evaluated. To examine each intersection the plane of focus was moved completely through the root and a record was made of any arbuscules, vesicles and hyphae intersected by the vertical crosshair. Records of intersections were stored in a multi-channel counter using the following categories: Negative (no fungal structures in the root), arbuscules (at least one arbuscule), vesicles (at least one vesicle) and mycorrhizal hyphae only (every effort was made to avoid making counts of other fungi). The proportion of each fungal structure was calculated as illustrated in Table 4.1.

Table 4.1 – Example of data obtained with the magnified intersections method

Root sample	Microscope slides	Number of intersections				
		Negative	Arbuscules	Vesicles	Hyphae only	Total
1	A	59	9	1	4	73
	B	54	7	0	6	67
	C	46	4	0	6	56
	Σ	159	20	1	16	196

Calculation of arbuscular colonisation (AC), Vesicular colonisation (VC) and Hyphal colonisation (HC) proceeds as follows. The value for AC is 20/196=0.1; for VC is 1/196=0.005 and for HC is (196-159)/196=0.19. (Adapted from McGonigle *et al.*,1990).

The magnified intersections method is preferable to the one previously used Giovannetti and Mosse (1986), because it allows a much better observation of fungal structures (Fig. 4.2), not only because roots are mounted in a slide instead of being disperse in a liquid medium but also because the magnification used is higher and the observation is much more detailed. Additionally if any possible difficulty comes into view, one can always change the magnification to 400x, make a more detailed observation and then come back to the 200x magnification and continue the slide observation. Besides, the discrimination within the several fungal structures allows more detailed information about the development of the colonisation.

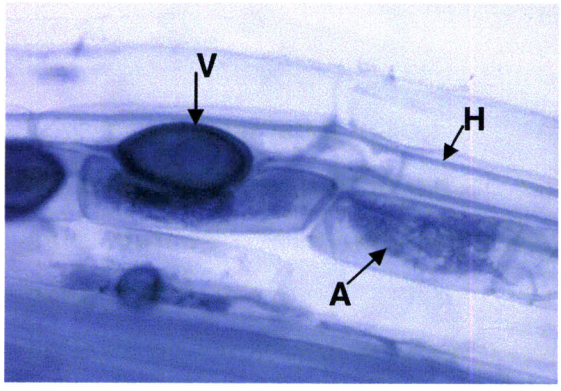

Figure 4.2 – Trypan Blue staining of AM colonisation in a wheat root with 3 weeks. A – Arbuscule, H – Hypha, V – Vesicle.

4.2.4 Statistical analysis

The observations followed a normal distribution confirmed by Shapiro-Wilk's W test (Shapiro *et al.*, 1968) and homogeneity of variances was confirmed by Levene's test (Conover *et al.*, 1981). Data were analyzed with MSTAT- C (version 1.42, Michigan State University) statistical package.

Step 1 of the experiment was analysed as two factors (soil disturbance and crop cycle number) with a randomized complete block design. Step 2 of the experiment was analysed as one factor (2 levels: disturbed and undisturbed soil) randomized complete block design combined over time (10, 21 and 35 days). When the F-test indicated that there were significant differences between treatments, means were compared using the Duncan test (α= 0.05).

4.3 Results

A noticeable decrease in all measured plant parameters occurred from the 1st to 2nd plant growth cycle. This decrease was particularly evident for dry weight and node length (Table 4.2). Thereafter, there was a gradual increase in all parameters. This effect might be related to growth conditions considering that temperature in this time of the year was increasing.

The Zn deficiency detected by the end of 4th cycle was corrected in the 5th cycle and all plant growth parameters also increased substantially in this

cycle. The most obvious effect was in shoot dry weight and shoot node length whereas the increase in plant height was less evident.

Consistent with the uniformity of soil disturbance on the beginning of 1st cycle there were no differences between any of the plant parameters measured at the end of this cycle. Significant differences developed during the 2nd cycle, at the end of which shoot height was significantly greater in disturbed than undisturbed soil, and this same trend continued for the 3rd cycle. It disappeared in the 4th cycle and the trend became inverted in the 5th cycle.

Table 4.2 – Effect of soil disturbance over 5 cycles on plant dry weight, shoot height and node length.

Plant parameters and Soil treatment	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle	SEM	CV%
Dry weight (g/pot)							
Undisturbed	0.44 e	0.26 g	0.29 fg	0.54 d	1.33 a	0.032	26.8
Disturbed	0.44 e	0.33 fg	0.36 ef	0.66 c	1.21 b		
Shoot height (cm/plant)							
Undisturbed	27.64 d	25.94 e	28.80 d	33.25 b	35.05 a	0.438	7
Disturbed	27.75 d	28.75 d	31.23 c	33.80 ab	34.30 ab		
Node length (cm/plant)							
Undisturbed	5.29 c	4.26 d	5.24 c	6.91 b	8.69 a	0.137	10.9
Disturbed	5.38 c	4.53 d	5.61 c	6.73 b	8.93 a		

Means followed by the same letter are not significantly different (α= 0.05). SEM - standard error of the mean, CV – Coefficient of variation.

No significant differences in node length between plants from undisturbed and disturbed soil were observed for any of the cycles.

After 1st cycle plant dry weight was greater in disturbed soil for all cycles, becoming significantly greater in the 4th cycle. However, after the Zn deficiency correction, the trend is completely inverted and in 5th cycle pant dry weight in undisturbed soil was significantly bigger. It can be speculated that if the application of Zn happed before, the difference between disturbed and undisturbed soil would have been significant in an earlier cycle.

It is important to remember that after the 5th cycle, clay pots were buried on the field, with the top at surface level, and remained there during the dry and hot season. Mean air temperature during August and September was 25 and 22°C, while the average values for a 30 years period were 25.5 and 23.1°C,

respectively. Maximum and minimum registered temperatures are showed in Table 4.3. There was no rainfall recorded.

Table 4.3 – Maximum and minimum air temperature during August and September 2005.

	August		September			August		September	
	Temperature (°C)		Temperature (°C)			Temperature (°C)		Temperature (°C)	
Day	Maximum	Minimum	Maximum	Minimum	Day	Maximum	Minimum	Maximum	Minimum
1	30.9	12.1	32.5	14.1	16	34.3	16.3	28.9	15.4
2	34.9	16.2	34.4	15.8	17	32.2	11.9	28.2	14.5
3	36.7	19.3	35.2	14.9	18	33.0	14.7	26.0	12.4
4	39.4	18.2	34.4	18.7	19	30.5	16.0	37.7	****
5	40.0	18.3	27.6	15.8	20	33.8	14.9	32.2	12.6
6	40.8	18.6	25.9	14.8	21	32.9	18.4	31.9	12.2
7	37.0	17.6	26.8	15.9	22	32.7	16.1	32.2	12.7
8	33.0	14.7	29.0	12.4	23	36.1	18.7	29.0	13.5
9	26.9	14.7	25.6	14.2	24	35.6	17.1	26.9	13.5
10	26.8	16.8	25.3	12.9	25	33.5	14.1	26.9	12.5
11	31.5	15.5	24.7	11.1	26	32.6	16.3	30.4	13.5
12	36.3	17.9	29.0	14.1	27	32.6	13.6	30.8	11.6
13	37.7	16.9	31.7	16.5	28	34.3	16.7	32.0	14.5
14	38.7	16.2	31.8	13.6	29	35.8	14.8	32.6	14.3
15	36.0	16.6	33.1	16.8	30	36.6	15.4	34.0	16.0
					31	29.4	15.6	-	-

(Source: Centro Geofísica de Évora, Mitra meteorological station)

Pots were removed from the soil and seedling sown in 6th October without any soil disturbance. In a field situation and under conventional tillage system, an additional soil disturbance by tillage would have occurred for seed bed preparation causing disruption of any extraradical mycelium developed during a previous crop. The option of doing the planting directly without additional soil disturbance was to be sure that any differences that developed between disturbance treatments resulted from differential mycelium survival over summer.

In Step 2 of the experiment, the evaluated parameters increased gradually with plant growth over time (Table 4.4). Slightly greater values of shoot height, node length and plant dry weight were found in plants coming from undisturbed soil treatment and harvested at 10 and 21 days after planting. In the sampling made 35 days after planting no differences between treatments in the measured plant growth parameters were significant.

For the first sampling date the values for AM colonisation parameters were very small, showing that AM colonisation was still insipient, and no differences between soil disturbance treatments in hyphal and arbuscular colonisation were significant at $p \leq 0.05$. The greater hyphal and arbuscular colonisation in plants from undisturbed soil treatment became significant 21 days after planting and at 35 days were still significantly greater in undisturbed soil.

Hyphal colonisation was always greater than arbuscular colonisation in agreement with the normal development of AM colonisation in young roots where colonisation is still spreading.

Arbuscular colonisation had exponential growth over time in both soil treatments. It is the parameter that more directly makes evident the establishment and functioning of mycorrhiza, since hyphal colonisation may take into account other fungi than AMF and vesicular colonisation is more informative in older mycorrhizal colonisations.

Table 4.4 – Effect of soil disturbance on wheat plants dry weight, shoot height, node length and arbuscular and hyphal colonisation, between 10 and 35 days after planting.

Previous soil treatment	Days after emergence	Plant parameters			AM colonisation parameters	
		Shoot height (cm/plant)	Node length (cm/pant)	Dry weight (g/pot)	Hyphal colon.	Arbuscular colon.
Undisturbed	10	20.92 c	4.25 c	0.11 b	0.10 c	0.04 d
Disturbed		20.53 c	4.08 c	0.09 b	0.07 c	0.02 d
Undisturbed	21	32.85 b	6.22 b	0.29 b	0.19 b	0.11 c
Disturbed		31.17 b	5.75 b	0.26 b	0.10 c	0.06 d
Undisturbed	35	38.41a	8.57 a	1.55 a	0.29 a	0.21 a
Disturbed		40.12 a	8.98 a	1.63 a	0.21 b	0.15 b
SEM		0.715	0.224	0.065	0.019	0.011
CV (%)		6.17	9.39	26.03	32.72	30.26

For each sampling period and soil disturbance treatment, means in the same column followed by the same letter are not significantly different ($\alpha = 0.05$). SEM - Standard error of the mean, CV – Coefficient of variation.

As expected, vesicular colonisation was not detected 10 days after planting since these structures are formed only when the colonisation is well established. At 21 and 35 days after planting some vesicular colonisation was observed. However values were very small and inconclusive and these data were omitted from further analysis.

All nutrients acquisition increased with plant development. Plant acquisition of all nutrients was similar in undisturbed and disturbed soil for the first two sampling dates (Table 4.5). A change in this trend occurred 35 days after planting. Acquisition of P, K, Cu and Mn by plants grown in disturbed soil was significantly greater than in undisturbed soil.

Table 4.5 – Effect of soil disturbance on plant nutrient acquisition at 10, 21 and 35 days after planting.

Soil Disturbance	Days after planting	Nutrients acquisition							
		P	K	Mg	Ca	Cu	Zn	Mn	Fe
		mg per pot				µg per pot			
Undisturbed	10	0.26 c	4.09 d	0.39 b	0.44 b	1.20 c	6.92 b	17.01 c	n.d.
Disturbed		0.23 c	3.57 d	0.35 b	0.39 b	1.27 c	6.71 b	14.32 c	n.d.
Undisturbed	21	0.55 c	12.29 c	0.92 b	1.12 b	2.76 c	21.16 b	50.61 c	36.01 b
Disturbed		0.43 c	9.02 cd	0.76 b	1.02 b	1.39 c	18.46 b	43.83 c	27.41 b
Undisturbed	35	2.44 b	46.38 b	3.65 a	6.17 a	8.72 b	99.27 a	303.67 b	121.54 a
Disturbed		3.12 a	55.53 a	3.87 a	6.06a	11.99 a	103.76 a	369.92 a	119.92 a
SEM		0.140	2.562	0.190	0.301	0.518	5.056	16.555	6.242
CV (%)		31.5	31.1	30.3	31.4	30.1	31.3	32.9	29.1

For each sampling period and soil disturbance treatment, means in the same column followed by the same letter are not significantly different (α= 0.05).
SEM - Standard error of the mean, CV – Coefficient of variation, n.d. – Not determined.

4.4 Discussion

A preliminary experiment, described in annexe 1, was preformed to elucidate if wheat was a suitable host plant to establish a differential AM potential through contrasting soil disturbance using Fairchild and Miller (1998) technique. After 4 cycles of disturbance plant development, all AM colonisation parameters and plant P content were significantly greater in undisturbed soil.

In step 1 of the experiment under discussion the generation of differential AM potential induced by soil disturbance didn't have any effect on the measured plant growth parameters.

As it was very laborious to gather the amount of soil needed for such an experiment the same soil was use in more than one experiment without any amendments, except for N, and later for Zn and S. A depletion of available nutrients, despite mycorrhization, might have somehow limited plant growth. However, in a similar experiment using recently collected soil from the same site and using the same host plant and P amendment, de Varennes and Goss (2007) also didn't find any differences in wheat development. This intermediate result didn't interfere with the planed progress of the experiment since the differential AM potential induced by soil disturbance was going to be tested in a later step.

After the soil had been through the Mediterranean hot and dry summer condition, AM colonisation was clearly favoured (faster and greater), in plants grown in undisturbed soil where extraradical mycelium was preserved, indicating that it was able to keep infectivity after Mediterranean summer, where matric potential can go lower than -28 MPa (Santos *et al.*, 2007) and maximum temperatures easily reach 40°C, despite the fact that in both soil treatments it was kept intact after the 5^{th} cycle of the first step of this experiment. These results confirm Jasper's *et al.* (1989b) work, one of the few concerning inoculum survival and soil disturbance developed in Mediterranean climate conditions and support observations of Li *et al.* (2006) by showing that the potential for colonisation in wheat is high and that the general belief that wheat is usually not well colonised is an over simplification.

Low temperatures, rather than high, are the usual concern in mycorrhizal research (Robinson *et al.*, 2001; Liu *et al.*, 2004; Aroca *et al.*, 2007) as temperature directly interferes with the functioning of the symbiosis by influencing the performance of chemical reactions. Entry *et al.* (2002) indicate that in general, AM formation takes place at temperatures ranging between 18 and 40°C with the optimum for most fungal-host species being close to 30°C. Not much is known in the literature about the influence of high temperature in soil inoculum survival. The presented results suggest that temperatures up to 40°C, combined with very low soil moisture content during a 2 months period, was not suffient to hamper extraradical mycelium survival. Furthermore, colonisation of a new crop was able to take place when temperature and moisture conditions became favourable.

