Resistance to Fusarium basal rot and response to arbuscular mycorrhizal fungi in *Allium*

Guillermo A. Galván Vivero

Thesis committee

Thesis supervisors

Prof. dr. R.F. Hoekstra
Professor of Genetics (Population and Quantitative Genetics)
Wageningen University

Prof. dr. Th.W. Kuyper
Personal Chair at the Sub-department of Soil Quality
Wageningen University

Thesis co-supervisors

Dr. ir. O. E. Scholten
Senior Researcher
Plant Research International, Wageningen

Dr. C. Kik
Head Curator
Center for Genetic Resources, Wageningen

Other members

Prof. dr. ir. P.C. Struik, Wageningen University
Prof. dr. ir. H.J. Bouwmeester, Wageningen University
Prof. dr. D. Pink, University of Warwick, Warwick, United Kingdom
Dr. H. Huits, Bejo Zaden BV, Warmenhuizen

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Thesis

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Contents

Abbreviations

Commonly used in this thesis

AFLP	Amplified fragment length polymorphism
AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
AUDPC	Area under disease progress curve
FBR	Fusarium basal rot disease
MB	Mycorrhizal benefit
MR	Mycorrhizal responsiveness
MV	Mycorrhizal breeding value
NM	Non-mycorrhizal
QTL	Quantitative trait loci
RF-hybrid	*A. roylei* x *A. fistulosum* parental genotype (PRI 91021-8)
REML	Residual maximum likelihood analysis

Thesis key-words

AFLP · *Allium cepa* · *A. fistulosum* · *A. roylei* · arbuscular mycorrhizal fungi · Fusarium basal rot · *Fusarium oxysporum* f. sp. *cepae* · *F. proliferatum* · *Glomus intraradices* · Japanese bunching onion · linkage mapping · onion · QTL · Welsh onion

Abstract

Onion (*Allium cepa* L.) cultivation in low input and organic farming systems is hampered by Fusarium basal rot (FBR) and the limited ability of onion to take up nutrients like phosphorus. The symbiosis with arbuscular mycorrhizal fungi (AMF) contributes to plant acquisition of phosphorus, among other benefits. This PhD research studied the potential contributions from *A. fistulosum* and *A. roylei* to breed onion cultivars with resistance to FBR and enhanced benefit from the symbiosis with AMF. The genetic basis of these traits was studied in an *A. cepa* x (*A. roylei* x *A. fistulosum*) population. A collection of *Fusarium* isolates was analysed using AFLP markers. The most abundant species was *F. oxysporum* (with isolates clustered in two clades) followed by *F. proliferatum.* The *Allium* species were screened for FBR resistance using one *F. oxysporum* isolate from each clade, and one *F. proliferatum* isolate. *Allium fistulosum* showed high levels of resistance to these three isolates and *A. roylei* intermediate levels of resistance. High level of resistance from *A. fistulosum* was dominantly expressed in the *A. roylei* x *A. fistulosum* hybrid and the tri-hybrid population. A molecular linkage map based on AFLP markers was developed for the *A. roylei x A. fistulosum* hybrid. A QTL for FBR resistance from *A. roylei* was mapped on chromosome 2, and a QTL from *A. fistulosum* on chromosome 8. Each QTL separately had significant effect on FBR but did not confer complete resistance, thus more QTLs from *A. fistulosum* remain to be discovered. Regarding *Allium*-AMF relationship, a first step of research studied genetic diversity and colonization levels of naturally occurring AMF, comparing organic and conventional onion farming in the Netherlands. All plants were colonized with 60% average arbuscular colonization. Onion yields were positively correlated with colonization. AMF phylotypes were identified by rDNA sequencing. The number of phylotypes per field ranged from one to six. Two *Glomus*-A phylotypes were the most abundant, whereas other phylotypes were infrequently found. Organic and conventional fields had similar number of phylotypes and Shannon diversity indices. A few organic and conventional fields had larger number of phylotypes, which suggested that specific environmental conditions or agricultural practices influence AMF diversity. The genetic basis for the response to AMF in the tri-hybrid *Allium* population was evaluated in two independent greenhouse experiments. The weights of mycorrhizal plants were significantly larger than the non-mycorrhizal plants. Mycorrhizal Responsiveness (MR) was negatively correlated with plant weight in the non-mycorrhizal condition and was therefore considered unsuitable as an index for plant breeding purposes. Two new indices were proposed: mycorrhizal benefit (MB) and mycorrhizal breeding value (MV). Tri-hybrid genotypes showed transgressive segregation for plant weight, MB, and MV. Two QTLs from *A. roylei* for these traits were detected on chromosomes 2 and 3. A QTL from *A. fistulosum* for MV (but not MB), plant weight and the number of stem-borne roots was found on linkage group 9. Positive correlations between plant weight, rooting system and benefit from mycorrhiza were observed, which open prospects to combine these traits in the development of more robust onion cultivars.

Chapter 1
General Introduction

Onion (*Allium cepa* L.) is one of the main vegetable crops worldwide with respect to its production and economical value. This is also the case for Uruguay and The Netherlands (FAOSTAT 2008). Onion cropping systems usually make use of large amounts of inputs, and high-yielding crops widely rely on chemical control of diseases and large use of fertilizers (Bosch-Serra and Currah 2002). In the last decades, a number of risks and negative consequences of the use of synthetic chemicals in agriculture have been identified (Lorbeer et al. 2002). Therefore, agricultural systems involving more sustainable ways of productions like organic and low-input agricultural systems gained interest (Lammerts van Bueren 2003). In organic and low-input agricultural systems, crop yield is more in balance with other considerations like sustainability of the agro-ecosystem, management of biodiversity, and reduced impact on environment (Rossing et al. 2007).

In this context, the search for a broader crop genetic background by combining or introducing new genetic variation enhances possibilities for more sustainable agricultural systems (Stuthman 2002). Furthermore, another biological interaction that has received increasing attention in sustainable agriculture is the symbiosis between crops and arbuscular mycorrhizal fungi (AMF). Arbuscular mycorrhizal fungi usually improve the performance of their host plant species under sub-optimal growing conditions (Atkinson et al. 2002, Van der Heijden et al. 2008), with benefits such us improved uptake of phosphorus and protection against diseases (Gosling et al. 2006). Plant genetic variation that could allow a better exploitation of the interaction with AMF by the host has been described for various crops, including onion (Powell et al. 1982). Genetic variation for benefit from the mycorrhizal symbiosis opens opportunities for breeding. However, the practical exploitation of this variation in plant breeding is still in its infancy.

This introductory chapter deals with general aspects comprising the background to this thesis research: utilization of species that are close relatives to onion in breeding, breeding for resistance to onion basal rot caused by *Fusarium* species, and the relevance of plant genetic variation in the benefit from AMF for developing more robust onion cultivars.

The use of allied species for onion breeding

Allium cepa L. (section *Cepa;* sensu Friesen et al. 2006) is a species domesticated probably 3000 – 4000 years B.C. and not found in the wild (Shigyo and Kik 2008). Onion is attacked by several air- and soil-borne diseases (Entwistle 1990, Maude 1990), and breeding for resistance has made use of the limited genetic variation present within the crop. Although some wild and cultivated species that are related to onion can be regarded as important reservoirs of resistance genes (Table 1), breeders only made limited use of them. The first successful example of the use of wild relatives in onion breeding was the introgression of resistance from *A. roylei* to downy mildew caused by *Peronospora destructor* (Scholten et al. 2007). This and other introgressions may lead to a breakthrough in the management of onion diseases in the near future (Lorbeer et al. 2002).

Many factors are responsible for the limited use of related species in onion breeding in comparison to other cultivated crop species such as potato. The most important factors are the long juvenile phase of onion, the long generation time, and the difficulties to make successful crosses. Especially the presence of interspecific barriers has despaired breeders (Kik 2002). Crosses between onion and other *Allium* species have been carried out, but few interspecific crosses yielded seeds and a subsequent offspring with variable levels of fertility. *Allium vavilovii* is completely interfertile with onion, whereas *A. fistulosum, A. altaicum, A. galanthum, A. pskemense*, and *A. roylei* show intermediate levels of interfertility (Van Raamsdonk et al. 2003). Hybrids between onion and more distant species have been obtained via embryo rescue, but proved to be sterile (Keller et al. 1996).

Molecular markers and *in situ* hybridization techniques were applied to analyse genome recombination in interspecific hybrids, and enabled the precise detection of introgressed chromosome segments (Stevenson et al. 1998, Khrustaleva and Kik 1998, 2000, Van Heusden et al. 2000a, Khrustaleva et al. 2005, Scholten et al. 2007).

The amplified fragment length polymorphism (AFLP®) marker system, which was applied in this thesis, was used before in *Allium* research to develop linkage maps (Van Heusden et al. 2000a, De Melo 2003), to locate QTLs (De Melo 2003, McCallum et al. 2006, 2007), to study recombination and chromosome organization (Khrustaleva et al. 2005) and to reconstruct phylogeny of the genus *Allium* (van Raamsdonk et al. 2000, 2003). The strength of AFLP systems is that it covers the whole genome, does not require prior DNA information, and gathers a large number of data points (Klaas and Friesen 2002).

Table 1. Resistance from *Allium fistulosum* and *A. roylei* to onion diseases (partly based on Kik 2002).

Disease	Response	References
Allium roylei		
Botrytis squamosa	High resistance (putatively one single dominant gen)	De Vries et al. 1992a Walters et al. 1995
Peronospora destructor	Complete resistance (one single gen, *Pd*1)	Kofoet et al. 1990 Van Heusden et al. 2000a Scholten et al. 2007
Colletotrichum gloeosporioides	Partial resistance (putatively polygenic)	Galván et al. 1997
Allium fistulosum		
Botrytis squamosa	High to complete resistance	Bergquist and Lorbeer 1971 Currah and Maude 1984 Walters et al. 1996
Fusarium oxysporum	High resistance	Holz and Knox-Davies 1974
Phoma terrestris	High resistance	Ludwig et al. 1992 Netzer et al. 1985 Porter and Jones 1933
Colletotrichum gloeosporioides	High resistance	Galván et al. 1997
Urocystis cepulae	Resistance	Felix 1933

Allium roylei Stearn

Allium roylei (Syn. *A. lilacinum* Royle ex Regel, non *A. lilacinum* Klotzsch) (2n=2x=16) was collected by V.B. Sharma from the Ku College in India at Mysore, Himalayas (McCollum 1982). This accession known originally as C502 and later as PI 243009 is still the single *A. roylei* source available worldwide, as further attempts to collect the species were unsuccessful (De Vries et al. 1992b).