The improved AM colonisation of wheat had no effect on plant development or increased nutrient acquisition, at least for the evaluated parameters and during the period under study, as previously reported by some authors (Karagiannidis *et al.*, 1998; Dekkers *et al.*, 2001; Mohammad *et al.*, 2005). Li *et al.* (2006) reported a wheat growth depression induced by AM colonisation at the early growth stage (~6 wk) that disappeared at maturity. These authors argued that vegetative growth, normally used as an index to evaluate the effectiveness of AM fungi on crops, is a poor predictor of reproductive growth for wheat.

For 21 days after planting, increased AM colonisation of plants from undisturbed soil treatment was associated with greater nutrient acquisition by these plants than by those from the disturbed soil, but after a further two weeks this trend was reversed.

Despite the large size of the pots used, after 35 days of plant development the available volume for root exploitation was limited, in addition the soil pH, between 6 and 7, did not favour P immobilization and both facts could have contributed to the lost of any possible AM effect on P scavenging. Thereafter, considering the limitations for plants development, some parasitic effect might have occurred in the plants with greater AM colonisation rate. Also different AMF might have been involved in root colonisation and caused the change in P acquisition. Braunberger *et al.* (1997) reported that only

mycorrhizas formed by *Glomus* spp. were observed in the early rewetting of the soil with autumn rains in a Mediterranean environment in the south-west of Western-Australia, whereas mycorrhizas of *Acaulospora*, *Scutellospora* spp. and fine endophytes, the latter being profusely present in wheat roots in this study (Fig. 4.3 A), were observed in a late break. For some *Glomus* species, spores are of minor importance as propagules and hyphae can be indifferent to soil dryness (Brundrett *et al.*, 1999; Jasper *et al.*, 1993), hence this genera tends to become the first coloniser of any plant root in a mixed soil inocula (Dodd *et al.*, 2000).

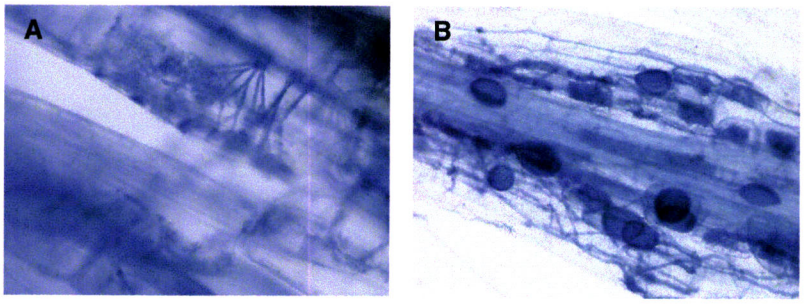

Figure 4.3 – Wheat root colonised with A - Fine endophytes (Morton, personal comun. July 2006) and B - *G. intraradices*.

By the time last sampling was made, infection started by spores should have already occurred and AMF with a preferable spore infecting strategy, like the ones belonging to *Gigaspora* and *Scutellospora* genera (Jasper *et al.*, 1993), may become more representative. In a study using 21 isolates Hart and Reader (2002) found some that colonised roots within 1 week, mainly *Glomaceae* isolates, while other isolates required 7 more weeks to colonise roots. These were the ones regenerating primarily from spores (i.e. members of the *Gigasporaceae*) because spore dormancy and specific environmental requirements for spore germination presumably slow the rate at which spore-regenerating can colonise roots. *G. intraradices*, a common species in this soil and frequently found in the studied wheat roots (Fig. 4.3 B), was described by Smith *et al.* (2004) as being highly efficient in delivery of P to three tested plant species, regardless of their responsiveness in terms of total plant dry weight or

P uptake. In the present study, *Glomus* spp are likely to have been the first colonisers, followed by other AMF groups, whose activity is not so evident at P acquisition level.

It should be remembered that the lack of plant response doesn't mean that AM colonising fungi make no contribution to P uptake. Mycorrhiza-inducible P transporter genes have been reported in wheat (Glassop *et al.*, 2005) and over 50% of P uptake by wheat plants may be absorbed via AM fungi (Li *et al.*, 2006), highlighting the fact that direct root uptake must be reduced because total P uptake is not increased.

From previous experiment (Chapter 3) the perception that soil disturbance interferes with AM fungal population of a particular site was evident. In this experiment the shift in P plant acquisition leads to the same idea, supporting the need for a deeper study on the AM fungal diversity induced by soil tillage regime. Apparently, beyond the biodiversity matter, there may be evident practical consequences on plant performance.

Wheat acquisition of trace elements Mn, Zn and Cu was lower in more greatly colonised plants. Karagiannidis *et al.* (1998) reported similar trend in the concentration of these trace elements when comparing mycorrhizal plants with non-inoculated ones. Lower shoot concentration of Mn in AM colonised wheat was also reported by Tarafdar and Marschner (1995) and Mohammad *et al.* (2005). A generally accepted theory supports that these metals are accumulated in the root region and they do not transfer to the shoots of the plants. Conversely Tarafdar and Marschner (1995) found shoot Zn concentration was enhanced in AM colonised wheat plants.

It is suggested by Mozafar *et al.* (2000) that not only the mycorrhizal fungus colonisation but also the concomitant colonisation of roots by non-mycorrhizal fungi needs to be taken into account in these kind of study. A cluster analysis revelled that concentrations of K and Zn were related to tillage and *Polymyxa* and that of Mn was related to *Gaeumannomyces-Phialophora* complex and mycorrhizal structures were not closely related to any nutrients in the wheat tops.

To take advantage of native AM, regardless of a direct effect on plant growth, it is necessary to understand nature of surviving propagules since it is

important to the management of plant growth in the cropping season. The temperature during wheat sowing date in Alentejo region (October, November) is quite low for the process of spore germination, making it even slower or inhibited. This experiment shows the ability of extraradical mycelium to survive Mediterranean summer season, enabling a faster AM colonisation in undisturbed soil conditions. The use of such information at filed level is important and can have practical implications: a) Earlier and greater mycorrhization occurs in undisturbed soil, consequently no-till or direct drilling systems should be preferred. b) The time lapse between the offset of autumn rain and sowing date and the presence of a host plant during this period may entail adjustments in weed control management to keep the best AM infectivity.

4.5 Conclusion

Consistent with the hypothesis tested, this pot experiment indicated that:

- Extraradical mycelium of native AMF can survive the dry and hot summer season when the host plant is absent and start new colonisations on the onset of the growing season.
- Soil disturbance reduces AM colonisation after the dry summer season and most probably influences the AMF population.
- Early AM colonisation is not necessarily associated with better initial plant development or early P nutrition in wheat.

5. Weed management and arbuscular mycorrhiza colonisation of wheat under Mediterranean climate

5.1 Introduction

Arbuscular mycorrhiza (AM) fungi colonize the roots of most agricultural crops and weeds (Yamato, 2004). In most of the cases AM fungi and weeds have co-evolved for longer time than AM fungi and crops, since crops are continuously manipulated by breeding programs that mostly ignore mycorrhiza formation as a selection trait.

AM plants develop an extensive extraradical mycelium network that enclose a multiplicity of roles such as exploiting larger volumes of soil for nutrient and water resources (Sieverding, 1991; Augé, 2004), enmeshing soil particles and contribute to soil aggregate stability (Tisdall and Oades, 1982, Goss and Kay, 2005), start new colonisations (Read *et al.*, 1976) and link AM colonised plants (Newman *et al.*, 1994). The facilitative effects mediated by the extraradical mycelium network of AM fungi suggest that special attention needs to be given to the agro-ecological functioning of weed communities.

It is generally considered that host species provide carbon compounds to the mycelium, which may use it to support formation of arbuscular mycorrhizas with other newly germinating second host species. Francis and Read, (1984) showed that transfer of carbon between plants connected by AM mycelium occurs primarily via the direct hyphal pathway. This allows the seedlings of the second host to receive nutrients and other mycorrhizal benefits while minimizing the energetic costs of mycorrhizal establishment to seedlings. For example weed communities in several cropping systems have been shown to enhance mycorrhizal colonisation and growth of subsequent crops, providing an alternative host between AM dependent crops (Kabir and Koid, 2000) or to maintain infective propagules over winter (Schreiner *et al.*, 2001). The negative impacts of fallow periods or the cultivation of non host crops on the AM inoculum might be mitigated by the presence of weeds on the field. Jansa *et al.* (2002) considered that the larger spore counts in no-till soil after a non-

mycorrhizal plant (canola), was due to the increased presence of mycorrhizal weed plants in the no-till plots. These weeds may have supported AM fungi development in their roots and also allowed some spore formation under the canola. In tilled plots, ploughing eliminated the majority of weeds and, therefore, AM fungi development during the growing of canola would be negligible. In an earlier study Miller and Jackson, (1998) showed that the number of AMF spores in soil was strongly correlated with the occurrence of weed hosts. In Dehérain plots Plenchette, (1989) also reported that when weeds were not controlled mycorrhizal infectivity was maintained. However this effect would be beneficial only if the species that received the additional resources was desirable, and would have economic importance under the prevailing conditions.

Abbott and Robson, (1981) suggested that the many factors associated with the differences in effectiveness of different AM fungi in stimulating plant growth result from their influence in the rapidity of infection rather than the ability of infected roots to take up phosphorus. Colonisation from spores is relatively slow because it has to go thought a successful biochemical dialogue between plant roots and the AM fungal spores, so contact with a common mycorrhizal network is the main method which seedlings are colonised by AMF in natural ecosystems (Read *et al.*, 1976). The existence of weed roots to support colonisation of a food crop may be important in that fast colonisation of the sown crop can take place from a well established mycelium network as soon as germination occurs. In natural ecosystems or no-till agricultural systems, where extraradical mycelium in not disturbed, young seedlings can germinate and "plug" into an already established AM fungi hyphal network which permeates the soil and links different plant species. The lack of specificity confers a great advantage for the success of AM fungi in mixed plant communities. Another benefit to the plant of interest is that carbon compounds from photosynthesis is needed only for maintenance of the AM fungi following colonisation and not for the development of the extraradical mycelium since it was pre-established (Dodd *et al.*, 2000). Although it is possible that AM fungi may have negative effects on the agro-ecological functioning of weed communities, simply by increasing abundance of problematic weeds (Jordan *et al.*, 2000) management of the existing weed population might provide an

important tool to guarantee a more rapid colonisation of a winter crop with the possible advantages of adequate early phosphorus nutrition.

Beside timing and energy advantages for crop AM colonisation, the mycelium interconnections among host species in a weed-crop mixture may also cause patterns of resource uptake and distribution among host species that differ qualitatively from those occurring in plant communities where AM fungi are absent (Moyer-Henry *et al.*, 2006). Some species may release nutrients into the AM fungal mycelium (Smith and Read, 1997) which may then be redistributed among other host species, enabling facilitative effects in crop-weed mixtures. The transfer of N, including that which has been symbiotically fixed, between colonised plants has often been described (e.g. van Kessel *et al.*, 1985; Frey and Schüepp, 1992; Moyer-Henry *et al.*, 2006) but P (Yao *et al.*, 2003) and other elements (Meding and Zasoski, 2008) also can be transferred. After selective weed control, nutrients acquired by the decomposing host weeds may be transferred to host crop or cover crop via the mycelium (Johansen and Jensen, 1996). Such processes may result in greater nutrient cycling and reduce competitive effects from non-host weeds. Properly timed control operations, such as sub-lethal post-emergence herbicide applications might be used to transfer nutrients from weeds to crops. In this scenario, the weeds might function as a temporary nutrient sink, restricting the competition for nutrients from non-host weeds and reducing leaching and other mechanisms of nutrient loss.

Facilitative functions may also occur in which one host species supports populations of mycorrhizal fungi that are beneficial to another plant species. Feldmann and Boyle (1999) studied the interaction of weed competition and AM fungi in a maize monoculture. They found decreases in the richness of AM fungal species and their effectiveness as symbionts in weed–free plots relative to those with weeds. Maize grew better in the presence of weeds and the authors concluded that the effective AM fungi over-compensate for any weed-mediated decrease in crop biomass. However, the benefit of enhanced AM fungi colonisation of maize observed by Galvez *et al.* (2001) in the absence of effective weed control did not translate into enhanced growth or yield. Abbott and Robson (1991) and also Baumgartner *et al.* (2005) reported a positive impact of weeds as host plants on the increase of AM fungi diversity. The

advantages of a diverse biological community are commonly known and excessive tillage to control weeds, associated with frequent cultivation of non-mycorrhizal crops, could hamper development of a diverse AM community (Gosling *et al.*, 2006).

The most commonly used mechanisms for weed control are mechanical disturbance and herbicide application, with crop rotation offering advantages that improve the effectiveness of these approaches as well as a longer-term alternative. They may all interfere with mycorrhiza formation, but to different extents.

Mechanical disturbance impacts the soil and affects AM through disruption of the hyphal network and the ability to start new AM colonisations. In addition deep ploughing (to more than 15 cm) hinders subsequent mycorrhiza formation by reducing propagule density in the rooting zone (Evans and Miller, 1990; Kabir *et al.*, 1998b).

The use of non-mycorrhizal crops in the rotations, like for or example plants belonging to the *Chenopodiaceae* and *Brassicaceae*, leads to a reduction in mycorrhizal propagules. The elimination of weeds which can act as AMF hosts is a factor that may be especially important in rotations involving non-mycorrhizal plants (Gosling *et al.*, 2006).

The effects on AM colonisation of two herbicides frequently used under no-till systems - glyphosate [N-(phosphonomethyl)glycine] and paraquate [1,1'-dimethyl-4.4'-bipyridilium ion] -, indicate that they are not seriously detrimental for the symbiosis. For example, soil application of glyphosate up to a dose equivalent to 10 L ha^{-1} had no effect on AM colonisation of a soybean crop seeded 10 days (Malty *et al.*, 2006) or 14 days (Silva *et al.*, 2006) after the herbicide application. Ryan *et al.* (1994) found no effect on AM colonisation levels of wheat after the application of a glyphosate base herbicide, used at field application rates. However, it has been described by Kaps and Kuhns (1987) glyphosate transfer between plants via AM fungi.

Abd-Alla *et al.* (2000) found that 60 days after application of a paraquat based herbicide in conjunction with cowpea or common bean, there were no significant differences in the AM colonisation rate of these crops or spore numbers in the soil compared with the control treatment.