However, recently new *A. roylei* accessions collected in the Western Himalaya (India) were reported, which were found in an altitude range of 2000-3500 m, and occasionally cultivated and used as a condiment (Pandey et al. 2008).

Allium roylei was successfully crossed with onion by Van der Meer and De Vries (1990). It is a conspicuous species, having high crossability with onion but with morphological characters typical of species belonging to section *Rhiziridium*

sensu lato: short plant with narrow filiform leaves, cylindrical leaf section sometimes solid, and reticulate bulb tunics (Fritsch and Friesen 2002). It was included in section *Cepa* based on nuclear and cpDNA markers, although carrying at the same time a considerable number of synapomorphies with *Rhiziridium* (Klaas and Friesen 2002, van Raamsdonk et al. 2003). As a consequence, it has been proposed that *A. roylei* may have a hybrid origin (*sensu* Hanelt 1990, Frisch and Friesen 2002), although that suggestion has been refuted by phylogenetic analysis on the basis of rDNA (Gurushidze et al. 2007).

Allium roylei is resistant to important onion diseases (Table 1). An AFLP linkage map was developed for this species, using a population obtained from a cross with *A. cepa* (van Heusden et al. 2000a). The resistance to downy mildew (*Pd1* gene) has been mapped in the distal region of the long arm of linkage group 2 (Van Heusden et al. 2000a), which was proven to be chromosome 3 via genetic analysis of monosomic addition lines (Van Heusden et al. 2000b).

Allium fistulosum L.

Allium fistulosum (2n=2x=16) or Welsh onion is a cultivated species. This species is also known as Japanese bunching onion, green onion, and scallion. In Asia, it is grown to a large extent and used as a typical ingredient in the local cuisine. *Allium fistulosum* does not develop bulbs (Inden and Asahira 1990).

Allium fistulosum is certainly the species where the largest number of attempts have been made to cross it with *A. cepa* in order to exploit disease resistances and physiological features (Kik 2002). Both species have the same chromosome number, similar cpDNA restriction patterns and similar karyotypes (Havey 1991), but apparently differ enough to produce an almost completely sterile inter-specific hybrid. Although some seed is obtained after selfing, the inter-specific hybrid backcross programs proved to be problematic, and onion cultivars with beneficial traits from *A. fistulosum* have been never developed (Villanueva Mosqueda et al. 2000). Heteromorphic bivalents and disturbed synapses in the centromeric region were observed during meiosis of interspecific hybrids, whilst chiasmata frequency was reduced in comparison with both parents (Stevenson et al. 1998). In backcrosses with onion, only a limited number of progeny plants were obtained with irregular segregation, due to the occurrence of nuclear-cytoplasmic incompatibility (Mangum and Peffley 2005).

In order to introgress *A. fistulosum* traits into the genetic basis of onion, *A. roylei* may act as a bridge species (Khrustaleva and Kik 1998, 2000). The intermediate size of the *A. roylei* genome (28-30 pg·cell^{-1}) between *A. cepa* (32-

33.5 pg·cell^{-1}) and *A. fistulosum* (22.5-23.5 pg·cell^{-1}) genomes may partly explain its effectiveness as bridge species (Ricroch et al. 2005).

The tri-hybrid *A. cepa x (A. roylei x A. fistulosum*) allows the simultaneous exploitation of two onion-related species, in a gene-pool approach, as coined by Hermsen (1992). Progeny plants of the first cross between *A. cepa* and the *A. roylei x A. fistulosum* hybrid showed high binding of bivalents arms (82.6%) with occasional inversions in meiosis, and intermediate pollen fertility (Khrustaleva and Kik 1998). The second backcross generation with onion showed high frequency of recombination points randomly distributed along the chromosomes (Khrustaleva and Kik 2000). Pollen fertility in progeny plants was variable, but seemed to be a trait that can be selected for. Although it has not been demonstrated that all the chromatin from *A. roylei* or *A. fistulosum* can be introgressed, the potential value of this bridge-cross population for onion breeding is evident (Kik 2002).

The tri-hybrid *A. cepa x (A. roylei x A. fistulosum*) was used to study the inheritance of rooting traits from *A. fistulosum,* traits that contribute to the development of more robust onion cultivars. QTLs were identified using an AFLP linkage map (De Melo 2003). This example of identification of QTLs and molecular markers linked to target traits can assist introgressions from *A. fistulosum* and *A. roylei* into the onion gene pool, by following specific chromosomal regions in segregating populations (Charcosset and Moreau 2004).

Breeding for resistance to Fusarium basal rot

Sources of resistance

The genetic analysis of resistance to Fusarium Basal Rot (FBR) in the tri-hybrid *A. cepa x (A. roylei x A. fistulosum)* is the first core subject of this thesis. FBR is spread worldwide in tropical and temperate regions, affecting onion and other cultivated *Allium* species. *Fusarium oxysporum* f. sp. *cepae* is regarded as the causal agent (Entwistle 1990). Other *Fusarium* species affecting onions have been also reported (Entwistle 1990, Du Toit et al. 2003). The pathogen infects the roots or the basal plate of the plant. Further infection of bulb scales only occurs late in the season, with major losses being observed during postharvest storage. Isolates of this pathogen may differ in aggressiveness (Villeveille 1996, Özer et al. 2003), but research on the presence of genetic variation in this onion pathogen has not been carried out before.

Cramer (2000) has reviewed FBR in the context of breeding for resistance. Onion host resistance has been exploited in several breeding programs located in different regions along the world. Among 331 onion cultivars in the United States, 52 were reported as having some FBR resistance by the vendors (Havey and Wehner 1999). They mostly belong to long day (LD) and intermediate day (ID) onion groups (Lopez and Cramer 2002). In order to explain the genetic basis of resistance to FBR, a number of hypotheses have been put forward (summarized by Cramer 2000), ranging from a single gene to multiple genes, including cytoplasmatic effects. However, no resistance gene was ever mapped on a molecular linkage map.

Although yield losses can be significantly reduced by host resistance, the response is not absolute, and the most widely grown cultivars are susceptible. Moreover, FBR resistant cultivars did not always behave as such when grown in different regions (Valdez and Galmarini, pers. com.). Breeders and growers are concerned about FBR, and higher levels of resistance will be welcome. *Allium* species related to onion may carry genes for resistance to FBR, but these have hardly been studied as sources of resistance.

Screening methods

The expression of *Fusarium* resistance in *A. cepa* accessions depends upon the conditions of the screening procedure. Plant age at the moment of inoculation or infection is a relevant factor of variation, as germinating seedlings and bulbing plants are much more susceptible than plants that are in the non-bulbing growing stage (Cramer 2000).

A seedling test under controlled conditions has been applied as a fast, easy and reliable procedure (Krueger et al. 1989, Lopez and Cramer 2002, Özer et al. 2003, Stadnik and Dhingra 1995). The necessary correlation with field resistance (e.g., reduced postharvest losses) was assumed, though scant information is available on this issue. The quality of the seed lots is also an important factor of variation, because accessions that have poorly germinating seeds behave as susceptible (Cramer 2000). An important constraint of seedling tests for genetic analysis is that susceptible genotypes die even before emergence, thus they cannot be fingerprinted.

Screening assays using adult plants under controlled conditions in the greenhouse have been described less frequently (Holz and Knox-Davies 1974, Stadnik and Dhingra 1995). Field screening is probably the most commonly applied test in commercial breeding companies. Resistance is evaluated at harvest by visual estimation of infections on onion basal plates. Slicing the basal plate to

examine internally developed infections (dark to brown areas within the basal disc) is an effective method to characterize the reaction of onion germplasm to FBR (Gutierrez and Cramer 2005). The evaluation of postharvest losses is used to a large extent by breeding companies (Cramer 2000).

Breeding onion for better exploiting the benefit from mycorrhiza

Arbuscular mycorrhizal fungi in sustainable agriculture

The analysis of the possibilities to enhance the benefit from arbuscular mycorrhizal fungi (AMF) in onion by means of breeding is the second core subject in this thesis. The focus in this sense is also on the contribution from *A. fistulosum* and *A. roylei* to broaden the genetic variation of the onion gene-pool.

Interactions with AMF are widespread among most plant families, comprising the majority of agricultural crop species. AMF are obligate symbionts with very low host specificity. As a result of the symbiosis, AMF take carbon and energy from the host plant, which usually grows better than a non-mycorrhizal plant due to the improved acquisition of nutrients (particularly phosphorus) and water through the fungal partner (Mosse 1973, Stribley 1990).

Industrialized agricultural systems developed during the 20th century disregarded the benefits from mycorrhiza (Barker et al. 2002). Under high-input agriculture, nutrients are available in large amounts, and AMF do not play a role in yield enhancement (Van der Heijden et al. 2008). Even more, AMF colonization can be inhibited by the high internal phosphorus status of the host plant or by high phosphorus availability in the soil (Hetrick et al. 1996, Ryan et al. 2000). However, in the context of organic and low-input agricultural systems there is room to re-consider the role of AMF, because availability of nutrients in these systems does not rely on external supplies, and shortages may frequently occur (Gosling et al. 2006; Ryan and Graham 2002). Furthermore, the symbiosis with AMF can result in other benefits for the crop in the frame of sustainable agriculture, like protective effect against pathogens (Pozo and Azcón-Aguilar 2007) and soil aggregation (Jastrow et al. 1998).