Dodd and Jeffries (1989) studied the effects of field application rates of four herbicides on spore germination and infection of wheat roots by three species of *Glomus* under low P availability. Some of the herbicides completely prevented spore germination while others had no effect. However wheat colonisation was not affected by the herbicides treatments suggesting that AM hyphal development was less susceptible to herbicides than was spore germination.

After reviewing the work of several authors, Douds *et al.* (2005) concluded that a more relaxed attitude toward weed management might increase both the diversity and effectiveness of the AMF community. In practice, the planting of weeds may not be necessary, but proper management of some existing mycorrhizal weed populations, could be profitable both financially and ecologically (Kabir and Koide, 2000).

In rainfed agriculture under Mediterranean climate, where the precipitation is irregular but concentrated in the winter period whereas summers are long, hot and dry, crop development is prohibited in the dry season so there are no available AM host crops. Wheat production and productivity in the Alentejo region is greatly hampered by these climatic conditions. A good early development is critical for successful wheat crop. The proper management of weed populations that emerge after the first autumn rains might provide a valuable tool to ensure a quick and efficient AM colonisation of wheat young seedlings.

This series of experiments was conducted to test the following hypotheses:

- Weeds can act as bridge between crop plant hosts, allowing quicker AM colonisation of the autumn-sown crop.
- The method of weed control affects the potential for the bridging of AM colonisation.
- Weeds need to grow for a certain period before they can act as AM bridge for the following crop.
- Early AM colonisation is beneficial for the autumn-sown crop.

Two pot experiments were carried out to test the proposed hypotheses.

5.2 Materials and methods

For these experiments fresh soil (Luvisol) was collected from the top 20 cm of arable field on the site where previous field experiment occurred in order to have some consistency on collected data (18 mg P_2O_5 kg^{-1}, 72 mg K_2O kg^{-1}, 24 mg NO_3 – N kg^{-1}, 13 mg OM g^{-1} and pH (water) 6,1). It was air dried and sieved (4 mm) before use.

Weeds used in the study were chosen according to their relevance to regional agronomy (Calado, 2005), namely ryegrass (*Lolium rigidum* Gaudin), wild oats (*Avena sterilis* L.) and littleseed canarygrass (*Phalaris minor* Retz.). Wheat (*Triticum aestium* L., var. Coa) was chosen as host plant due to its importance as small grain crop in the region and the previous work on the crop.

5.2.1 Experiment 1

Soil was packed in 6L plastic pots, with a bulk density of approximately 1.3 g cm^{-1} and watered from the base to field capacity. On the 3rd February 2005 one pre-germinated seed of each weed species was planted in each of 30 pots. Pots were kept in green house and watered to weight from the base (Fig. 5.1).

Figure 5.1 – View of the experiment in the greenhouse. Weeds development after 2 months.

For the control treatment, with no weeds, 10 soil-filled pots with were left in the greenhouse without any manipulation. The greenhouse heat system was activated and the minimum temperature adjusted to 17°C.

Two months later, on 6th April, 2005 weeds were controlled. In two separate sets of 10 pots, the contact herbicide, paraquate (Gramoxone) and the systemic herbicide, glyphosate (Roundup) were applied in the usual dose for field applications, 10 mL L^{-1} (Fig. 5.2).

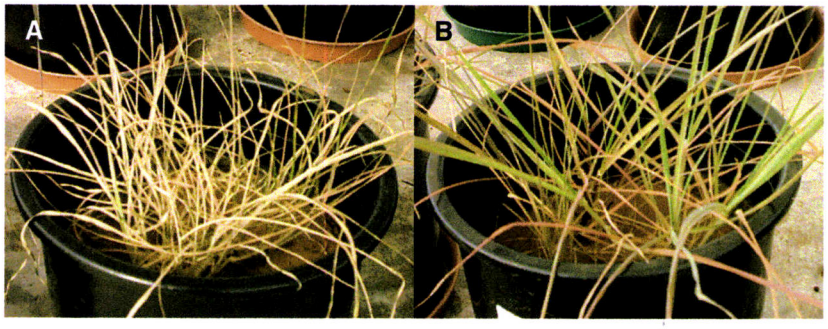

Figure 5.2 – Weeds aspect 48 hours after the application of paraquate (A) and glyphosate (B).

In another set of 10 pots, weeds were controlled by soil disturbance. The soil was removed as two layers and passed separately through a 4 mm sieve. All root material separated on the sieve was cut into 2 cm long segments and mixed into the soil of the appropriate layer. Soil was repacked in the pots and arranged in the same two layers. Shoots were cut into small fragments an mixed in the top layer (Fig. 5.3)

Figure 5.3 – Steps of weed control by soil disturbance.

On 18th April, 4 pre-germinated wheat plants were placed in each pot, including the ones that had been kept weed-free. Plants were kept in the greenhouse and were watered to weight from the base of the pot. To detect the

early evidence of AM colonisation plants were sampled at 14 and 21 days after planting. At each sampling date shoots from 5 pots per treatment were excised, their length measured before being dried at 70°C for 48 hours and then weighed. Plants were ground and analyzed for nutrient content by colorimetry after ashing the samples at 500°C, dissolving in 0.3 M HCl and mass spectrometry. These analyses were made in the Agricultural Chemistry and Environment Department, Agronomy Institute of Lisbon. Nutrient acquisition was calculated multiplying nutrient concentration by the shoot dry weight.

Roots were carefully removed from five pots per treatment. A random sample of fine roots was excised and taken to the laboratory. Thicker roots and the ones profusely developed at the bottom of the pot were avoided because these are usually very poorly colonised by AMF. In the laboratory, the roots were washed and stained with Trypan Blue according to the procedure described in Section 3.2.2.1 and kept in 50% (v/v) glycerol-water for observation of mycorrhizal colonisation under a compound light microscope. Three microscope slides were prepared for the root sample taken from each pot. Assessment of AM colonisation was done by the magnified intersections method (McGonigle *et al.*, 1990) as previously explained in Section 4.2.3.

5.2.2 Experiment 2

The soil from the previous experiment was used. It was again passed through a 4 mm sieve and thoroughly mixed before potting.

Based on the results of experiment 1, some adjustments were made to the experimental design. Specifically, the duration of weed development was shortened and the level of their colonisation by AMF determined, the nutrient content of the soil was amended and the wheat was allowed to grow for longer.

Weeds were planted in 9th March 2006 and develop for 1 month before wheat was planted. In 10th April 2006 weed control was made just with a systemic glyphosate herbicide (Roundup) or by soil disturbance. At this time in 5 of the pots, weed shoots separated by plant species were excised, dried at 70°C for 48 hours and weighed.

AM colonisation was assed in these plants. Roots were carefully taken and because it was not possible to discriminate between plant species a random sample was excised and brought to the laboratory. In the laboratory roots were washed and stained with Trypan Blue according to the procedure described in Section 3.2.2.1 and kept in 50% (v/v) glycerol-water for observation of mycorrhizal colonisation under a compound light microscope. Three microscope slides were prepared for the root sample taken from each pot. Assessment of AM colonisation was done by the magnified intersections method (McGonigle *et al.*, 1990) as previously explained in Section 4.2.3.

The control pots with no weeds were twice watered to field capacity over a 10 day period to promote nutrient leaching before wheat was planted.

Pre-germinated wheat seedlings were planted on 12th May. One week later, 10 mL of 1M NH_4NO_3 were applied to each pot together with 100 mL of distilled water, equivalent to 50 mg N kg^{-1} dry soil or 120 kg of N ha^{-1}. At the same time 320 µL of 1M $ZnSO_4.7H_2O$ were applied per pot, equivalent to 3.4 mg Zn and 1.7 mg S kg^{-1} dry soil or 8 kg of Zn and 4 Kg of S ha^{-1}).

Wheat was sampled 21 and 28 days after planting. The subsequent procedures were the same as described for experiment 1.

5.2.3 Statistical analysis

The observations followed a normal distribution confirmed by Shapiro-Wilk's W test (Shapiro *et al.*, 1968) and homogeneity of variances was confirmed by Levene's test (Conover *et al.*, 1981). Data were analyzed with MSTAT-C (version 1.42, Michigan State University) statistical package.

Experiment 1 was analysed as two factors (weed control and time of wheat growth) in a randomized complete block design for AM colonisation parameters and as one factor (weed control) combined over time (14 and 21 days of wheat growth) for plant growth parameters and nutrient acquisition.

Experiment 2 was analysed as two factor (weed control and time of wheat growth) complete randomized block design for plant growth and AM colonisation parameters, and as one factor (weed control) combined over time (21 and 28 days of wheat growth) for plant nutrient acquisition. When F-test

indicated that there were significant differences between treatments, means were compared using Duncan test (α= 0.05).

5.3 Results

5.3.1 Experiment 1

Due to an incorrect procedure, wheat plants harvested 14 days after planting grown after paraquate control of weeds were lost. All plant growth parameters increased gradually with plant development over time in every treatment and were significantly greater in the control treatment with no weeds (Table 5.1).

Table 5.1– Effect of weeds development and control either by soil disturbance, systemic (Glyphosate) or contact (Paraquate) herbicide, on wheat plant and AM colonisation parameters at 14 and 21 days after emergence.

Measured parameters	Days after planting	No Weeds	Weed control			SEM	CV%
			Glyphosate	Paraquate	Disturbance		
Dry Weight (g/pot)	14	0.17 b	0.04 c	n.d.	0.07 c	0.023	35.95
	21	0.48 a	0.10 bc	0.08 c	0.07 c		
Node length (cm/plant)	14	3.95 bc	2.65 de	n.d.	2.08 e	0.297	19.17
	21	6.39 a	4.08 b	3.13 cd	1.98 e		
Plant height (cm/plant)	14	24.83 b	13.32 d	n.d.	11.59 d	1.057	12.55
	21	33.37 a	19.57 c	16.9 c	12.18 d		
Hyphal colonisation	14	0.07 d	0.68 b	0.75 ab	0.48 c	0.040	17.47
	21	0.09 d	0.84 a	0.77 ab	0.46 c		
Arbuscular colonisation	14	0.02 c	0.35 a	0.36 a	0.08 bc	0.034	36.32
	21	0.02 c	0.40 a	0.33 a	0.14 b		

For each measured parameter means followed by the same letter are significantly different (α=0.05). SEM– Standard error of the mean, CV – Coefficient of variation, n.d. – Not determined

This was also the treatment where AM colonisation parameters, hyphal and arbuscule colonisation, were significantly lower for both sampling dates. When soil disturbance was used to control weeds the subsequent wheat development for all the measured plant parameters was smaller, when compared to the use of any of the herbicides. Node length and plant height, this difference becomes significant 21 days after planting. AM colonisation parameters are also smaller in disturbance treatment and the differences are highly significant from the first sampling date, for hyphal and particularly arbuscular colonisation. For the weed disturbance treatment, the increase in wheat plant growth parameters between 14 and 21 days after planting was not significant.

Comparisons between the different herbicides tested in the plant growth parameters are not possible at 14 days after planting. However, 21 days after planting when the glyphosate-based herbicide was applied, there was a significant increase in node length. Differences in AM colonisation parameters between the different herbicides were not significant at 14 days after planting but in the following sampling date both hyphal and arbuscule colonisation were bigger when the systemic herbicide was applied.

For the treatments where weeds had grown, there was a positive correlation between hyphal and arbuscular colonisation with plant height and node length (Figs. 5.4 and 5.5).

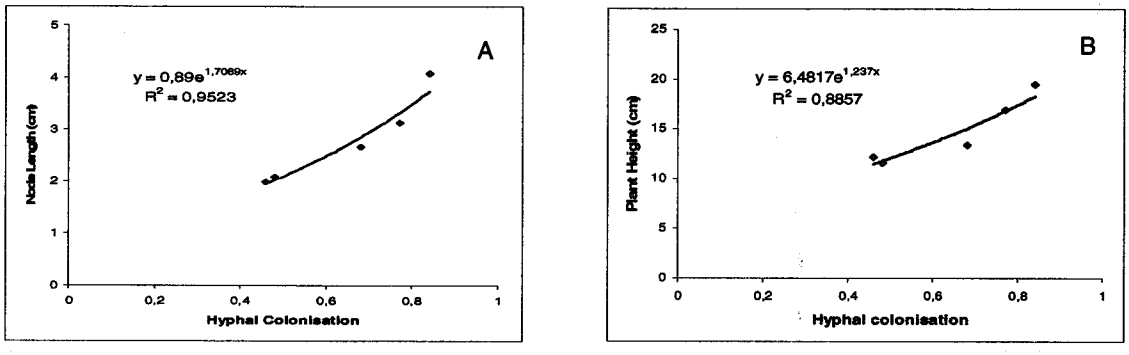

Figure 5.4 – Correlation between hyphal colonisation and A - Node length and B – Plant height. (Critical r=0.878 for n=5 and α=0.05).

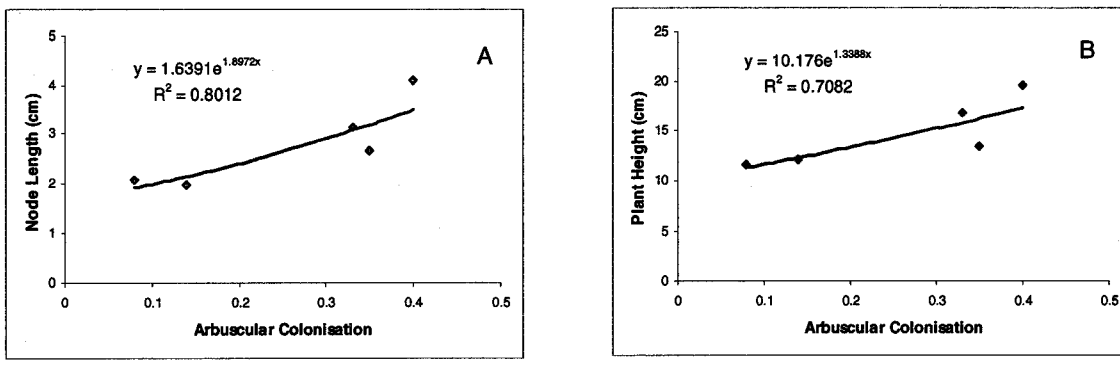

Figure 5.5 – Correlation between arbuscular colonisation and A - Node length (Critical r=0.878 for n=5 and α=0.05) and B – Plant height (Critical r=0.805 for n=5 and α=0.1).