Agriculture and diversity of arbuscular mycorrhizal fungi

Agricultural soils hold lower AMF diversity than natural ecosystems such as woodlands and grasslands (Helgason et al. 1998). An analysis of diversity in arable lands showed that the number of AMF genera and species was negatively

related to the degree of intensification of the agro-ecosystem (Oehl et al. 2003). Several factors have a negative impact on AMF communities, like frequent tillage, rotation with non-mycorrhizal crop species, the occurrence of bare fallow periods, and the use of fertilizers and fungicides (Lekberg and Koide 2005, Gosling et al. 2006). For example, species of *Acaulospora* and *Scutellospora* were rare in soil samples from highly intensively managed soils (Oehl et al. 2003). As AMF diversity decreases, some *Glomus* species become dominant in agricultural soils, particularly *G. mosseae* (Helgason et al. 1998) and *G. intraradices* (Mathimaran et al. 2005). One of the explanations is that these species tolerate tillage-induced disruption of the hyphae, and have an abundant and fast production of spores (Boddington and Dodd 2000, Jansa et al. 2002).

Does AMF species diversity contribute to agricultural yields? AMF species diversity is correlated with aboveground diversity in plant communities in natural ecosystems (Van der Heijden et al 1998). In agricultural systems, hypothesis on the role of AMF diversity refers to the complementary action of taxa differing in their functionality and optimal ecological conditions, with differences in timing of activity during the growing season (Merryweather and Fitter 1998, Hijri et al. 2007) and soil depth (Oehl et al. 2005). In several studies, organic agricultural systems had crops with larger AMF colonization and inoculum potential than crops in conventional agricultural systems. These differences were often explained by the lower P availability of the soils (Dann et al. 1996, Ryan et al. 2000, 2002). Whether the adoption of organic farming may lead to a difference in AMF diversity, however, has been studied to a lesser extent (Hijri et al. 2007). Chapter 4 of this thesis aims to contribute to this topic.

Plant genetic variation for the benefit from mycorrhiza

Host plant genetic variation for the benefit from mycorrhiza has been described between cultivated species (Plenchette et al. 1983), as well as between cultivars and breeding lines in several cultivated species. Although the development of cultivars with enhanced response to AMF was claimed as a way to achieve successful and stable yields in low-input agriculture (Ryan and Graham 2002, Barker et al. 2002), the benefit from mycorrhiza has not been a selection criterion applied in breeding programs yet. A factor preventing the practical utilization could be the difficulties to measure the benefit from mycorrhiza when comparing plant genotypes, as discussed in Chapter 5.

The interaction between plants and AMF is partly determined by the genetic background of both partners. These genetic backgrounds determine, firstly, a compatible interaction (Gollotte et al. 2002, Barker et al. 2002). In a time-course process, this involves recognition events, the establishment of the symbiosis, and

its functionality. Genetic variation among plant genotypes for the benefit from mycorrhiza could be the result of differences in recognition events, which may be translated into differences in the timing and degree of colonization. Root cells harbouring arbuscules are completely re-programmed in their functioning (Massoumou et al. 2007), and differences among genotypes may occur.

Another hypothesis explaining differences among plant genotypes is that they differ in the functioning of the symbiosis. Although primarily supported by specific biochemical pathways involving carbon and mineral transfer, particularly phosphorus (Strack et al. 2003), this efficiency should be finally expressed in an agricultural context as increased plant biomass.

Genetic variation in the efficiency of the symbiosis could also be influenced by plant traits, like the morphology of the rooting system (Lynch 2007). The larger and denser the rooting system, the lower the benefit will be from mycorrhiza because the plant itself can take up enough nutrients from the soil (Zhu et al. 2001, Wright et al. 2005). Plants of *Andropogon gerardii* with smaller rooting system invested proportionately more in the symbiotic association than plants with larger rooting system (Schultz et al. 2001). Plant potential growth and efficiency of the plant to take up nutrients may also diminish the benefit from mycorrhiza (Kaeppler et al. 2000). Genetic variation for such plant traits may interfere with evaluation of the benefit from mycorrhiza and the set up of breeding schemes.

The benefit from mycorrhiza can be measured as the difference in total plant biomass (fresh weight, dry weight) in comparison to a non-mycorrhizal control. 'Mycorrhizal responsiveness' (Janos 2007) is a commonly used index. It is a dimensionless ratio, based on the difference in plant dry weight between a mycorrhizal and non-mycorrhizal plant, in relation to biomass of mycorrhizal (Plenchette et al. 1983) or non-mycorrhizal (Baon et al. 1993) plant:

$$MR = \frac{DW_{AM} - DW_{NM}}{DW_{NM}} \times 100$$

Because the main contribution of AMF is the enhanced P acquisition by the plant, the benefit from mycorrhiza can also be expressed in the literature as the improvement in P content (P mass fraction x biomass) (Smith 2000, Janos 2007). Nevertheless, improvements in phosphorus uptake do not automatically result in an increased plant growth (Li et al. 2006).

As responsiveness of a plant genotype depends upon the P availability in the soil (Mosse 1973, Stribley 1990, Wright et al. 2005), it was suggested that the phosphorus concentration in the soil should be indicated when responsiveness is evaluated (Plenchette et al. 1983). Furthermore, Janos (2007) warned that the comparison of plant species or genotypes at a single phosphorus level may be misleading, because genotypes may differ in their response curves along a gradient of P levels in the soil. However, screening a large set of genotypes in a range of P levels could be expensive. Furthermore, P level is not the only environmental factor influencing the benefit from mycorrhiza (Sawers et al. 2008). Therefore, screening in a single environment with one P level can be a valuable first step in a breeding programme.

As discussed before, plant genotypes differ also in the ability to grow in a P-deficient environment in the absence of AMF (Wright et al. 2005). Janos (2007) proposed to exclusively use the expression AMF dependency (which had earlier been treated as equivalent to mycorrhizal responsiveness) to indicate the threshold P level below which a genotype cannot grow without mycorrhiza (Figure 1).

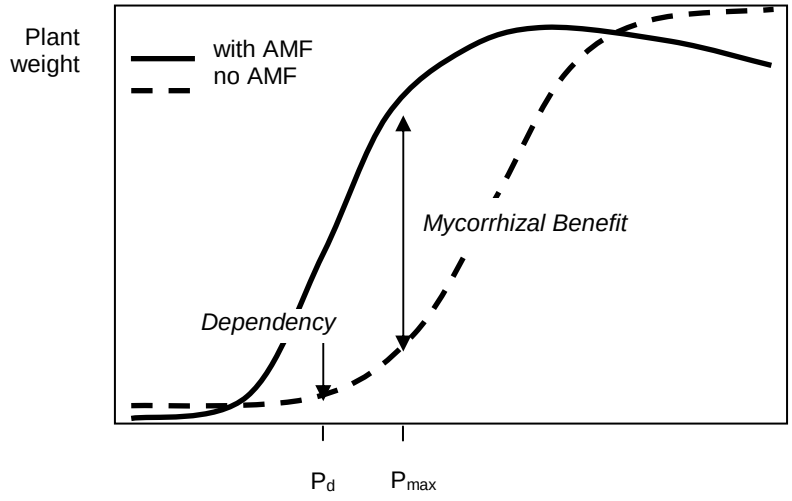

Figure 1. Typical plant response curves along a gradient of P levels with and without mycorrhiza (based on Janos 2007). Mycorrhizal responsiveness varies as a function of the P level in the soil. Mycorrhizal benefit (MB), which was defined in this thesis as the weight difference between plants with and without mycorrhiza, varies with P level in the soil. The soil P level where benefit is largest is P_{max}. Dependency is the minimum P level in the soil (P_d) below which the plant is not able to grow without mycorrhiza.

Genetic analysis of the benefit from mycorrhiza

Few studies have analyzed the genetic basis of the benefit from mycorrhiza. Hetrick et al. (1993, 1995) studied the genetic basis of AMF responsiveness in wheat. As most modern wheat cultivars had lower responsiveness than old accessions, they suggested that modern breeding activities in wheat could inadvertently have selected against AMF responsiveness. This indirect selection would be driven by environments not conducive to AMF (e.g. high nutrient availability, intensive tillage, use of fungicides) which lead to a low responsiveness, as plant genes involved in this interaction are not selected for (Hetrick et al. 1993). However, an alternative explanation could be that low responsiveness of modern cultivars may be the result of their ability to grow in a nutrient-deficient environment in the absence of AMF, by selection for yield stability across different environmental conditions (Kaeppler et al. 2000, Sawers et al. 2008).

Kaeppler et al. (2000) studied the genetic basis for AMF responsiveness in maize using inbred lines in a soil with low P availability. Responsiveness was negatively correlated with plant weight under non-mycorrhizal conditions. This means that inbred lines with larger biomass in the non-mycorrhizal control had lower responsiveness, because growth improvement provided by the action of AMF was proportionally lower. The use of ratio variables with a non-constant denominator may lead to incorrect conclusions because of the effects of other traits acting as co-variables (Righetti et al. 2007). This problem associated with the use of ratios should be taken into account when measuring the benefit from mycorrhiza for breeding purposes, and will therefore be discussed in the framework of this thesis.

Variation in AMF responsiveness was reported among onion cultivars (Powell et al. 1982). *Allium fistulosum* highly benefitted from the inoculation with AMF, and differences between cultivars were observed as well (Tawaraya et al. 2001). Thus, the question arises whether species related to onion such as *A. fistulosum* or *A. roylei* may contribute to enhanced benefit from mycorrhiza in onion by broadening the genetic basis for this trait. This issue will also be addressed in this research.

Aims and outline of this thesis

This thesis aims to contribute to a more sustainable agriculture via the development of onion cultivars that harbour a high resistance to Fusarium basal rot, and an improved benefit from arbuscular mycorrhizal fungi. To this end, the diversity of *Fusarium* species pathogenic to onion was studied, and the resistance to FBR in *A. fistulosum* and *A. roylei* was analyzed. Furthermore, the diversity of AMF communities in agricultural fields was analyzed, and the genetic basis of the exploitation of AMF in *Allium* species was studied.