The presence of weeds impaired the acquisition of all nutrients by wheat plants sampled 21 days after planting (Table 5.2). P acquisition by wheat was unaffected by the method of weed control 14 days after planting. However, by 21 days, the mechanical disturbance method reduced P acquisition relative to that when glyphosate was used.

When weeds were present there was a positive correlation between arbuscular colonisation and P acquisition (Fig. 5.6).

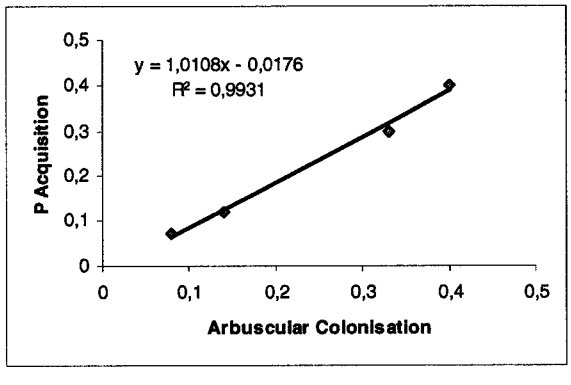

Figure 5.6 – Correlation between arbuscular colonisation and P acquisition for wheat when weeds could grow before sowing. (Critical r=0.991, for n=5 and α=0.001).

The method of weed control had no significant impact on the acquisition of K, Mg, Ca, Zn, Mn and Fe by wheat. However, Cu acquisition appeared to be enhanced when mechanical disturbance was used for control weeds relative to glyphosate.

In summary, the results indicate that wheat AM colonisation was improved when weeds had been allowed to develop prior to sowing, and

especially when control was by herbicide rather than soil disturbance, particularly when glyphosate was used. Therefore weeds can act as very efficient bridge but the method of controlling them, particularly the use of soil disturbance limits that capacity. This pattern of results was similar to that obtained for the acquisition of P and other nutrients.

According to Purdue Plant and Soil Analysis Laboratory (Vitosh *et al.*, 1995) the nutrient levels found in wheat leaves 21 days after planting (vegetative stage) were in the medium level range for all the evaluated nutrients, except for K which can be considered high.

Table 5.2 – Effect of weed control on wheat nutrient acquisition at 14 and 21 days after planting.

Weed control	Days after planting	Nutrients acquisition							
		P	K	Mg	Ca	Cu	Zn	Mn	Fe
		mg per pot				µg per pot			
No Weeds	14	0.41 b	6.49 b	0.63 b	0.94 b	1.45 bc	6.61 b	13.22 b	24.79 c
	21	1.18 a	18.57 a	1.33 a	2.31 a	3.46 a	14.79 a	54.14 a	60.43 b
Glyphosate	14	0.04 d	1.23 c	0.13 c	0.19 c	0.66 c	3.08 c	3.30 c	9.91 c
	21	0.40 b	3.19 c	0.23 c	0.41 c	1.27 bc	3.82 c	9.34 bc	15.28 c
Paraquate	14	0.14 cd	1.75 c	0.18 c	0.23 c	0.92 bc	3.22 c	4.84 bc	n.d
	21	0.30 bc	2.64 c	0.23 c	0.35 c	1.06 bc	3.62 c	8.08 bc	12.12 c
Disturbance	14	0.07 d	1.57 c	0.15 c	0.25 c	1.70 b	3.19 c	4.25 c	n.d.
	21	0.12 cd	2.15 c	0.19 c	0.34 c	1.29 bc	3.00 c	8.15 bc	13.73 c
SEM		0.060	0.936	0.073	0.121	0.236	0.850	2.581	4.688
CV (%)		40.3	44.5	42.5	43.3	35.7	36.7	43.8	31.0

Means in the same column followed by the same letter are not significantly different (α= 0.05). SEM - Standard error of the mean, CV – Coefficient of variation, n.d. – Not determined.

5.3.2 Experiment 2

Only one germinated seed of each weed species under study was placed in every pot, however species develop differently according to their biology and in one month, the dry weight accomplished by the different weed species was very diverse. The dry weight of wild oats was 4 times that of the small-seeded canarygrass and almost twice that of ryegrass (Fig. 5.7). The hyphal and arbuscular colonisation of weed roots one month after planting was 0.16 (±0.023) and 0.11 (±0.008) respectively. This was relatively small in comparison with the AM colonisation of wheat roots with the same time of development (see Section 4.3).

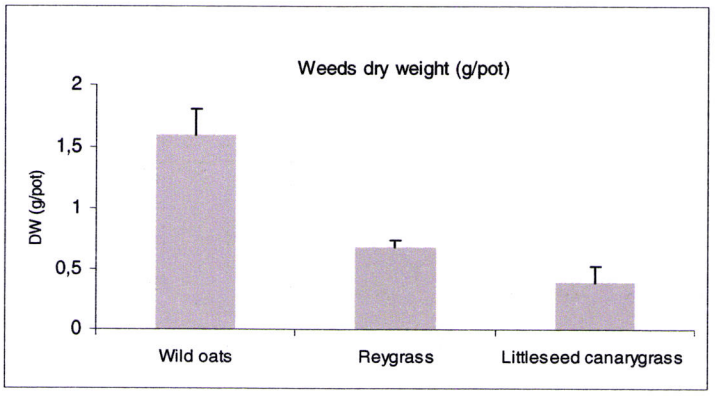

Figure 5.7 – Weeds dry weight one month after planting. Error bars are the standard error of the mean.

Dry weight was the only plant parameter where significant differences between treatments were found (Table 5.3). At the first sampling date these differences were not very evident, but by 28 days after planting, growth of wheat plants was better in the absence of weeds. The other measured plant growth parameters (node length and plant height) were unaffected by treatment at each sampling date, values increased with time.

When weeds were allowed to grow for one month but were then controlled with herbicide, the hyphal and arbuscule colonisation of subsequent wheat plants was significantly greater than if weeds were controlled by soil disturbance. This result was clear from the first sampling date, 21 days after planting.

In the pots where wheat was planted without any previous weed development, the hyphal colonisation, although smaller, was not significantly different from the one observed where weeds were controlled by herbicide application, in both sampling dates. It was always significantly bigger than that observed where mechanical disturbance was used to control weeds.

At the first sampling date, arbuscule colonisation of wheat in the "no weeds" treatment was similar to that observed where mechanical disturbance was used for weed control, but significantly smaller than that found if herbicides had been used. In the second sampling date arbuscular colonisation of wheat in "no weeds" treatment becomes significantly larger than in the mechanical disturbance treatment but similar to that in for the herbicide treatments (Table 5.3).

Table 5.3 - Effect of weed control either by herbicide or soil disturbance, on wheat dry weight, node length, plant height and hyphal and arbuscular colonisation at 21 and 28 days after planting.

Measured parameters	Days after planting	No Weeds	Weed control		SEM	CV%
			Herbicide	Disturbance		
Dry weight (g/pot)	21	1.32 c	1.22 cd	1.02 d	0.097	10.5
	28	3.43 a	2.72 b	2.65 b		
Node length (cm/plant)	21	9.88 b	10.33 b	10.25 b	0.723	10
	28	21.52 a	22.38 a	23.07 a		
Plant height (cm/plant)	21	36.23 b	35.57 b	34.96 b	0.792	4.4
	28	44.93 a	43.66 a	45.06 a		
Hyphal colonisation	21	0.32 c	0.39 bc	0.22 d	0.031	19.4
	28	0.43 ab	0.48 a	0.29 cd		
Arbuscular colonisation	21	0.21 c	0.28 ab	0.18 c	0.025	21.8
	28	0.30 ab	0.33 a	0.23 dc		

For each measured parameter means followed by the same letter are significantly different (α=0.05). SEM – Standard error of the mean, CV – Coefficient of variation.

Table 5.4 – Effect of weed control on wheat nutrient acquisition 21 and 28 days after planting.

	Days after planting	Nutrients acquisition							
		P	K	Mg	Ca	Cu	Zn	Mn	Fe
		mg per pot				µg per pot			
No Weeds	21	2.84 d	44.65 d	3.18 d	4.20 de	7.32 b	78.14 c	149.29 c	136.37 c
	28	7.38 a	84.42 a	8.94 a	11.71 a	13.31 a	195.97 a	399.5 a	256.72 a
Herbicide	21	2.70 d	40.45 d	3.29 d	4.58 d	6.01 c	103.45 b	176.17 c	105.46 d
	28	6.20 b	59.60 c	7.94 b	10.08 b	6.02 c	182.10 a	362.69 ab	237.78 a
Disturbance	21	2.03 e	32.23 e	2.36 e	3.24 e	6.70 dc	70.28 c	145.03 c	89.80 d
	28	5.53 c	67.83 b	6.14 c	7.06 c	4.38 d	190.13 a	336.38 b	197.41 b
SEM		0.208	2.292	0.257	0.325	0.270	6.334	12.262	7.838
CV (%)		10.5	9.34	10.8	10.7	8.27	10.4	10.5	10.3

Means in the same column followed by the same letter are not significantly different (α= 0.05). SEM - Standard error of the mean, CV – Coefficient of variation.

The acquisition of all the measured nutrients, except Zn, Mn and Fe, was significantly greater in wheat plants sampled 28 days after planting and grown in the pots with no previous weeds development and associated soil nutrient depletion (Table 5.4).

When weeds had been present, P acquisition by wheat was greater where herbicides rather than mechanical disturbance was used for weed control. It should be noted that at the first sampling date there were no significant differences in P uptake between the "no weeds" and the "herbicide" treatments.

Weed control by herbicides also favoured the acquisition of Mg and Ca by wheat on both sampling dates, but this was only true for K acquisition at the first sampling date. Thereafter, K acquisition was better if mechanical disturbance rather than herbicides was used to control weeds.

For micronutrient acquisition by wheat the pattern of results was very variable. When herbicide was used to control weeds, Cu acquisition was favoured. Acquisition of Zn was better in wheat grown after the herbicide control of weeds for the first sampling date, but 28 days after planting any differences disappeared. Uptake of Mn and Fe was similar in that on the first sampling date there were no significant differences between treatments, except for a better Fe acquisition in "no weeds" treatment, but by 28 days after sowing the use of mechanical disturbance to control weed development was clearly less favourable for the acquisition of these nutrients than was the use of herbicides.

Overall, 21 days after planting, wheat nutrient acquisition was similar in the "no weeds" and herbicide treatments and both were better then in the mechanical disturbance treatment. On the second sampling date, 28 days after planting, the herbicide treatment was much better for wheat nutrient acquisition then soil disturbance. These results are consistent with the results for AM colonisation (Table 5.3).

According to Purdue Plant and Soil Analysis Laboratory (Vitosh *et al.*, 1995) the nutrient levels found in wheat leaves 28 days after planting (vegetative stage) were in the medium level range for all the evaluated nutrients, except for Cu which can be considered low.

5.4 Discussion

5.4.1 Experiment 1

AM colonisation parameters were noticeably greater in wheat plants grown after weeds than after a bare fallow, indicating that weeds as host plants did develop AM inoculum, as described by Miller and Jackson (1998). This was evident from the first sampling date, only 14 days after wheat planting.

The method used for weed control strongly interfered with the colonisation of the subsequent crop. Mechanical disturbance was clearly the less acceptable method for weed control if the effect of AM colonisation is to be captured in the following crop. This is consistent with the treatment disrupting the extraradical mycelium and preventing it from immediately being able to initiate new colonisation. Despite a possible transfer of the herbicides to the wheat via AM fungi, specially glyphosate because it is systemic, and also the short time lapse between the application of herbicides and wheat planting (about 12 days) these were the treatments where wheat demonstrated the largest AM colonisation rates. In comparing the two herbicides used, glyphosate seems to have been the less detrimental for a later AM colonisation, consistent with the results of Ryan *et al.* (1994), Malty *et al.* (2006) and Silva *et al.* (2006).

Despite a greater AM colonisation of wheat grown in the pots with previous weed development, neither plant growth parameters nor nutrient acquisition reflected the same trend. Wheat plant attained greater dry weight, node length and plant height as well as better acquisition of all the studied nutrients in the "no weeds" treatment than where weeds had been grown. Nutrient depletion that occurred in the pots with previous weed growth was likely the reason. The root development of 3 different species of weeds, during two months in a 6L pot, easily explored the available soil volume and used available resources. In the middle picture of Figure 5.3, the profuse development of weeds roots is evident. This aspect was not accurately controlled in experiment 1 and in some way impaired any effect of AM

colonisation on plant performance, given the inverse relation between AM fungal infectivity and effectiveness and the availability of soil resources (Dickson *et al.*, 1999; Kahiluoto *et al.*, 2000; Sorensen *et al.*, 2005). In the glyphosate treatments, where weeds likely depleted the nutrient supply, better wheat growth and nutrient acquisition was associated with a greater AM colonisation rate.

5.4.2 Experiment 2

To eliminate any possible problems associated with soil depletion, and to determine if there was a minimum period required for weeds to grow before they can act as an AM bridge for the following crop, weeds were only allowed to develop for one month.

Another specific objective of Experiment 2 was to assess the effects of the treatments over a longer period, therefore the final sampling was made 28 days after planting.

Given the small difference in the measured parameters observed between the tested herbicides in Experiment 1 it was decided to use only one in this experiment. Glyphosate was chosen, as it was the one that allowed wheat to perform better.

The large differences in plant growth parameters observed in this experiment in comparison with the previous one was likely related to the different source of wheat seeds, although the same variety was used.

On both sampling dates, when weeds were grown (even if for only one month) and then controlled with glyphosate, the subsequent AM colonisation of wheat was greater than when weeds had been absent or if weeds had been controlled by soil disturbance. In the case of mechanical disturbance it seems reasonable to assume that the extraradical mycelium was disrupted such that the early AM colonisation of wheat was severely reduced. This was consistent with the results of the previous experiment. When weeds were AM colonised and developed an extraradical mycelium network, once they are controlled with herbicide the extraradical mycelium is kept intact and can mediate a number of

facilitative effects, such as a faster and potentially more effective colonisation of the succeeding crop (Abbott and Robson, 1981), wheat in this case, with reduced energetic costs as they can be partially supported by the failing weed root system (Francis and Read, 1984; Johansen and Jensen, 1996). The faster AM colonisation supported an earlier P acquisition, as described by Vivekanandan and Fixen (1991), McGonigle and Miller (1996b) or McGonigle *et al.* (1999). The provision of adequate P nutrition early in crop development is usually directly related to an improved final grain yield (Gavito and Miller, 1998b) conferring a comparative advantage to the crop in the initial growth period, independently of soil P content (Fairchild and Miller, 1990; McGonigle and Miller, 1996b) although not always (Vivekanandan and Fixen, 1991).