In **Chapter 2** the resistance to Fusarium basal rot in *Allium* species was investigated using different *Fusarium* isolates. A collection of *Fusarium* isolates from Uruguay, The Netherlands and other countries and regions of the world is analyzed using AFLP markers. This is the first analysis of genetic diversity of this onion pathogen based on molecular markers, and the first comprehensive screening of onion related species for resistance to FBR.

Chapter 3 presents the genetic analysis of the resistance to Fusarium basal rot in a tri-hybrid population of *A. cepa x (A. roylei x A. fistulosum)*. A greenhouse screening was performed using clonally propagated *in vitro* plant material. A molecular linkage map based on AFLP markers was developed for the *A. roylei x A. fistulosum* parent, on which QTLs for resistance to FBR were located.

In **Chapter 4**, the diversity of AMF in onion cultivation in The Netherlands is described, with the aim to compare organic and conventional cultivation. Besides the analysis of AMF diversity, this chapter gives insight in the relevance of AMF in agricultural soils in the Netherlands, and its relevance for onion cultivation.

Chapter 5 presents an evaluation of the benefit from mycorrhiza in onion and related *Allium* species. A genetic analysis was carried out based on the evaluation of the response to AMF of tri-hybrid genotypes. New indices to measure the response to AMF are proposed, and QTLs for these indices are located on the AFLP linkage map of the *A. roylei x A. fistulosum* parent. The potential value of this benefit in relation to measured plant traits is discussed. Besides experimental data and results for *A. fistulosum* and *A. roylei*, this research contributes to a theoretical framework for breeding for the benefit from mycorrhiza.

A general discussion of the findings of this thesis is presented in **Chapter 6.** The value of the tri-hybrid population for onion breeding is discussed, based on the results obtained in this PhD research. Other topics addressed are the genetic variation in *Fusarium* isolates pathogenic to onion, the significance of AMF diversity for agriculture, and the benefit from mycorrhiza as a goal in breeding programmes. Emerging future research lines are indicated, like introgressions schemes for the traits investigated in this study, and the analysis of the interaction between AMF and *Fusarium.*

Chapter 2

Genetic variation among *Fusarium* isolates from onion and resistance to Fusarium basal rot in related *Allium* species†

Abstract

The aim of this research was to study levels of resistance to Fusarium basal rot in onion cultivars and related *Allium* species, by using genetically different *Fusarium* isolates. In order to select genetically different isolates for disease testing, a collection of 61 *Fusarium* isolates, 43 of them from onion (*Allium cepa*), was analysed using amplified fragment length polymorphism (AFLP) markers. Onion isolates were collected in the Netherlands (15 isolates) and Uruguay (9 isolates), and received from other countries and fungal collections (19 isolates). From these isolates, 29 were identified as *F. oxysporum*, 10 as *F. proliferatum*, whereas the remaining 4 isolates belonged to *F. avenaceum* and *F. culmorum*. The taxonomic status of the species was confirmed by morphological examination, by DNA sequencing of the elongation factor 1-α gene, and by the use of species-specific primers for *Fusarium oxysporum*, *F. proliferatum*, and *F. culmorum*. Within *F. oxysporum*, isolates clustered in two clades suggesting different origins of *F. oxysporum* forms pathogenic to onion. These clades were present in each sampled region. Onion and six related *Allium* species were screened for resistance to Fusarium basal rot using one *F. oxysporum* isolate from each clade, and one *F. proliferatum* isolate. High levels of resistance to each isolate were found in *A. fistulosum* and *A. schoenoprasum* accessions, whereas *A. pskemense*, *A. roylei* and *A. galanthum* showed intermediate levels of resistance. Among five *A. cepa* cultivars, 'Rossa Savonese' was also intermediate resistant. Regarding the current feasibility for introgression, *A. fistulosum*, *A. roylei* and *A. galanthum* were identified as potential sources for the transfer of resistance to *Fusarium* into onion.

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Introduction

Fusarium oxysporum Schlecht.: Fr. f. sp. *cepae* (H.N. Hansen) Snyder & Hansen causes basal rot of onion (*Allium cepa* L.) (Entwistle 1990). The fungus infects the roots or the basal plate of the bulbs. Further infection of bulb scales occurs later in the season, and most severe losses are found in post-harvest storage. The fungus is spread worldwide, and also infects other cultivated *Allium* species, such as garlic (Entwistle 1990).

The forma specialis *cepae* is one of the host-specific groups within *F. oxysporum*, a complex and diverse species with large diversity in specific host-ranges as well as non-pathogenic forms (Kistler 1997). Numerous studies have been conducted to describe the genetic diversity of this species, although no markers related to pathogenicity were found (Baayen et al. 2000a, Recorbet et al. 2003). Comparisons of sequences of the elongation factor 1α and the mitochondrial small subunit rDNA led to the identification of three different clades (O'Donnell 1998, Baayen et al. 2000a), each consisting of isolates from several formae speciales. Studies on genetic diversity of *Fusarium* isolates from onion are not yet available.

Variation in aggressiveness between *F. oxysporum* isolates pathogenic on onion was reported before (Villeveille 1996, Özer et al. 2003, Valdez et al. 2004). Variation among *Fusarium* isolates might explain differences in response of resistant selections, as was suggested by C. Galmarini and J. Valdez (*pers. com.*) when partially resistant onion cultivars bred in the USA appeared to be susceptible in Argentina. This observation might be an indication that variation among isolates exists and could be a factor towards the targeted selection for resistance against specific isolates.

Recently *F. proliferatum* (Mats.) Nirenberg was found affecting onion (du Toit et al. 2003, Stankovic et al. 2007) and garlic (Dugan et al. 2003). Other *Fusarium* species were reported in the past as minor onion pathogens (Entwistle 1990), but *F. oxysporum* is the most frequently found species causing onion basal rot.

Within *A. cepa*, only partial resistance to *F. oxysporum* f. sp. *cepae* has been found, which is being exploited in breeding programmes (Cramer 2000). Although this has resulted in the development of cultivars with reduced post-harvest and yield losses, breeding efforts are ongoing, as there is still a need for further improvement.

In species related to onion (*Allium* Section *Cepa* (Mill.) Prokh.) high levels of resistance to several diseases have been found (Kik 2002). Resistance to Fusarium basal rot was reported in *A. fistulosum* (Abawi and Lorbeer 1971, Holz and Knox-Davies 1974). More recent reports, however, showed that *A. fistulosum* can be affected by *F. oxysporum* (Shinmura et al. 1998; Navia and Gómez 1999) and *F. redolens* (Shinmura 2002). No reports are available about screening for resistance to Fusarium basal rot in other *Allium* species related to onion.

The aim of the current research was to study levels of resistance in onion cultivars and related *Allium* species to genetically different *Fusarium* isolates. In order to know whether or not isolates differ genetically and belong to different species, a collection of *Fusarium* isolates originating from onions grown in different regions of the world was studied by the use of amplified fragment length polymorphism markers (AFLP). The taxonomic status of the isolates was investigated morphologically and confirmed by DNA sequencing of the elongation factor 1-α gene, and by the use of species-specific primers for *Fusarium oxysporum, F. proliferatum,* and *F. culmorum.* Two *F. oxysporum* isolates, one from each clade, and one *F. proliferatum* isolate, were taken to screen for levels of resistance to Fusarium basal rot in onion cultivars and six related *Allium* species.

Materials and Methods

Fungal collection

A collection of *Fusarium* isolates was set up by sampling onion fields and storage sheds in Uruguay in 2003, and the Netherlands in 2004, as well as by kind supply from researchers and institutes from various countries (Table 2). This collection includes 43 isolates from onion, three from garlic (*A. sativum*) and one from shallot (*A. cepa* common group *aggregatum*). In addition, *Fusarium* isolates from other crops were included as controls (Table 2).

Slices of basal plates or diseased roots, 5 to 6 cm in length, were surface disinfected by immersion for one minute in 70 % ethanol, one minute in NaOCl (15 $g \cdot l^{-1}$), two times in sterile water, and incubated in Petri dishes on a blotter (25°C, 3-7 days). From rotten bulbs, pieces of mycelium were isolated with a needle from the borders of the lesions that appeared in rotten bulbs. Hyphal-tip colonies from root lesions, basal rot, or bulb rot, were first isolated in water-agar (34 g $agar \cdot l^{-1}$), and then maintained on potato dextrose agar (PDA, Oxoid Ltd, England).

Table 2. Collection of *Fusarium* isolates from onion and other host species included in the study of genetic diversity of *Fusarium* using AFLP markers.

Identification [a]	Test of pathogenicity [b]	Date of collection	Country and place of collection	Host, organ
***Fusarium oxysporum* isolated from onion (f. sp. *cepae*) and *Allium* crops**				
93.816 [d]	+	1993	The Netherlands	Onion
CBS 148.25 [e]	+	1925	n.i. [c]	Onion
CBS 192.35 [e]	+	1935	Germany	Onion
CBS 193.35 [e]	+	1935	Germany	Onion
DSM 62306 [f]	+	n.i.	United States, California	Onion, bulb
EZA [g]	+	2004	Australia	Onion
Fo Ech [h]	+	n.i.	France	Shallot
Foc 06 [i]	+	n.i.	Turkey	Onion, seed
Hue-2	n.d.	2004	Spain, Huelva	Garlic, basal plate
Hue-3	n.d.	2004	Spain, Huelva	Garlic, basal plate
Hue-5	n.d.	2004	Spain, Huelva	Garlic, basal plate
LJC 10081 [j]	+	2004	Argentina, Buenos Aires	Onion, fleshy scales
LJC 10045 [j]	+	2004	Argentina	Onion, fleshy scales
LJC 10164 [j]	+	n.i.	United States, Texas	Onion
LJC 10165 [j]	+	n.i.	United States, Texas	Onion
LJC 10159 [j]	+	n.i.	United States	Onion
NL 102-1	+	2004	The Netherlands, Schoondijke	Onion, root
NL 102-2	n.d.	2004	The Netherlands, Schoondijke	Onion, root
NL 104-2	+	2004	The Netherlands, Kerkwerve	Onion, root
NL 106-2	+	2004	The Netherlands, IJzendijke	Onion, root
NL 106-3	n.d.	2004	The Netherlands, IJzendijke	Onion, root
NL 106-4	+	2004	The Netherlands, IJzendijke	Onion, root
NL 109-2	+	2004	The Netherlands, Langeweg	Onion, root
NL 132	+	2004	The Netherlands, Wageningen	Onion, basal plate
NM 1 [k]	+	1999	United States, New Mexico	Onion
NM 2-4 [k]	+	2004	United States, New Mexico	Onion
NM 2-5 [k]	+	2004	United States, New Mexico	Onion
NM 2-7 [k]	+	2004	United States, New Mexico	Onion
UR 07	n.d.	2003	Uruguay, Canelones, La Paloma	Onion, root
UR 16	+	2003	Uruguay, Canelones, Progreso	Onion, fleshy scales
UR 17-3	+	2004	Uruguay, Canelón Grande	Onion, fleshy scales
UR 17-5	+	2004	Uruguay, Canelón Grande	Onion, fleshy scales
UR 17-8	+	2004	Uruguay, Canelón Grande	Onion, fleshy scales
F. oxysporum* f. sp. *lilii				
Fol 11 [d]	n.d.	n.i.	The Netherlands	Lily
Fol 4 [d]	n.d.	n.i.	The Netherlands	Lily
F. oxysporum* f. sp. *lagenariae				
UR 13 [l]	n.d.	n.i.	Uruguay	Pumpkin