Except for K and Zn, the wheat acquisition of all the other measured nutrients, including trace elements, was also improved in the plants with a greater AM colonisation, partially contradicting Karagiannidis *et al.* (1998), but in agreement with Tarafdar and Marschner (1995).

Compared to the previous experiment, the level of colonisation of wheat in "no weeds" treatment was unexpectedly large, however it must be remembered that the soil used in this experiment was the same that was used on the previous one. It was sieved, mixed together and potted again for Experiment 2. The improvement of native inoculum promoted by plants growth (weeds and wheat) during Experiment 1 was redistributed to all pots and likely benefited AM colonisation of all plants grown in Experiment 2. Additionally these are most certainly fast colonising forms of inoculum (mycelia and colonised roots) given that the short period of plant growth during Experiment 1 was not long enough to satisfactorily promote spore development (Brundrett *et al.*, 1999). Another consequence of reusing the soil was the fact that despite some nutrient amendments, depletion of some nutrient during Experiment 1 was not offset, as can be observed by the level of P found in the soil at the end of both experiments, 14 mg P_2O_5 kg^{-1} (Egner-Rhiem). Overall, Experiment 2 started with better level of quick colonising forms of AM inoculum and lower level of nutrients in the soil, therefore favouring mycorrhiza formation. This likely explains why in the first sampling date, the young wheat plants from the "glyphosate" treatment that had been able to "plug" into an already established

AM fungi hyphal network have an unquestionably better AM colonisation than did those from the "no weeds" or "disturbed" treatments. However, these initial differences moderated over time as other sources of inoculum takeover.

Despite the efforts to control soil nutrient levels in this experiment, wheat still had better nutrient acquisition and achieved greater dry weight in the absence of weeds. However, on the first sampling date, when roots had probably not yet completely explored the soil volume and there was still a potential for extraradical mycelium to scavenge of nutrients, there were no differences between treatments, indicating that the mycorrhization could overcome the disadvantage of having less available nutrients in "glyphosate" treatment. In the second sampling date, as roots develop in a confined volume, the advantage conferred by the extraradical mycelium for nutrient acquisition became less important and plants grow better and acquire more nutrients where they were more available, i.e. in "no weeds" treatment.

What can eventually be identified as constraints in the present study, were created by the experimental process. In the field the absence of weeds is rare, although the extent of weed development depends on several factors. Also the nutrient depletion they can cause is negligible when scaling up to field level and in view of their normal growth and decay cycles.

The results encouraged further investigation of the advantages of AM bridging promoted by weeds. The study of other weed species and the effects on AMF diversity would be worth pursuing. Pot studies, with a few design adjustments, together with field experiments would be appropriate scales of investigation.

In rainfed agriculture under Mediterranean conditions, the beginning of cropping season for small grains is very much dependent on the timing of first autumn rains. After the dry and hot summer no crops and very few weeds are present, it is the late September and October rain that stimulates biological activity, such as weed germination and host plants are again available for AM colonisation. The multiplicity of roles they can play in agro-ecosystem in relation to arbuscular mycorrhiza formation, in particular the promotion of a quick colonisation of the following crop and the associated advantages, requires

careful planning of the timing and method for their control if the benefits are to be captured for crop production.

5.5 Conclusion

Consistent with the hypotheses tested, these pot experiments indicated that:

- Weeds can act as bridge between host plants allowing a quicker AM colonisation of the following crop. Particularly if the initial AM inoculum is not exceptionally abundant or if slower colonizing forms (spores) are dominant.
- The method of weed control affects succeeding AM colonisation. The use of herbicides (i.e. glyphosate) is a better option because extraradical mycelium is kept intact and new AM colonisations are favoured (faster and greater).
- One month growth of weeds is enough to establish a mycelium network that can act as an AM bridge for the following crop.
- Early AM colonisation is beneficial to a following crop in terms of early nutrient acquisition.

6. Impact of no-till vs. conventional tillage on arbuscular mycorrhiza fungal community of a Mediterranean soil

6.1 Introduction

Arbuscular mycorrhiza fungi (AMF) exhibit high functional diversity, inclusively at intraspecific level (Koide, 2000; Munkvold *et al.*, 2004). AMF differ in the manner and extent to which they colonise roots (Abbott and Grazy, 1994; Hart and Reader, 2002; Drew *et al.*, 2006) and do not all contribute equally to nutrient uptake and plant growth (Abbott and Robson, 1984; Smith *et al.*, 2004).

According to Douds and Millner (1999), an understanding of the impacts of agronomic practices upon communities of AM fungi would help to ensure an opportunity for the utilization of the symbiosis and contribute to the success of sustainable practices. Any factor (e.g. host – symbiont combinations) causing differential reproduction and survival of arbuscular mycorrhizal fungi (e.g. sporulation rates) will operate as a selective force on the composition of soil population and have consequences for the dynamic and diversity of the fungal community. Therefore knowledge of the different factors influencing the population biology of arbuscular mycorrhizal fungi is essential in any attempt to use them in sustainable agriculture (Bethlenfalvay and Linderman, 1992).

Soil disturbance is certainly one of these factors which by disruption of extraradical mycelium (Evans and Miller, 1990) and mixing of surface residues within the soil profile (Abbott and Robson, 1991; Kabir *et al.*, 1998b) selectively interferes with the different AMF according to their life and colonising strategies, promoting or tearing down specific groups. In general *Glomus* and *Acaulosposra* isolates colonise from all inoculum types, whereas *Gigaspora* and *Scutellospora* isolates colonise mainly from spores and to a limited degree from root fragments (Abbott *et al.*, 1992; Brundrett *et al.*, 1999; Klironomos and Hart, 2002).

It would be expected that AMF mainly depending on extraradical mycelium to start new colonisations to be favoured in no-till systems whereas

AMF that depend mostly on spores for that purpose to be less affected by soil disturbance. However, evidence from the literature suggests that this is too simplistic.

Boddington and Dodd (2000b) demonstrated that disturbance of pre-established extraradical mycelium reduced the formation of mycorrhizas by *Gigaspora rosea* but increased that by *G. manihotis* on *Desmodium ovalifolium* plants. Jansa *et al.* (2002), on the basis of morphological and molecular identification after a long-term (13 years) reduced tillage, found an apparent increase in the incidence of *Gigaspora, Scutellospora* and *Entrophospora* in these soils, whereas in conventionally tilled soils, almost all the AM fungi present belonged to the genus *Glomus*. Apparently *Glomus* spp tend to survive perturbations and hence they prevail in highly disturbed agricultural systems (Douds *et al.*,1995; Dodd *et al.*, 2000), leading to the suggestion that different AM fungal species vary in their adaptation to soil disturbance.

Comparing the impact of different land use intensities on AMF diversity, Oehl *et al.* (2003) found different behaviour among AMF species. The rate of root colonisation by AMF was greatest with inocula from the permanent grasslands, structurally comparable to no-till system, and least with those from high-input sites under monocropping. In contrast, AMF spore formation was slower with the former inocula when compared to the latter inocula. These authors conclude that the increased land use intensity was correlated with a decrease in AMF species richness and a preferential selection of species that colonise roots slowly but formed spores rapidly.

Jansa *et al.* (2002) made further progress using a nested PCR (polymerase chain reaction) approach, and produced the first report on community structure of AM fungi in the roots of a field-grown crop plant (maize) as affected by soil tillage. Their results showed that the presence of the genus *Scutellospora* was strongly reduced in maize roots from mouldboard ploughed and chisel ploughed soils relative to other forms of soil preparation. Fungi from the suborder *Glomineae* were prevalent in roots from ploughed soils, but were also present in the roots from other tillage treatments.

Jansa *et al.* (2002) propose that these changes in community of AM fungi colonizing maize roots might have been due to differences in tolerance to the

tillage-induced disruption of the hyphae between AM fungi species, together with changes in the nutrient content of the soil, microbial activity and changes in weed populations in response to tillage. Putting in evidence the complexity of factors regulating AMF diversity.

Bever *et al.* (2001) developed a comprehensive study on the AM fungal community dynamics at an abandoned 1 ha agricultural field, these authors hypothesized that the high fungal diversity found in their field site was due to the fact that species are ecologically distinct and occupy different niches. Individual fungi would therefore be competitively superior in their specific niche, and the presence of multiple niches in a habitat results in the active maintenance of a speciose fungal community. The results of Bever *et al.* (2001) suggested that plant host, seasonality and edaphic factors are possible environmental variables on which AMF differentiate. Li *et al.* (2007), studying cultivated land, suggest that the impact of habitat on AMF communities were greater than of the host preference to AMF.

Although Jansa *et al.* (2002) did not find significant differences in AM fungi diversity among different soil tillage treatments, the community structure was profoundly affected by the tillage treatment and Boddington and Dodd (2000a), Oehl *et al.* (2003) and Hijri *et al.* (2006) concluded that species richness can be reduced by soil disturbance or intensive tillage.

What seems to be consistent across studies of AMF diversity is the fact that for a given site the closer the environment is to "natural" conditions the more diverse is the AM fungal community (Helgason *et al.*, 1998; Oehl *et al.* 2005; Wu *et al.*, 2007; Tchabi *et al*, 2008). Given that there are pronounced plant x fungus interactions (Klironomos *et al.*, 2001, Pivato *et al*, 2007) with seasonal variation (Allen *et al.*, 1995; Daniell *et al.*, 2001) and evidence of many synergistic and complementary effects in co-occurring fungal species (van Tuinen *et al.*, 1998a; Gustafson and Casper, 2006; Jansa *et al.*, 2008), AM fungal diversity has practical implications, which makes the host-fungus combination pivotal to the quantitative outcome of any impact of the symbiosis. According to the prevailing environmental conditions, a diverse AM fungal community would be expected to increase the successful combinations of symbionts.

Plenchette *et al.* (2005) support the view that we need to be able to characterize AM fungi and mycorrhiza as easily as soil chemical properties, such as exchangeable cations. This approach would allow AM fungal characterization to be integrated into an agronomic diagnostic approach, and would help us establish when mycorrhizal development leads to poorer productivity. However, for the purpose of assessing the impact of any biotic or abiotic stress factor, measurement or evaluation of diversity in AM fungi communities in field soils, at least at the species level, presents a variety of challenges.

Spore characters and their various states of expression, especially spore wall structure (e.g. number of layers, size, colour, refractivity, flexibility, histological reactivity, ornamentation) and developmental sequence with associated morphological features, have long been the basis for species-level taxonomy (Morton and Benny, 1990). Species identification and its appropriate application are further challenged by the obligate dependence of these fungi on host plant. Spores collected from the field loose or change the appearance of their structural characters in response to root pigments, soil chemistry, temperature, moisture and microbial activity, making it difficult to properly identify field collected spores from their morphology alone (Sanders, 2004). For the purpose of identification this necessitates the establishment of trap cultures to obtain healthy spores with clear structural characteristics. Depending on the set of plants chosen for the trap culture and the growing conditions, this technique may allow detection of different members of the community but can interfere with the composition and diversity of associated AM fungi (Bever *et al.*, 2001; Jansa *et al.*, 2002; Mathimaran *et al.*, 2005).

There is no clear relationship between functional diversity of organisms and the morphological diversity of spores used to delineate species (Douds and Millner 1999). Life-history traits that are important for the AM symbiosis (e.g. amount and architecture of external hyphae, proportional fungal biomass as arbuscules versus vesicles and absorptive and transport capacity of hyphae) are not directly linked to any apparent character trait used to distinguish species (Morton and Bentivenga 1994).

The morphology of fungal structures that can be detected in roots is in general very similar and doesn't necessarily reflect the extent to which different fungal species colonize the root systems. For a long time the lack of reliable methods for the identification of fungal species that colonize a root greatly limited our ability to characterise changes in mycorrhizal populations (Miller *et al.*, 1994). One of the most important objectives in AM research has been the need to find good and practical methods for describing communities. Serological (Hepper *et al.*, 1988; Cordier *et al.*, 1996), enzymatic (Hepper *et al.*, 1986; Dodd *et al.*, 1996), fatty acid profile (Graham *et al.*, 1995) and many modern DNA based molecular tools have been developed and continuously improved over the last decade, and now constitute a powerful approach to detect and evaluate this diversity, within roots (Clapp *et al.*, 1995; van Tuinen *et al.*, 1998a; Gollotte *et al.*, 2004) or directly in the soil (van Tuinen *et al.*, 2004). The greatest advantage of being able to identify AM fungal diversity based on soil analyse would be that as all forms of inoculum (spores, extraradical mycelium and colonised root fragments) can be present in the samples, biological bias is avoided and species identification is independent of spore formation.

Most authors used the systemic approach of detecting ribosomal sequences. Given an adequate database of sequences and knowledge of phylogeny, this approach allows the specificity of probes or PCR primers to be tailored to a species, genera or any other level of taxonomy (Redecker, 2002).

Based on the knowledge that mycorrhiza formation is favoured in NT relative to CT systems (Evans and Miller,1988; McGonigle and Miller, 1996a; Kabir *et al.*, 1997a; Kabir *et al.*, 1998b; Boddington and Dodd, 2000a; Galvez *et al.*, 2001; Borie *et al.*, 2006; Castillo *et al.*, 2006) and that a more diverse AM fungal community offers more possibilities for functional AM symbiosis to establish, this study of the impact of tillage systems on AM fungal diversity and community structure of a agricultural field was undertaken for an agronomic system of the Alentejo. It aimed at gaining an understanding of diversity and community structure under conditions where AMF versatility and resilience is constantly challenged by the unpredictable Mediterranean climatic conditions and marginal soils under cropping.

The experiment tested the following hypotheses:

- Soil disturbance and mixing of surface residues induced by tillage will influence the community of indigenous AM fungi.
- The effect of soil disturbance on AM fungal diversity can be detected directly on field soil, by nested polymerase chain reaction (PCR).

6.2 Materials and methods

6.2.1 Sampling procedure

AMF propagules and spore densities are known to exhibit a strong spatial structuring at small scales (Bever *et al.*, 2001; Wolfe *et al.*, 2007), consequently the spatial heterogeneity of AMF distribution makes sampling procedures for evaluating diversity, a complex problem. The results of Whitcomb and Stutz (2007) clearly indicate that sampling intensity and strategy can affect perceptions of AMF community structure. However in an agricultural field the patchy distribution is in part reduced as the same host plant is dominant across a field and the pattern of cropping rows helps to determine the location of plant roots.

The site selected was at Revilheira farm, where previous experiments had been carried out (see section 3.2.1 for description), and samples were collected after wheat had been harvested in June 2004.

Soil samples were taken from two adjacent field plots to reduce soil (physical and chemical characteristics) and field (slope, shade) variability. One plot had been cultivated by conventional tillage (mouldboard ploughed and then disk harrowed) was designated as the disturbed (D) treatment, and the other plot, which had not been tilled for the last 9 years, was identified as the undisturbed (U) treatment. From each plot (6 X 17 m), ten soil cores of approximately 200 mL (0-15 cm) were taken at random across the plot and then mixed to produce a single composite sample. This sample was sieved and a sub-sample kept at 4°C until used.

6.2.2 Nested polymerase chain reaction

To increase the sensitivity of detection, a nested polymerase chain reaction (PCR) was performed. This technique involves the successive use of two sets of primers, the second set was intended to amplify a secondary target within the product of the first run. The great advantage of this technique is the high sensitivity of the final amplification, as it is very unlikely that any unwanted DNA fragments contain binding sites for both new primers. In addition, the high sensitivity of the reaction allows for a positive reaction to develop using relatively crude preparations of DNA. Finally, the first amplification is the only reaction performed directly on the biological material, and thereafter a wide range of analyses, using taxon-specific primers, can be made on the same sample. In this way, even a small sample of mycorrhizal root or soil DNA can be analysed for the presence of a variety of AM fungi (van Tuinen *et al.*, 1998a, 2004).

6.2.3 Laboratory procedures for ribotypes definition

The laboratory analysis was undertaken at the Plant Microbe and Environment laboratory (INRA CNRS, Université de Bourgogne) in Dijon, France, over two summers (2004 and 2005).

In the summer of 2004, three 200 mg sub-samples of the composite sample from each field plot were used to isolate total DNA according to Martin-Laurent *et al.* (2001) (see Annexe 2 for the detailed protocol).

One µl of the purified DNA and a 1/10 dilution was used to perform the first PCR amplification of the 5' end of the large sub-unit (LSU) rDNA with the eukaryotic specific primers LR1 and NDL22 (van Tuinen *et al.*, 1998b) designed to border the D1 and D2 variable domains of the 5' end of the large ribosomal subunit encoding gene.

Reactions were performed in a final volume of 20 µl containing 2 µL 10 X PCR buffer with 1.5 mM $MgCl_2$ (Qbiogen), 2.5 mM dNTP, 10 µM of each primer,

0.5 U per 100 µl of *Taq* polymerase (Qbiogen) and an aliquote (1 µL) of soil DNA in ultra-pure water. Each reaction was overlaid with mineral oil and amplification was performed in a thermal cycler (Biometra T3000) programmed as follows: initial denaturation cycle at 93°C (3min), annealing at 56°C (1min), extension at 72°C (1min) followed by 29 cycles of denaturation at 93°C (1min), annealing at 56°C (1min) and extension at 72°C (1min). The last cycle was followed by a final extension at 72°C for 5 min.

For this PCR 1 µL/reaction of the T4 bacteriophage gene 32 product (T4 gp 32) (Q-Bio Gene) was added. It is reported to act by binding to single-stranded DNA in a cooperative manner thereby destabilizing double stranded DNA, increasing the efficiency of the amplification.

The amplification product was diluted 1/500 and used as template for the second PCR with the primers LR1 and the fungal specific primer FRL2 (Trouvelot *et al.*, 1999). The PCR conditions were similar except for the number of cycles, 28 this time, and the annealing temperature being 60°C.

The second PCR products were separeted on a 1.4% agarose gel in TAE buffer (40 mM Tris, pH 7.8, 20 mM acetic acid and 2 mM EDTA) and visualised under UV light after ethidium bromide staining (50 µg L^{-1}) (Figure 6.1).

The product of the second PCR was pooled together and cloned using the TOPO TA Cloning® kit for sequencing (ref. 45-0030 or 45-0641, Invitogen).

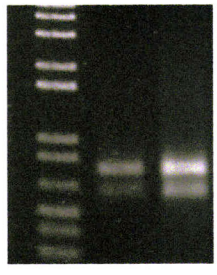

Figure 6.1 – Second PCR (LR1- FLR2 primers) product. Lower band is comprised of other fungi and the upper band is material from Glomeromycota.

Forty eight positive clones (white colonies) were analysed by PCR, using the LR1-FLR2 primers. The inserts with the expected size for AMF, ranging between 650 and 750 bp were selected (Figure 6.2). These clones were multiplied in liquid LB medium (ref.12780-052 Invitrogen) with ampicillin (0.5 µg mL^{-1}) and the plasmid purified using kit (Nucleo Spin® Plasmid, Machery-Nagel). The plasmids were sent for sequencing by MWG (Germany).

To have a larger number of sequences for analysis, in the summer of 2005 this work was repeated, using the soil collected previously. A similar approach was undertaken but with a few protocol adjustments. The primers adopted for the first PCR were LR1 and FLR2. In previous year these had only been used for the second PCR. This procedure allowed better specificity in the first amplifications and was possible because an AMF specific primer (FLR4) was had been developed by Gollotte *et al.* (2004), and was available for use in the second PCR.

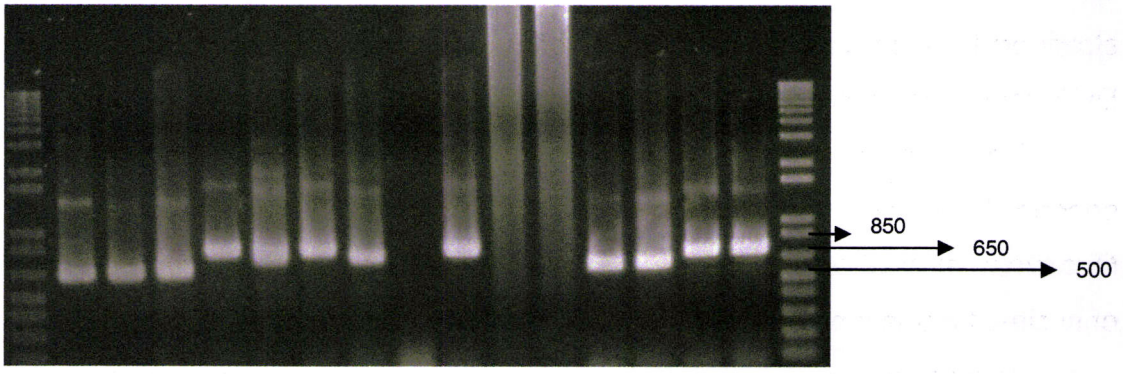

Figure 6.2 – Clones with the insert of required size (> 750 bp) marked with white harrow.

6.3 Results

Totalled over both years, 83 sequences of good quality were obtained, 36 from the undisturbed treatment and 47 from the disturbed soil. All sequences were checked through a BLASTN analysis (Altschul et al., 1997) for the presence of chimeric sequences, which can be generated during this procedure, and aligned with ClustalW. Four chimeric clones in total were detected and withdrawn from the analysis. Blast searches in the GeneBank database showed that all sequences obtained in this study belonged to the Glomeromycota, confirming the specificity of the LR1-FLR4 primer pair for detection of Glomeromycota. The detection of *Scutellospora* and *Paraglomus*, also confirmed that all the Glomeromycota families are amplified. This had been questioned by Mummey and Rillig (2007). The sequences were aligned with ClustalW and the alignment was optimized manually using the Se-Al v 2.0

software (University Oxford). Phylogenetic analyses were performed using the neighbour joining (NJ) algorithm included in the ClustalW programme, using *Mortierella multidivaricata* as an out-group. Positions with gaps were ignored and the reliability of the internal branches of the NJ tree was assessed using the bootstrap method with 1,000 replicates. Tree files were drawn using njplot (http://biom3.univ-lyon1.fr) and the sequences grouped together in ribotypes on the bootstrap values, with a threshold of more than 950‰ (Fig. 6.3).

The number of ribotypes in the different clusters is reported in Table 6.1 and Figure 6.4. With the exception of one ribotype, identified as a *Scutellospora*, all the other ribotypes belonged to the *Glomineae*, and mainly to *Glomaceae*. Four ribotypes could be identified at the species level, namely *G. mosseae*, *G. intraradices*, *G. claroideum-etunicatum* and *G. occultum* basionym of *Paraglomus occultum*. According to the data obtained only 2 of the ribotypes found (*G. mosseae*, *G. intraradices*) were present in both soil types, 6 could only be found in undisturbed soil and 3 only in disturbed soil. The AM fungal diversity was higher in undisturbed soil, where 8 different ribotypes were identified, when compared to disturbed soil with only 5 ribotypes recognized.

Table 6.1 – Ribotypes identified in undisturbed and disturbed soil in total for both years.

Ribotypes	Undisturbed soil	Disturbed soil
G. mosseae	3	7
Glomus I	1	0
Glomus II	0	3
Glomus III	3	0
Glomus IV	1	0
G. intraradices	24	32
Glomus V	1	0
G. claroid.-etuni.	0	2
Glomus VI	1	0
Scutellospora sp.	2	0
P. occultum	0	3
total	36	47

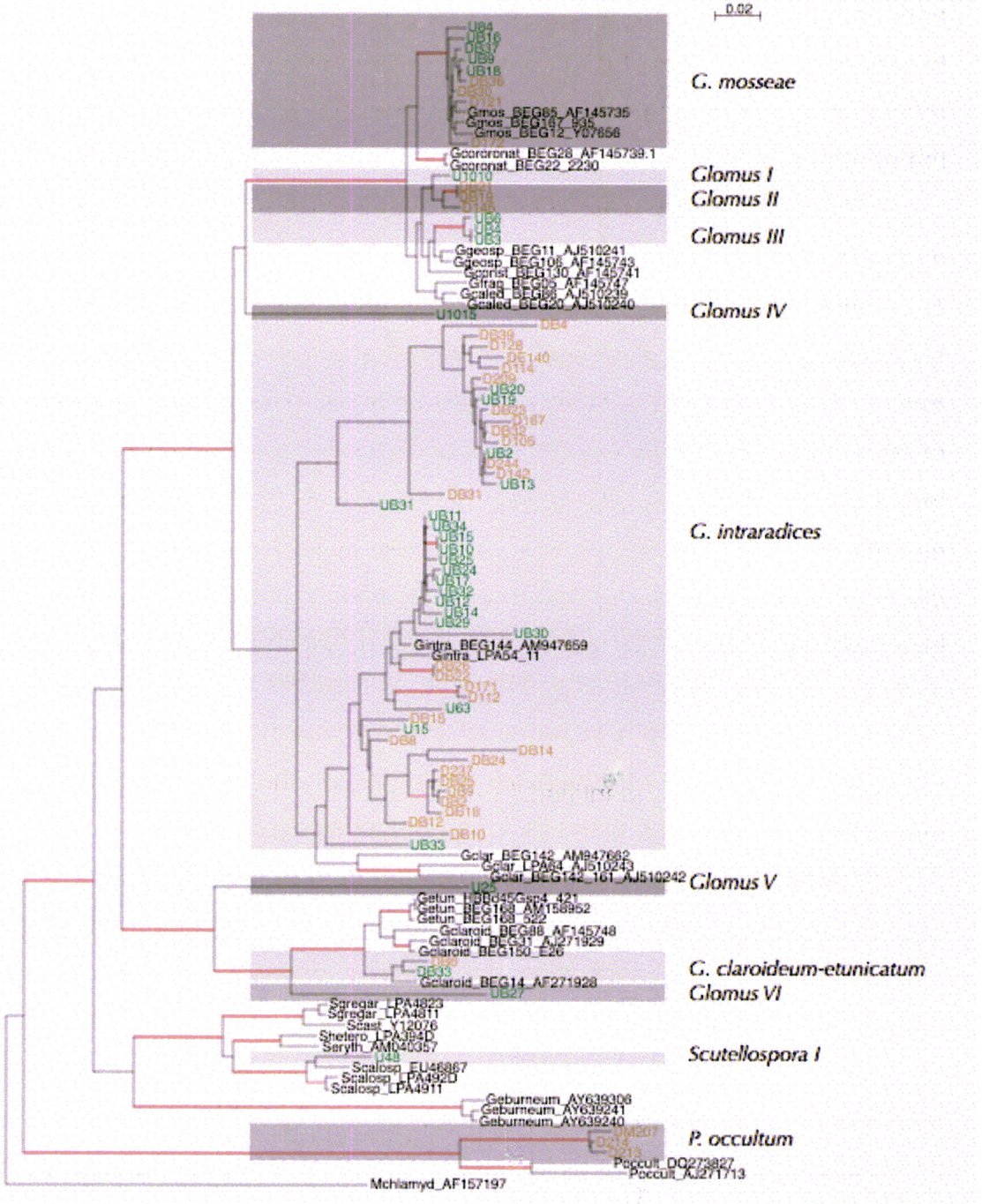

Figure 6.3 – Neighbor-joining tree representing AM fungal sequences from undisturbed (in green) soil and from disturbed soil (in brown), obtained by nested PCR, in comparison with known sequences.

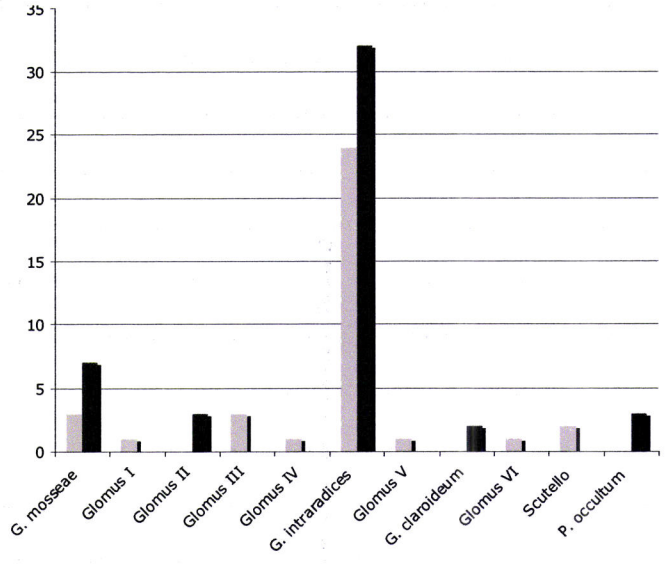

Figure 6.4
Ribotype distribution and frequency in undisturbed (grey bars) and disturbed (black bars) soil.