Table 2 (*cont.*)

Identification [a]	Test of pathogenicity [b]	Date of collection	Country and place of collection	Host, organ
F. oxysporum* f. sp. *loti				
UR 15 [l]	n.d.	n.i.	Uruguay	Birds-foot trefoil
F. oxysporum* f. sp. *tulipae				
Fot 10 [d]	n.d.	2003	The Netherlands	Tulip
Fot 13 [d]	n.d.	2003	The Netherlands	Tulip
Fot 47 [d]	n.d.	2003	The Netherlands	Tulip
Fot 67 [d]	n.d.	2003	The Netherlands	Tulip
Fot Yoko3 [d]	n.d.	2003	The Netherlands	Tulip
Fusarium proliferatum				
LJC 10013 [j]	+	2004	Argentina, San Juan, Pocito	Onion, fleshy scales
LJC 10023 [j]	+	2004	Argentina, San Juan, Pocito	Onion, fleshy scales
LJC 10033 [j]	+	2004	Argentina, Mendoza, Maipú	Onion, fleshy scales
NL 109-1	n.d.	2004	The Netherlands, IJzendijke	Onion, root
NL 131-1	+	2004	The Netherlands, Wageningen	Onion, basal plate
NL 131-2	+	2004	The Netherlands, Wageningen	Onion, basal plate
NL 131-3	+	2004	The Netherlands, Wageningen	Onion, basal plate
UR 01	+	2003	Uruguay, Canelones, Las Piedras	Onion, fleshy scales
UR 03	n.d.	2003	Uruguay, Villa Nueva Sauce	Onion, fleshy scales
UR 06	n.d.	2003	Uruguay, Villa Nueva Sauce	Onion, seed
Fusarium equiseti				
UR 09 [l]	—	2003	Uruguay, Canelones, Progreso	Pumpkin
Fusarium verticillioides				
MRC 826 [m]	n.d.	n.d.	South Africa	Maize, kernels
Fusarium avenaceum				
UR 04 [l]	+	2003	Uruguay, Canelones, Canelón Grande	Onion, fleshy scales
UR 10 [l]	n.d.	2003	Uruguay, Canelones, Progreso	Pumpkin
Fusarium graminearum				
Fg 820 [d]	n.d.	n.i.	The Netherlands	Wheat, kernels
Fusarium culmorum				
IPO39 [d]	+	n.i.	The Netherlands	Wheat, kernels
NL 110-1	+	2004	The Netherlands, Zeeland, Stroodorp	Onion, root
NL 110-2	+	2004	The Netherlands, Zeeland, Stroodorp	Onion, root
NL 110-3	+	2004	The Netherlands, Zeeland, Stroodorp	Onion, root

[a] In the identification codes, a figure behind a slash distinguishes isolates obtained from different plants or bulbs in a single field or storage shed. [b] (+) pathogenic; (—) non-pathogenic; (n.d.) not determined. (n.i.) no information available. [d] Plant Research International, Wageningen Univ., the Netherlands. [e] Provided by Centraalbureau voor Schimmelcultures, the Netherlands; [f] by Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; [g] by Dr. K. Posthuma, Enza Zaden, Enkhuizen, The Netherlands; [h] by Dr. C. Alabouvette and Dr. N. Gautheron, C.M.S.E.- INRA Dijon, France; [i] by Dr. N. Özer, Univ. of Trakya, Turkey; [j] by Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Argentina; [k] by Dr. C. Cramer and Dr. Muhyi, New Mexico St. Univ., USA. [l] Laboratorio de Fitopatología, Fac. de Agronomía, Univ. de la República, Uruguay. [m] Provided by Dr. W. Marasas, M.R.C, South Africa.

Pathogenicity was tested on onion cultivar ´Texas Early Grano 502´ by the seedling test described by Krueger et al. (1989). Three replications per isolate were tested, each consisting of 30 seeds sown in heat-sterilized sand, and inoculated with a suspension of conidia ($1 \cdot 10^5$ spores·ml^{-1}, $1 \cdot 10^4$ spores·g^{-1} dry sand). Isolates were considered pathogenic when the number of emerged seedlings significantly differed from a non-inoculated control (analysis of variance, $p<0.05$). Pathogenic isolates were included in this research, as well as six isolates that were not tested (Table 2).

Amplified Fragment Length Polymorphism analysis

Fungal isolates were multiplied in 100 ml flasks containing 50 ml of potato dextrose broth (PDB, Difco, Madison, USA), and grown for five to seven days in a shaker rotating at 100 rpm in the dark, at 18 °C. Fungal tissue was harvested, dried by vacuum filtration through a nylon membrane, and lyophilized overnight. For AFLP analysis, fungal DNA was isolated from 10 mg of lyophilized mycelium ground in 2 ml tubes. Cells were lysed by incubation with 450 µl Puregene Cell Lysis Solution D5002 (Gentra Sys., Minneapolis, USA) for one hour at 65°C, followed by addition of 150 µl of Puregene Protein Precipitation Solution D-5003, and centrifugation at 14.000 rpm for 5 min. DNA was precipitated by mixing the supernatant with 300 µl chilled isopropanol, and centrifugation at 14.000 rpm for 10 min. at 4°C. DNA pellets were washed with 300 µl 70% ethanol, dried and dissolved in 100 µl Tris-EDTA. RNA was degraded with 5 µl of RNAse A solution (37°C, 30 min). 400 ng of DNA suspension was used for AFLP reactions.

AFLP® fingerprinting (Keygene B.V., The Netherlands) was done as described by Vos et al. (1995) using 400 ng of fungal DNA and two combinations of restriction enzymes: *Eco*RI-*Mse*I and *Eco*RI-*Pst*I. Pre-amplification was performed in a volume of 20 µl using primers without extra-nucleotides: E_{00} (5'-GACTGCGTACCAATTC-3') – P_{00}L (5'-GTAGACTGCGTACATGCAG-3') and E_{00} – M_{00} (5'-GATGAGTCCTGAGTAA-3'). For selective amplification three primer combinations were used: E01 (E_{00}+A) – P16 (P_{00}L +CC); E15 (E_{00}+CA) – M14 (M_{00}+AT); and E15 – M23 (M_{00}+TA).

The primers P16 and E15 were fluorochrome-labeled IRD700 (Westburg BV, Leusden, The Netherlands). Reactions were performed in 10 µl containing 5 µl aliquot of the pre-amp template (v/v 1/20), 50 ng unlabelled primer, 0.5 pmol labeled primer, 0.2 mM of all four dNTPs, and 0.2 U Taq polymerase (SuperTaq, Enzym Tech., The Netherlands) in PCR buffer (Superbuffer, Enzym Tech., The

Netherlands). From each sample, 0.5 µl was loaded on a 5.5% denaturing polyacrylamide gel (5.5% Gel Matrix, KB Plus, Westburg, The Netherlands), and gel electrophoresis was performed in a Li-Cor DNA Analysis System (Li-Cor Biosc., Lincoln, USA). Images were scored manually for the presence or absence of bands, mainly in the range of 100-500 bp, and the data were transformed into binary matrices.

Cluster analyses were performed in TREECON 1.3b (Van der Peer and De Wachter 1994) using Nei and Li's (1979) dissimilarity coefficient and UPGMA (Unweighted pair-group method using arithmetic averages). In comparative studies, the combination of these methods resulted in the best fit of the tree to the distance matrix, expressed by the highest cophenetic correlation (Mace et al. 1999, Koopman et al. 2001). Support values for the nodes in the trees were calculated in 1000 bootstrap replicates. In each replicate, the original data set is re-sampled, and a new tree is constructed based on the re-sampled data set. Subsequently, the bootstrap value for a certain node in the original tree is calculated as the percentage of trees from the re-sampled data sets that show that particular node (Felsenstein, 1985). The data matrices were analyzed separately for each primer combination, and combined for all three primer combinations.

Determination of fungal species

Species-specific primers were used to confirm the taxonomic position of isolates in the collection. *Fusarium oxysporum* isolates were identified using the primer set CLOX1F/2R (Mulè et al. 2004), which generates a 534 bp product; *F. proliferatum* using the primer set TH5F/6R, generating a 330 bp product (Waalwijk et al. 2003); and *F. culmorum* using the primer set Fc01F/R, giving a 570 bp product (Nicholson et al. 1998).

In addition, DNA amplification and sequencing of the Translation Elongation Factor 1α gene (EF-1α) was performed, according to Geiser et al. (2004). A set of 23 isolates were selected as representatives of clusters along the phenetic tree obtained by AFLP (see Figure 2). The EF-1α gene was amplified using primers combination ef1/ef2, which generate a 660 bp product. The amplified template was sequenced, the 23 sequences were submitted to BLAST query using the database http://fusarium.cbio.psu.edu, and their phylogenetic relationships were analysed. The 23 sequences were deposited in the National Center for Biotechnology Information (NCBI) databases (Accession Numbers EU220393 to EU220415).

Table 3. *Allium* accessions included in the screening for resistance to Fusarium basal rot.

Name	Accession or Cultivar	Origin
Section *Cepa*		
Allium cepa	Texas E. G. 502 (SD) [a]	Vikima Seeds, Denmark
	Pantanoso del Sauce (ID)	Universidad de la República, Montevideo, Uruguay
	Rossa Savonese (LD)	Bavicchi SPA, Italy
	Rijnsburger (LLD)	Bejo Zaden, The Netherlands
	Jumbo (LLD)	Syngenta Seeds, The Netherlands
Allium fistulosum	PRI 97166	H.B. Odessa 84236, Ukraine
	W37802	Botanical Garden, Wageningen University, The Netherlands
	W00501	Botanical Garden, Wageningen University, The Netherlands
	UR 2003-1	Cultivated, Maldonado, Uruguay
Allium vavilovii	PRI 97202	H. B. Chorog, wild origin, Tajikistan
Allium roylei	PRI 98202	USDA Beltsville C 502, USA
Allium galanthum	PRI 99358	USDA Beltsville 82550, USA
Allium pskemense	CGN 23459	H. B. Alma-Ata 65448, Kazakhstan
Section *Schoenoprasum*		
A. schoenoprasum	CGN 21442	Centre for Genetic Resources, The Netherlands

[a] Onion cultivar types, according to the daylenght requirement for bulbing. SD: short day (about 10-12 h); ID: intermediate day (12-13 h); LD: long day (13-14 h); LLD: long-long day onion types (more than 14 h).

Screening for resistance in onion and related Allium species

Three *Fusarium* isolates were selected to study the response of five onion cultivars and nine accessions from six related *Allium* species (Table 3). The *Fusarium* isolates were selected on the basis of the AFLP phenetic tree. One isolate was taken from each of the major groups, namely UR17-5 and NM2-4 belonging to each clade of *F. oxysporum*, and isolate NL131-2 belonging to *F. proliferatum* (Figure 2). *Allium* species belong to Section *Cepa*, except *A. schoenoprasum*, which belongs to the closely related Section *Schoenoprasum* (van Raamsdonk et al. 2003). Because of the limited number of available

seedlings, *A. schoenoprasum, A. pskemense,* and *A. vavilovii* accessions were only tested with isolates UR17-5 and NL131-2.

Seedlings were grown in sterilized pot-soil and transplanted 38 days after sowing to 0.5 litre pots containing the same substrate (one plant per pot). The experimental layout consisted of twelve plants per accession-isolate combination, and twelve water-inoculated controls. Plants were randomized within each *Fusarium* treatment, separated from the other treatments to prevent cross contamination. *Allium vavilovii, A. pskemense* and *A. schoenoprasum* were tested with two isolates because of shortage of seedlings.

Each *Fusarium* inoculum was produced as a suspension of conidia obtained from 10-15 day-old colonies grown on PDA, filtered through cheese-cloth, and adjusted to $3 \cdot 10^5$ conidia·ml $^{-1}$. All plants were inoculated twice, 10 and 21 days after transplanting, by pouring each time 40 ml of the suspension into each pot. Average daily temperature during the test ranged from 22 to 27°C.

Plants were harvested by carefully washing the soil from the roots under running tap water. Onion cultivars 'Texas E.G. 502' and 'Pantanoso del Sauce CRS' were evaluated 67 days after transplanting, because they showed plant maturity. Other accessions were assessed between 91 and 98 days after transplanting. A disease index (0-3) was established to score the plants based on the necrotic proportion of the basal plate, as follows: 0, no symptoms; 1, slightly infected (less than 20% of the basal plate was infected); 2, moderately infected (20-50%); 3, highly infected (more than 50%) and rotten bulbs or plants. The presence of *Fusarium* infections in basal plates was confirmed by examination of the developed colonies after incubation in a moist chamber at 27°C during seven days, and observations under dissection microscope.

Analysis of the disease index data concerned the fitting of a Proportional Odds Model using Genstat 9th Ed. (Lawes Agricultural Trust, Rothamsted Exp. St., UK, 2006). The disease infection was scored on an ordinal scale of four classes. Such ordinal data cannot be analyzed under the assumption of normality. These data can be modelled by reference to an underlying latent variable and threshold values associated with the ordinal scores (Proportional Odds Model, McCullagh and Nelder 1999). These parameters (threshold values and means) were estimated by the maximum likelihood method (Cox and Hinkley 1979) and the result is presented as an analysis of deviance (Table 5). The classes need not to represent equidistant measures of infection. In order to improve the balance in the number of observations among the classes, scores 2 and 3 (moderate and severe infections) were merged into one class. In addition, *A. schoenoprasum* was excluded for its biased scores (as no *Fusarium* infection was scored in this accession).

At harvest, also the number of roots per plant was investigated. It was hypothesized that a dense rooting system may influence the ability of a plant to survive *Fusarium* infection. The relationship between Fusarium basal rot index and the number of stem-borne roots was analyzed using linear regression.

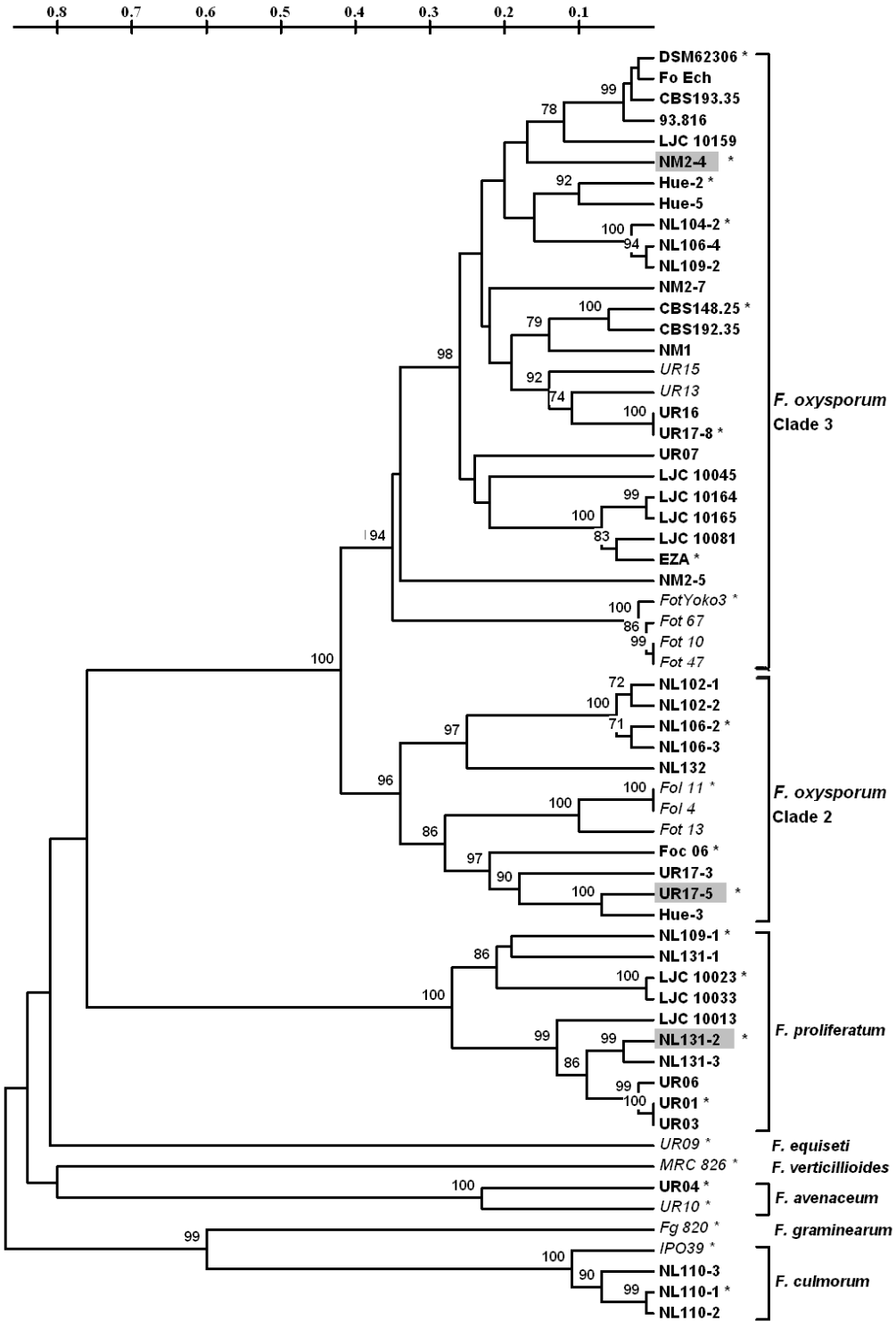
Results

Cluster analysis of Fusarium isolates

The AFLP analysis of the fungal collection yielded a total of 470 bands: 126 for E01-P16, 167 for E15-M14 and 177 for E15-M23. Figure 2 shows the phenetic relationships among *Fusarium* isolates for the combined data set, including all three primer combinations. Trees generated for the individual primer combinations (data not shown) had a similar topology on the species level, indicating consistency among the data from the individual primer combinations. The majority of the onion isolates (89 %) clustered in two main groups with high bootstrap support, namely the *F. oxysporum* and the *F. proliferatum* groups (Figure 2). The Nei & Li distance between these groups was 0.77. The *F. oxysporum* group comprised 43 isolates, 29 derived from onion, one from shallot, and three from garlic (33 *Allium* isolates in total). The other *F. oxysporum* isolates were obtained from tulip, lily, pumpkin and birds-foot and were included initially as controls. This group, with bootstrap values 100, 100, and 99% for the individual primer combinations, was confirmed as *F. oxysporum* by morphological characteristics, by testing with the species-specific primers, and by sequencing the EF-1α gene.