6.4 Discussion

Differences between treatments in the frequency of the ribotypes present in soil, confirms that AMF are differently vulnerable to soil disturbance. This appears to be true, both in terms of the community structure, as the same ribotypes show different frequencies depending on the level of soil disturbance, and in terms of diversity, given that the ribotypes present in the undisturbed soil were not always the same as those isolated from disturbed soil.

Ribotype diversity found in the undisturbed system was clearly greater than in disturbed soil, indicating that species richness may be reduced by mechanical disturbance as described by Boddington and Dodd (2000a), Oehl *et al.* (2003) and Hijri *et al.* (2006). The belowground diversity is an essential component of ecosystem health (Bever *et al.*, 2001) and the use of non-disturbing soil management techniques, such as no-till, clearly favours it.

Over both soil treatments, 11 ribotypes were recorded for this site, most belonging to Glomus group. These results are consistent with those from other studies as the number of species, or different sequences (depending on the techniques used) found in different agricultural soils is quite variable, ranging from 9 to 18 (Daniell *et al.*,2001; Jansa *et al.*, 2002; Oehl *et al.*, 2003; Mathimaran *et al.*, 2005).

Rarefaction curves were constructed with the freeware program Analytical Rarefaction 1.3 (www.uga.edu~strata/software) to determine whether the number of sequences tested sufficiently represents Glomeromycota diversity in the studied soils. In these curves the number of sequences analysed were plotted against the cumulative number of ribotypes (Fig. 6.5). In undisturbed soil and although the maximum of the diversity was not reached, it was estimated that with 500 sequences analysed, 14 different ribotypes would be detected, suggesting nearly 60% of the diversity of the Glomeromycota present in this soil was already described. On the other hand, in disturbed soil, the rarefaction curve almost reached the plateau indicating that diversity seemed to be better covered. The similar estimation for 500 analysed sequences indicated that more than 70% of the Glomeromycota present in this soil were already identified. These results are in a similar range as those found by Pivato *et al.* (2007) in another farming system, suggesting that the analysis of 45-50 clones/sequences per treatment provide a reasonable coverage of AM fungal diversity.

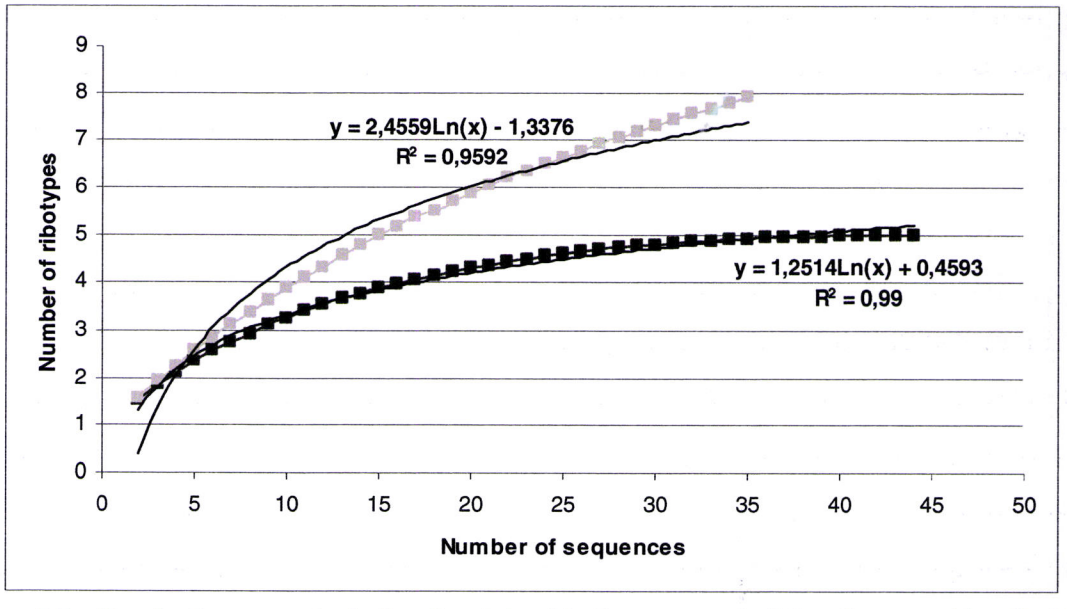

Figure 6.5 - Rarefaction curve including the data of both years and all the ribotypes identified in undisturbed (grey) and disturbed (black) soil.

Despite the multiple difficulties associated to the sampling procedures for description of AMF diversity, it seems that the process followed in the present

work (field and laboratory sampling) may constitute a good first approach for the study of the effect of tillage system on AM fungal diversity.

The ribotype corresponding to *Glomus intraradices* was clearly the most abundant ribotype found in both experimental conditions, confirming van Tuinen (personal communication, July 2005) unpublished data, that in contrast with other isolates from the Glomaceae, the ones within this taxonomic group give evidence of high molecular diversity. Also Mathimaran *et al.* (2005) found a high dominance of *G. intraradices* spores in a soil community associated with wheat as host plant.

This study expresses how helpful molecular identification methods can be for a more global view of the diversity of a particular AM fungal community, even if no direct correlation between sequence identity and species identity can be made, as each spore harbours different sequence types. For this reason a high threshold of the bootstrap values (>950‰) was used to group the ribotypes groups. Until there is more knowledge in the field of the phylogenetic species concept, taxa have to be bundled in groups as shown in this study, under the assumption that all taxa in the respective group share at least some ecological or physiological characteristics (Redecker, 2002).

The data obtained allows the generation of ribotype specific primers (Turnau *et al.*, 2001), which will give the opportunity to perform a more detailed study on the distribution of selected taxa or ribotypes in the roots of wheat plants through out the growth season and help elucidate the functional diversity of these fungi.

Agricultural practices such as tillage, produce changes in the soil chemical, physical and biological properties, and can modify the ecological niches available for occupancy by the soil biota. All these changes can influence in various ways the performance of the symbiotic relationship between the higher plant and the AM fungi, the potential for the production of inoculum for new mycorrhizas, and consequently effecting changes in the balance of indigenous AM fungal communities. The molecular tools have been very important in broadening our understanding of these changes, as well as building a greater awareness of the consequences, which the choice of management practice imposes on AM development.

The goal of sustainable management is to create a more spatially heterogeneous habitat that offers the potential for more diverse and balanced systems to establish, in which natural occurring organisms, like AM fungi, can express their potential, evolved over millions of years of co-existence with most terrestrial plants (Simon *et al.* 1993, Redecker *et al.* 2000).

By favouring AM fungal diversity, management practices like no-till, clearly have the possibility of encouraging arbuscular mycorrhizas as the possible combinations between host plant and AMF are wider and, depending on eventual biotic or abiotic stresses, the chances of having a functional AM are greater.

6.5 Conclusion

Consistent with the hypotheses tested, the experiment indicated that

- Soil disturbance and mixing of surface residues induced by tillage reduces the diversity (number of ribotypes) and influences the community structure (frequency of each ribotype) of indigenous AM fungi.
- The effect of soil disturbance on AM fungal diversity can be detected directly on field soil, by nested PCR.

7. General conclusions

AMF establish symbioses with the majority of plant species and influence a number of key processes in terrestrial ecosystems, such as primary productivity, nutrient cycling and physico-chemical properties of soil (Allen, 1991; Rillig, 1994). Many benefits accrue to plants from their association with arbuscular mycorrhizal fungi (Gupta *et al.*, 2000), even if at times these benefits are not obviously evident or easily quantifiable.

In the last decade with the arrival of new molecular and biochemical techniques many advances have been made in the AMF study, but the integration of these findings in a broader reality enclosing community and ecosystems dynamics is essential. As stated by Miller and Kling (2000), without such framework, the use of new technologies might in reality only be adding unnecessary digits beyond the decimal point. Experiments must look at combination of organisms and simulate conditions that are known to co-occur in the field if they are to provide useful information about the field performance of AMF (Mozafar *et al.*, 2000; Gange and Brown, 2001) and contribute to the desired integration of knowledge. The present work tried to follow these principles. The objectives were not focussed on the straight improvement of crop yield considering the complexity of integrating the mycorrhizal symbioses in a crop, as demonstrated by Hetrick *et al.* (1993) when studying mycotrophy in winter wheat. The objectives were focused on having the perception of the potential value of AM under Mediterranean climate and how they could be managed within the agricultural systems of Alentejo region assuming that if AM colonisation is present, benefits would be, directly or indirectly, sooner or later, captured by the crop.

According to Abbott *et al.* (1995) the potential to make effective use of the mycorrhizal symbiosis in Mediterranean agriculture depended on developing further understanding of several key relationships between the components of the symbiosis and the soil condition; that is on the factors that limit plant growth and the extent and timing of AM formation (Abbott and Robson, 1991). Thus, beside plant growth parameters, the AM features evaluated in this thesis were

colonisation rate and timing of occurrence. AM fungal diversity studies were also carried out as fungi differ in the manner and extent to which they colonise roots (Abbott and Grazy, 1994), and these have practical implications due to pronounced plant x fungus interactions (Klironomos *et al.*, 2001).

Agro-ecosystems are managed biological systems that may involve the use of several agricultural practices such as tillage, crop rotation or weed management that affect ecological niches available to the soil biota including mycorrhizal symbiosis. The potential level of colonisation will depend on how agricultural practices affect the properties of the soil and on the response of the fungi present to changes induced by these practice. Knowledge of the likely extent to which AM form can be based on an understanding of the impact of agricultural practices on the fungi associated with particular hosts (Abbott and Robson, 1994; Thompson, 1994).

7.1 Most relevant findings

The main conclusions of the present work can be described as flows:

- Soil disturbance negatively affects the establishment of AM.
- AM extraradical mycelium can remain infective after the hot and dry Mediterranean summer.
- Weeds can act as a bridge for AM colonisation of crops.
- Soils disturbance affects the AMF community of the soil.
- Wheat can take nutritional advantage of AM colonisation.

In the context of Mediterranean agriculture there is a potential value for AM and it could and should be considered in the agro-ecosystem management given that the transfer of inoculum from one crop to the following one in the next cropping season was established. The transfer occurred through the summer survival of extraradical mycelium, and from weeds to crops through mycelia facilitative effects.

Soil disturbance associated with tillage systems promotes the disruption of extradicular mycelium and the AM colonisation of new crops is mainly assured by spore germination. Under these circumstances the ability of a preceding crop in a rotation to produce spores is the only way of improving inoculum potential at a particular site. However this will always be a less efficient mean to start new AM colonisations as spore germination is slower than that effected by contact with a common mycorrhizal network. Furthermore the temperature during the period of sowing the wheat crop (November) is too cool for the process of spore germination to be rapid. This makes colonisation from spores even slower so AM colonisation of wheat after seeding is predominantly dependent on extraradical mycelium.

Knowing that extraradical mycelium can survive over the Mediterranean summer means that the use of no-till or direct drilling systems that support a greater AMF diversity and maintain the extraradical mycelium intact, together with the choice of the crops included in rotations, provides opportunities for agro-ecosystem management to benefit from the presence of mycorrhiza. Additionally, seeing that the weed mediated AM transfer is more efficient than the transfer from one crop to the following cropping season, an adequate weed management (i.e. allowing weeds to growth until extraradical mycelium is well established, the use of herbicide to control them and preservation of extraradical mycelium network) can strongly benefit the initial colonisation of the crop.

In view of the fact that wheat can actually take advantages of AM colonisation and knowing that under Mediterranean conditions the initial development of the crop is fundamental to stabilize the biomass of the crop at flowering, there is an unquestionable potential value for AM under properly managed agricultural systems in Alentejo region. Management practices like no-till, crop rotation and a watchful weed control clearly have the possibility of encouraging arbuscular mycorrhiza.

It should also be emphasised the important role AM might have under marginal biotic or abiotic conditions since they can confer important, or even decisive, comparative advantages to their host, acting like a kind of "life insurance" in the sense that the benefits granted by mycorrhization may not be

obvious but under a disturbing situation the AM colonised plants are the ones that better tolerate it. This may be particularly important in the Alentejo's agronomic context taking into consideration the great variability of Mediterranean climate and the marginal soils under cropping.

The interest in extensive farming systems, environmental sustainability and the economics of production focuses attention on the identification of agronomic management practices that may allow controlled manipulation of the fungal community and capitalization of mutualistic effects possible through mycorrhizal formation. In addition, they encourage evaluation of the use of local AM fungal inoculum as an option for mycorrhiza promotion and development in sustainable crop production.

7.2 Directions for future research

According to the results of this study, in the Alentejo region and under Mediterranean climate conditions, it is evident the beneficial effect of no-till practices on preserving extraradical mycelium and the consequent improved AM infectivity despite Mediterranean summer conditions. The timing and method for weed control and the role they can play in keeping or increasing AM infectivity on the cropping season was also observed. In order to have a deeper knowledge of the potential these findings enclose and the contribution they can have in the stability of yield potential, new objectives should be targeted and novel hypothesis investigated.

- The screening of different common weeds of the region as AM starters before the crop cultivation should be undertaken. Simultaneously their susceptibility to herbicide control must be considered.
- Despite the importance of wheat as small grain crop in the region, other crops like fodder crops, lupines or other small grain crops can be addressed in the perspective of an adequate crop rotation design adapted to make effective use of AM.
- The assessment of the AM fungal diversity associated to different field situations should be simultaneously preformed, considering it can have

functional significant consequences as different AMF don't have equal contributions and the usual approach of assessing the mycorrhizal root as a whole, without considering the diversity of fungi within the colonised roots, limits the ability to assess contributions of these fungi in field soils. Also the identification of the different AMF isolates or species colonising the crop roots along the vegetation cycle may provide useful information about the functional diversity of these fungi.

- The increase of irrigated area in Alentejo region partially changes the agriculture scenario allowing for new possibilities in terms of novel varieties with different cycles length, other crops and different timings for many field operations. How AM fit in this new agronomic reality has to be studied.