Within *F. oxysporum*, isolates clustered in two main clusters supported by the topology of all three primer combinations. Isolates obtained in Uruguay were present in both clusters, and isolates obtained in the Netherlands too (Figure 2).

Figure 2 (opposite page). UPGMA dendrogram of genetic relationships among *Fusarium* isolates, based on AFLP markers from three primer combinations. Bootstrap values greater than 70% (1000 replicates) are shown above the branches. Nei and Li distances are shown on top. Isolates from onion or *Allium* are presented in bold font, and the other ones in italics. Isolates used in the screening for resistance are indicated by gray shaded boxes, whereas those isolates followed by an asterisk were sequenced for the elongation factor 1α gene. *F. oxysporum* Clades were termed according to O'Donnell et al. (1998).


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
DSM62306 *
Fo Ech
CBS193.35
93.816
LJC 10159
NM2-4 *
Hue-2 *
Hue-5
NL104-2 *
NL106-4
NL109-2
NM2-7
CBS148.25 *
CBS192.35
NM1
UR15
UR13
UR16
UR17-8 *
UR07
LJC 10045
LJC 10164
LJC 10165
LJC 10081
EZA *
NM2-5
FotYoko3 *
Fot 67
Fot 10
Fot 47
F. oxysporum Clade 3
NL102-1
NL102-2
NL106-2 *
NL106-3
NL132
Fol 11 *
Fol 4
Fot 13
Foc 06 *
UR17-3
UR17-5 *
Hue-3
F. oxysporum Clade 2
NL109-1 *
NL131-1
LJC 10023 *
LJC 10033
LJC 10013
NL131-2 *
NL131-3
UR06
UR01 *
UR03
F. proliferatum
UR09 *
F. equiseti
MRC 826 *
F. verticillioides
UR04 *
UR10 *
F. avenaceum
Fg 820 *
F. graminearum
IPO39 *
NL110-3
NL110-1 *
NL110-2
F. culmorum

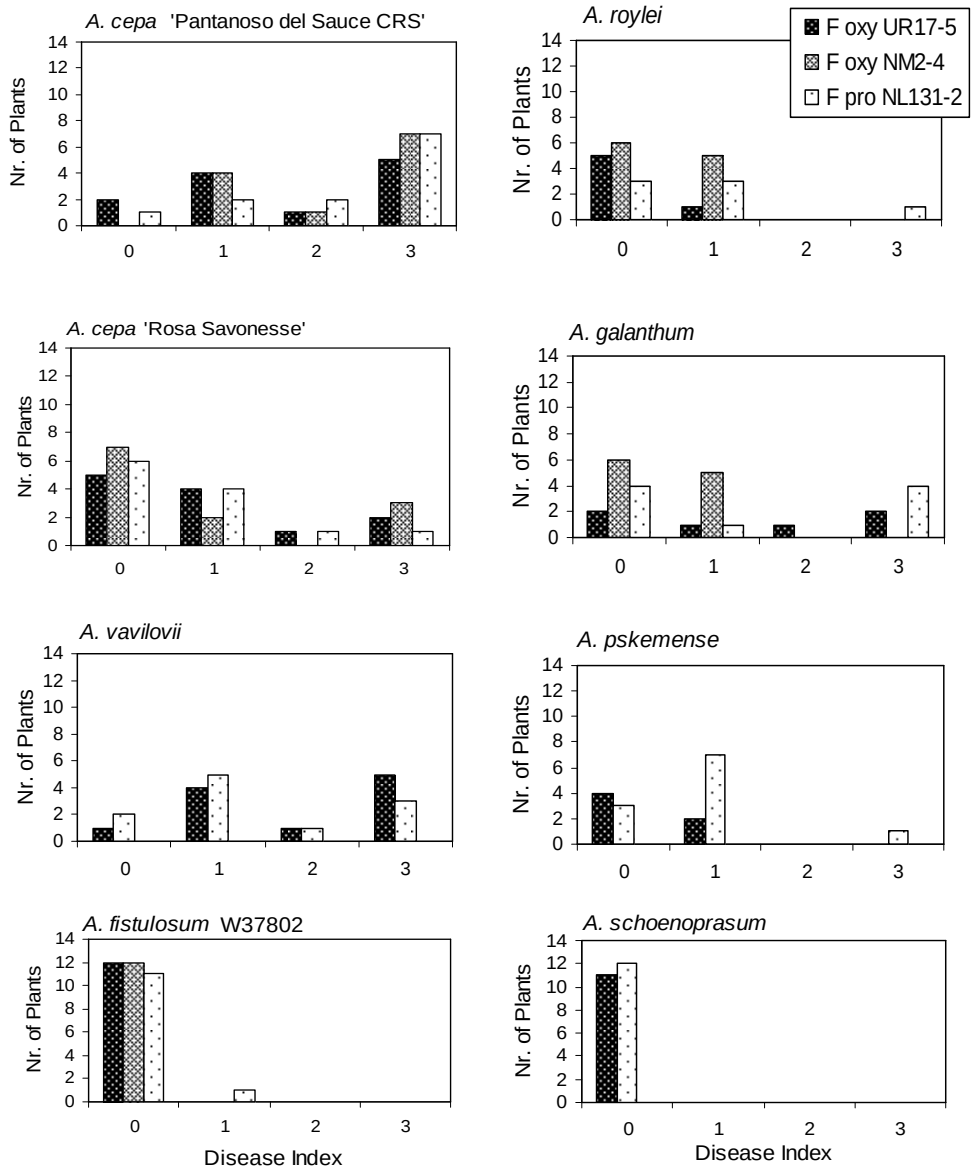


Figure 3. Division of numbers of plants per *Allium* accession scored in each disease index (DI) class after inoculation of individual plants by *Fusarium oxysporum* isolates UR17-5 and NM2-4, and *F. proliferatum* NL-131-2. DI classes are based on the estimation of the affected proportion of the basal plate: (0) no symptoms; (1) slight infection, <20%; (2) moderate infection, 20-50%; (3) severe infection (>50%) and rotten plants.

Table 4. Summary of all pairwise differences of specific *Allium-Fusarium* combinations, based on estimated distribution means of the Proportional Odds Model.

***Allium* Accessions**	**Nr of plants**	**Groups [a]**								
NM 2-4 (*Fusarium oxysporum* Clade 3) [b]										
A. fistulosum W37802	12	a								
A. fistulosum W00501	12	a								
A. fistulosum PRI97166	12	a	b							
A. fistulosum UR2003-1	12	a	b	c						
A. galanthum	11	a	b	c						
A. roylei	7	a	b	c						
A. cepa cv. 'Rossa Savonese'	12		b	c						
A. cepa cv. 'Texas EG502'	12			c	d	e	f	g		
A. cepa cv. 'Rijnsburger'	11				d	e	f	g	h	i
A. cepa cv. 'Pantanoso del Sauce'	12							g	h	i
A. cepa cv. 'Jumbo'	12								h	i
UR17-5 (*Fusarium oxysporum* Clade 2)										
Allium schoenoprasum [c]	11	-								
A. fistulosum W37802	12	a								
A. fistulosum UR2003-1	12	a								
A. fistulosum W00501	12	a								
A. roylei	6	a	b							
A. pskemense	6	a	b	c						
A. fistulosum PRI97166	12	a	b	c						
A. cepa cv. 'Rossa Savonese'	12		b	c	d					
A. cepa cv. 'Jumbo'	12			c	d	e	f	g	h	
A. galanthum	6			c	d	e	f	g	h	i
A. cepa cv. 'Pantanoso del Sauce'	10				d	e	f	g	h	i
A. vavilovii	10					e	f	g	h	i
A. cepa cv. 'Texas EG502'	12							g	h	i
A. cepa cv. 'Rijnsburger'	11									i
NL131-2 (*Fusarium proliferatum)*										
Allium schoenoprasum [c]	12	-								
A. fistulosum W00501	12	a								
A. fistulosum UR2003-1	12	a								
A. fistulosum W37802	12	a								
A. fistulosum PRI97166	12	a	b	c						
A. cepa cv. 'Rossa Savonese'	12		b	c						
A. roylei	7		b	c	d					
A. pskemense	11		b	c	d	e				
A. cepa cv. 'Rijnsburger'	12			c	d	e	f			
A. galanthum	9			c	d	e	f			
A. vavilovii	11			c	d	e	f	g	h	
A. cepa cv. 'Texas EG502'	12						f	g	h	i
A. cepa cv. 'Pantanoso del Sauce'	12								h	i
A. cepa cv. 'Jumbo'	12								h	i

[a] Combinations in a group with the same letter do not differ mutually. Comparisons between combinations with no letter in common differ significantly at the level of 5%, ranking from most resistant (level a) to most susceptible (level i) reactions. [b] *A. schoenoprasum, A. pskemense*, and *A. vavilovii* accessions were only tested with isolates UR17-5 and NL131-2. [c] Because of the requirements of the statistical model, *A. schoenoprasum* was not included for its biased scores (no *Fusarium* infected plants were observed in this accession).

Table 5. Accumulated analysis of deviance for the distribution over disease index classes (Proportional Odds Model), testing the effects of *Allium* accessions, *Fusarium* isolates and their interaction.

Sequentially added terms to the model	d.f.	Deviance	Mean Deviance	Deviance Ratio	Chi-Square
Fusarium isolates	2	7.2	3.60	3.60	0.027
Allium accessions	12	140.6	11.72	11.72	<.001
Isolate x Accession	16	27.8	1.74	1.74	0.034
Residual	30	39.0	1.30		
Total	60	214.6	3.58		

Some closely related onion isolates (Nei & Li distance lower than 0.10) originated from the same region: e.g. isolates NL102-1, NL106-2, -3 (The Netherlands). However, isolates originating from different countries also showed high genetic similarity, e.g. EZA (Australia) and LJC-10081 (Argentina). In addition, high similarity was found for some isolates obtained from different host plants and locations, e.g. UR17-5 (onion, Uruguay) and Hue 03 (garlic, Spain).