Re-using the same soil in pot experiments can clearly be problematic. The control of soil nutrient depletion from one experiment to the other is not easy, unless more sophisticated equipment like specific probes and retention columns is available. Also the increase of AM natural inoculum potential may be a confounding factor on the results and depending on the specific objectives of the experiment this has to be considered in the study protocol.

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Annexe 1 – Cycles of disturbance preliminary experiment

Objectives

This preliminary experiment was based on the "Cycles of Disturbance Technique" developed by Fairchild and Miller (1988) and was preformed to appreciate if wheat growth could be a good indicator of AM inoculum differential potential.

Using this technique, and after 3 or 4 cycles of disturbance, greater colonisation rates are consistently observed in maize and soybean plants coming from undisturbed soil pots (Goss and de Varennes, 2002; McGonigle *et al.*, 2003; Antunes *et al.*,2006). However there was no previous information on how suitable wheat would be as AM host plant to achieve the desired differential AM inoculum potential. Considering this technique uses the naturally occurring inoculum and allows a common history of inoculum and host plant throughout the successive cycles, the host plant chosen to perform it, is very important in the interpretation of results. For a number of reasons wheat was the host plant that was preferred for this study, which is why it was important to evaluate its suitability as an AM host plant in the "Cycles of Disturbance Technique".

Soil

The soil, a Luvisol, was collected from the same site as the field investigation. It was air dried and sieved using a cereal winnowing-machine (4mm sieve).
The chemical characteristics of the soil are the following:

P_2O_5 – 25 mg kg^{-1}

K_2O – 76 mg kg^{-1}

NO_3 – 14 mg kg^{-1}

Mg - > 125 mg kg^{-1}

Total N – 63 mg g^{-1}

pH (H_2O) - 6.2

OM – 6 mg g-1

Experimental procedure

- The soil was packed into plastic pots (6 kg per pot).
- Pre-germinated wheat seeds were placed per pot, after one week seedlings were thinned to 2 plants per pot. The same variety (Coa) was selected as used in the field investigation.
- Plants watered with distilled water to 20% of field capacity (constant weight) every 2 days.
- Three weeks after planting shoots were excised and measured, dried at 70°C for 48 hours and weighed.
- In half of the pots (13 pots) the soil was removed as two 10 cm layers and passed separately through a 4 mm sieve. All root material separated on the sieve was cut into 2 cm long segments and mixed into the soil of the appropriate layer. Soil was repacked into the pots and arranged in the same two layers at the same bulk density.
- In the other half pots (13 pots) the soil remains undisturbed.
- On the next day or in the same day, each of the 26 pots was reseeded with 4 pre-germinated seeds.
- 50 mg N kg^{-1} (120 kg N ha^{-1}) – Ammonium nitrate at the start of the each cycle applied one week after transplant, 10.7 mL of 1M solution of NH_4NO_3 diluted on 100ml of distilled water.

Greenhouse conditions

- Temperature - Between 9 and 30°C. After March, the cooling system was kept on.
- Photosynthetic Active Radiation (PAR) during last cycle - 350 µmol m^{-2} s^{-1}. With shade protection was required to maintain temperatures at or below 30 °C.

Parameters measured

- After the 4^{th} cycle root system and soil samples were collected and from both treatments (Undisturbed and Disturbed).

- 3rd and 4th cycle shoot material was ground and prepared for P analysis.
- Roots were stained with Trypan blue and colonisation parameters were accessed (arbuscular, vesicular and hyphal colonisation) according to using the intersection method (McGonigle *et al.*, 1990). 2 slides per pot were observed.

Results

Data were analyzed with MSTAT-C (version 1.42, Michigan State University) statistical package. The observations followed a normal distribution confirmed by Shapiro-Wilk's W test (Shapiro *et al.*, 1968) and homogeneity of variances was confirmed by Levene's test (Conover *et al.*, 1981).The experiment was analysed as two factors (soil disturbance and cycle) randomized complete block design.

Table A– Effect of soil disturbance on plant height and dry weight over each cycle, and arbuscular, hyphal and vesicular colonisation after 4 cycles of disturbance.

Soil Disturbance	Cycles of disturbance	Plant parameters		AM colonisation		
		Shoot height (cm)	Shoot dry weight (g/pot)	Hyphal	Arbuscular	Vesicular
Undisturbed	1	25.52 d	0.12 cd	n.d.		
Disturbed		24.8 d	0.10 d			
Undisturbed	2	27.31 c	0.15 ab	n.d.		
Disturbed		25.30 d	0.15 ab			
Undisturbed	3	25.35 d	0.13 bc	n.d.		
Disturbed		25.22 d	0.13 bc			
Undisturbed	4	33.48 a	0.17a	0.20 a	0.13 a	0.01a
Disturbed		31.09 b	0.14 abc	0.07 b	0.03 b	0.01 a
SE		0.566	0.07	0.018	0.014	0.001
CV (%)		7.19	19.51	45.69	61.01	105.93

Means followed by the same letter are not significantly different (α= 0.05). SEM - Standard error of the mean, CV – Coefficient of variation

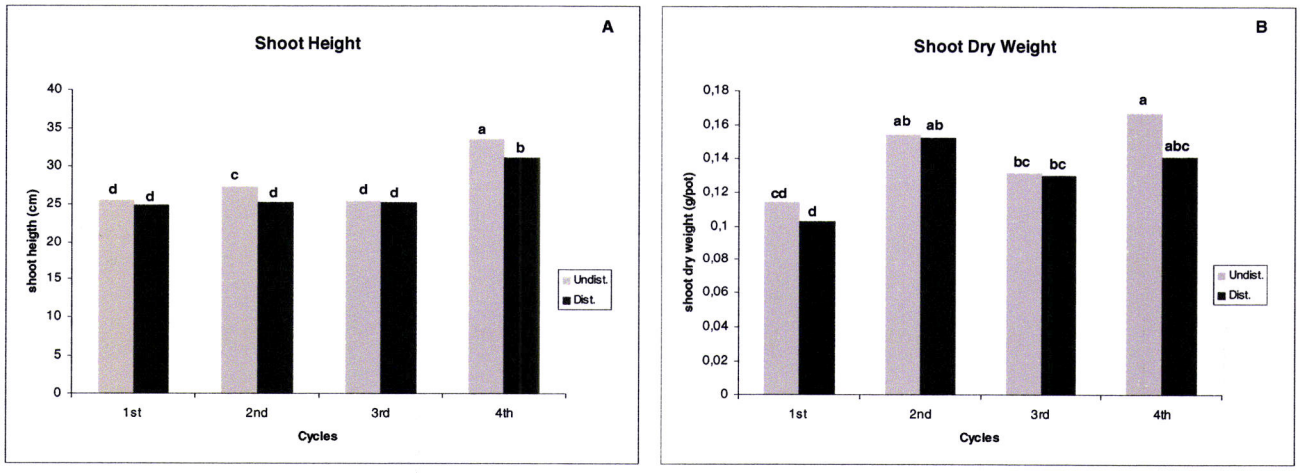


Figure a– Effect of soil disturbance on plant growth over 4 cycles of disturbance. A - Shoot height (cm) and B - Shoot dry weight (g/pot). Columns with the same letter are not significantly different (α= 0.05).

- After 4 cycles there is an effect of differential soil disturbance on plant height and shoot dry weight (Fig. a).
- In undisturbed soil, plants were significantly taller and shoot dry weight greater.
- All AM colonisation parameters (hyphal, arbuscular and vesicular) are significantly affected by soil disturbance. They are greater in undisturbed soil (figure b).
- 4 wheat cycles of 3 weeks are enough to establish a significant differential soil disturbance.
- P plant content from undisturbed soil is higher on 4th cycle (figure c - A).
- There are no differences in N plant content in either of the cycles (figure c - B).

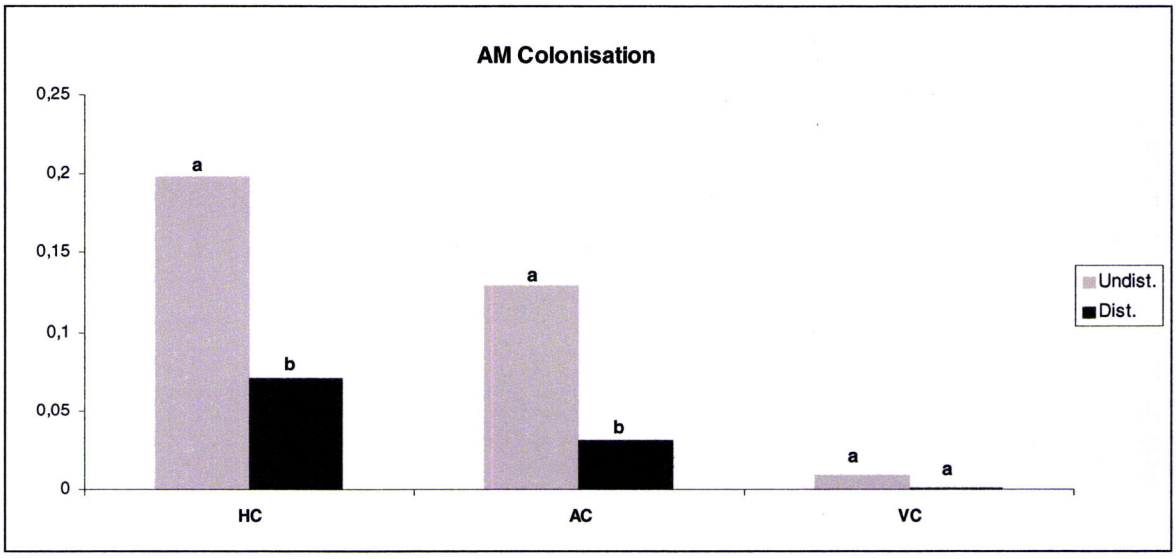
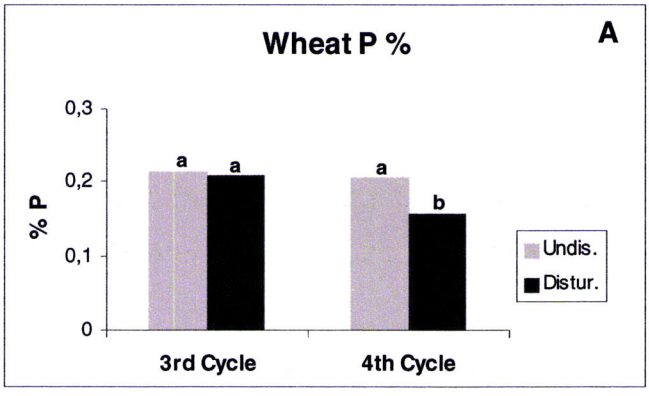


Figure b – AM colonisation parameters after 4 cycles, in undisturbed and disturbed soil. HC - Hyphal Colonisation, AC - Arbuscular Colonisation, VC - Vesicular colonisation. Columns with the same letter are not significantly different (α= 0.05).



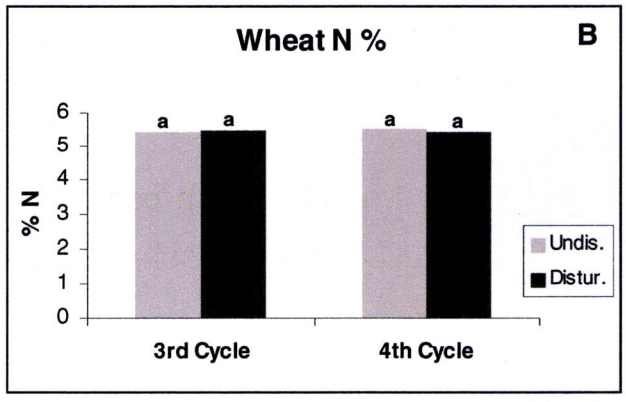


Figure c – Wheat nutrient content in undisturbed and disturbed soil. A – Phosphorus (%) and B - Nitrogen (%) on 3rd and 4th cycle. Columns with the same letter are not significantly different (α= 0.05).

During slide observation it became apparent that there were several different types of arbuscules present. They were characterized by the way they retained Trypan blue, some arbuscules retained very little stain, and by their morphology (different thickness and brunching of hyphae). The variability suggested the presence of different species colonizing the same root system simultaneously. No quantification of these differences was made.

Conclusion

Wheat is a suitable host plant to develop the "Cycles of Disturbance Technique" considering that after 4 cycles it captures the effect of differential AM inoculum potential, showing consistent differences not only in AM colonisation parameters but also in plant growth parameters and P acquisition.

Annexe 2 - DNA extraction from soil

According to Martin-Laurent *et al.* (2001)
Applied and Environmental Microbiology 67:2354-2359.

1. Weight 0.20 g of soil
2. Add 0.5 g of 106µm (facultative) and 2 of 1mm of diameter glass beads
3. Add 1 ml extraction buffer (Tris-HCl 100 mM pH8; EDTA 100 mM pH8; NaCl 100 mM; 1% PVPP- Polyvinilpyrrolidone-; 2% SDS)
4. Shake the tubes in a Mini Beader™ 1600Hz 30" (precool the holders at –20°C)
5. Incubate 10' at 70°C
6. Centrifuge 14000g 1' at 4°C
7. Recover the supernatant and add 1/10 vol. sodium acetate 5M pH 5.5
8. Incubate 10' on ice
9. Centrifuge 14000g, 5' at 4°C
10. Recover the supernatant and add 1 vol. Isopropanol at –20°C
11. Incubate 15' at –20°C
12. Centrifuge 14000g, 30' at 4°C
13. Discards the supernatant with care
14. Wash the pellet with 75% alcohol (do not resuspend the DNA), ≅ 1mL
15. Centrifuge 5' at 14000g and dry the pellet 15' at 37°C
16. Resuspend the pellet in 50 µL H2O or TE pH8 (better)
17. Add the resuspended pellet (16) on the PVPP column and spin 2' 1000g
18. Add the pass through to the sepharose 4B column and spin 2' 1000g
19. Recover the passthrough (DNA) and store at –20°C until use

Preparation of a PVPP column

- Prepare a PVPP column using Micro Bin-Spin column (Bio-Rad 732-6204)
- Fill the column with PVPP till the neck
- Add 400 µl H_2O
- Spin 2' 1000g 10°C

- Add400 µl H_2O
- Spin 2' 1000g 10°C
- *Preparation of a Sepharose 4B column (same procedure as above)*
- Add 1 ml sepharose 4B
- Spin 2' 1000g
- Add 50 µl TE pH8
- Spin 2' 1000g