The *F. proliferatum* group (bootstrap 100% in each primer combination) consisted of ten onion isolates originating from Argentina, Uruguay, and The Netherlands. Species identification was confirmed by morphological characteristics, species-specific primers, and sequencing the EF-1α gene. The isolates clustered closely together (largest Nei & Li distance 0.27). *F. proliferatum* isolates were obtained from infected seeds, and from infected basal plates of bulbs collected in the field and after storage. When collecting bulbs it was not possible to discriminate between symptoms caused by *F. proliferatum* or *F. oxysporum*.

Three isolates collected in one farm in the Netherlands (NL110-1, -2, -3) joined with *F. culmorum* isolate IPO-39. This taxonomic position was confirmed also by morphological characteristics, by testing with the species-specific primers, and by sequencing the EF-1α gene. The onion-isolate UR04 and the pumpkin-isolate UR10, both from Uruguay, were identified as *F. avenaceum* by sequencing the EF-1α region.

Resistance in Allium species

Three *Fusarium* isolates were used to screen for levels of resistance in onion cultivars and six related *Allium* species. For the *Allium* accessions, large and

significant differences were observed in the overall distribution of the number of plants over the disease index (DI) scores (Table 4, Figure 3).

In addition, significant differences between isolates and significant isolate x accession interactions were observed, but not as apparent as the difference between *Allium* accessions (Table 5). The aggressiveness of each isolate was very much dependent on specific isolate-accession combinations, although the onion cv. 'Rijnsburger' was the only one with a distribution of DI scores significantly different between isolates (Table 4). In general, *A. schoenoprasum* and the four accessions of *A. fistulosum* had consistently the lowest DI scores when evaluated for their resistance to the three *Fusarium* isolates (Table 4, Figure 3). On the other extreme of the spectrum, four of the five onion cultivars and *A. vavilovii* had consistently the highest DI scores. Among them, onion cv. 'Pantanoso del Sauce' was the most diseased, being different from all *A. fistulosum* accessions for the three isolates. Interestingly, variation in DI scores was found within the onion germplasm tested, as cv. 'Rossa Savonese' contained plants resistant to all three *Fusarium* isolates. Considering the resistance observed in other wild relatives of onion, namely *A. roylei*, *A. pskemense*, and *A. galanthum*, DI scores were intermediate between those of *A. fistulosum*−*A. schoenoprasum*, and those of the four susceptible onion cultivars. The mean number of roots for each accession in the controls was found to be negatively correlated with the disease indices (linear regression, data not shown; NL131-2: $p<0.001$; NM2-4: $p<0.065$; UR17-5: $p<0.016$). For example, large numbers of roots were found in *A. fistulosum* accessions (29 roots per plant on average) and *A. cepa* cv. 'Rossa Savonese' (28 roots per plant), whereas 'Pantanoso del Sauce', 'Texas E.G. 502', 'Rijnsburger', and 'Jumbo' had 8, 8, 10, and 14 roots per plant respectively.

Discussion

To identify sources of resistance, bioassays should be done with the relevant fungal species or strains. Therefore, in the first part of this research we studied genetic diversity in a collection of *Fusarium* isolates from onion to select genetically different isolates. AFLP fingerprinting has been applied to study pathogen diversity in several pathosystems, being a technique able to distinguish clusters of isolates at the species level. Examples of comparative AFLP studies are *Fusarium* in asparagus (Baayen et al. 2000b) and in cassava (Bandyopadhyay et al. 2006). In the first part of this research, we showed that a significant part of the diversity in AFLP markers was found among species. Four species were identified that originated from onion: *F. oxysporum, F. proliferatum, F. avenaceum*, and *F. culmorum*. AFLP clustering was in complete agreement with

the species identification using the species-specific markers, and the sequences of the EF-1α gene.

Fusarium oxysporum is assumed to be the causal agent of onion basal rot, and was indeed predominantly present in the collection. Our results showed a significant diversity among *F. oxysporum* isolates from onion, grouped in two main clades, namely Clade 2 and Clade 3 (O'Donnell et al. 1998), as supported by the position of isolates Fol4 in Clade 2, and Fol10 in Clade 3 determined earlier by Baayen et al. (2000a) (Figure 2). Some *F. oxysporum* isolates collected in one region were found to cluster closely together, suggesting a clonal origin (e.g., NL109-2, NL106-4). In contrast, diversity was also observed at the regional level. For example, isolates from a sampled region fell in different *F. oxysporum* clades (e.g., NL109-2, NL106-3), leading to genetic variation in a single location or a single field. The finding of multiple vegetative compatibility groups (VCG) within f. sp. c*epae* (Swift et al. 2002), likewise, indicated genetic variation in this forma specialis, because VCG can be considered as distinct clonal lineages in a *F. oxysporum* population (Kistler 1997).

This diversity suggests that various *F. oxysporum* strains may have evolved towards pathogenicity in onion. In the same way, Baayen et al. (2000a) postulated non-monophyletic origins for various formae speciales of this species. In addition, they found closely related isolates that originated from different host species (Baayen et al. 2000a), as was found in the present research when comparing isolates from onion with those from pumpkin, lily, and other host species. Although our observations are based only on AFLP markers, Baayen et al. (2000a) showed that similarities based on nuclear and mitochondrial DNA sequences corresponded with those based on AFLP markers.

Genetically similar *F. oxysporum* isolates were found originating from different countries (e.g., Hue-3 from Spain and UR17-5 from Uruguay) as well as from different collection times (e.g., CBS-193.35 collected in 1935 in Germany, and 93.816 collected in 1993 in The Netherlands). These findings may be the result of human activities, such as the transportation of seeds and bulbs with immigration drifts and commerce. However, this can only be regarded as a hypothesis.

Fusarium proliferatum was also present in a substantial proportion of onion samples originating from different continents. This species was reported on onion in USA (du Toit et al. 2003), Serbia (Stankovic et al. 2007), and Argentina (J. Valdez, *pers. com.*). In garlic, this species was reported in Hungary (Simay, 1990) and USA (Dugan et al. 2003). In addition to these reports, our results support that *F. proliferatum* is another relevant species causing Fusarium basal rot.

Fusarium culmorum, found on a farm in the Netherlands, has been reported before as a pathogenic species of onion in Mexico (Montes et al. 2003). In leek (*Allium porrum* L.), *F. culmorum* is known to cause leek rot disease (Blancard et al. 2003; Koike et al. 2003). *Fusarium avenaceum* is known to cause rot of pumpkin fruits (*Cucurbita* sp.), which are cultivated in rotations with onion and garlic (P.H. González, Uruguay, *unpublished data*). It is not known to which extent *F. culmorum* and *F. avenaceum* could be relevant species in the basal rot disease of onion, or merely have onion as an alternative host (Dhingra and Coelho 2001).In order to test the resistance of onion cultivars and *Allium* species, we selected for a first and general screening one strain from each *F. oxysporum* sub-group, and one from *F. proliferatum.* All tested onion cultivars appeared susceptible to *Fusarium* basal rot, with moderate to high levels of infections on average. An exception was 'Rossa Savonese', confirming results obtained by Özer et al. (2004). The high number of roots per plant of this accession might partially explain this response. Every onion accession had also plants without any infection. As onion is an outcrossing crop, each accession is genetically heterogeneous, which may explain the finding of resistant plants within the set of cultivars tested. In that case, recurrent selection of non-infected plants may gradually result in populations with larger proportions of plants resistant to *Fusarium.* This approach has resulted in the successful development of selections with higher levels of resistance (Gutierrez and Cramer 2005, Cramer 2006). Alternatively, non-infected plants may be the result of escapes from infection during the screening assay.

Allium fistulosum accessions were affected at a very low incidence. These results are in agreement with Holz and Knox-Davies (1974), who found very low levels of infection in *A. fistulosum* caused by a *F. oxysporum* f.sp. *cepae* isolate, compared to a set of onion cultivars. One of the differences between *A. cepa* and *A. fistulosum* concerns the rooting system, which is much denser and larger for the latter (De Melo 2003). *Allium fistulosum* also differs from *A. cepa* in the lack of bulbing and dormancy. These morpho-physiological differences might influence the response to *Fusarium* basal rot, leading to mechanisms of escape. *Allium fistulosum* keeps actively growing, developing new roots, and as a result may overcome *Fusarium* root infections. In contrast, *A. cepa* stops growing after bulb formation, and root and basal plate tissues become senescent. However, the complete absence of *Fusarium* symptoms in a large number of *A. fistulosum* plants may suggest the presence of true resistance against *Fusarium* isolates from onion.

High level of resistance was also found in *A. schoenoprasum*, where only root infections were observed. Intermediate levels of resistance were found in *A.*

roylei, A. galanthum, and *A. pskemense*. *Allium vavilovii,* which is closely related to the cultivated *A. cepa* (Van Raamsdonk et al. 2003), showed a larger proportion of susceptible plants. This accession has a denser root system than onion, showing that plants with a larger and denser rooting system may also be susceptible.

Within *A. cepa*, studies have been done to investigate the inheritance of resistance to *Fusarium oxysporum* f. sp. *cepae* and a range of hypotheses have been proposed (reviewed by Cramer, 2000). As no detailed mapping of any source of resistance to Fusarium basal rot on a molecular linkage map has been developed yet, however, it remains difficult to predict how complicated it will be to introgress the resistance present in wild relatives into onion elite lines. Transfer of resistance from *A. schoenoprasum* and *A. pskemense* would be very difficult, as Van Raamsdonk et al. (2003) showed that interspecific hybridization between these species and onion did not result in viable progenies. For *A. fistulosum, A. roylei* and *A. galanthum* better opportunities are present (Kik 2002).

Chapter 3

The genetic basis of resistance to Fusarium basal rot in the tri-hybrid population *Allium cepa* x *(A. roylei* x *A. fistulosum)*[†]

Abstract

Allium fistulosum and *A. roylei* are potential sources of resistance to Fusarium basal rot (FBR) in onion. The genetic basis of resistance to FBR was studied in an inter-specific tri-hybrid population *A. cepa* x (*A. roylei* x *A. fistulosum*). An offspring of 83 genotypes was clonally propagated *in vitro* to obtain replications for the screening assay. A greenhouse test was performed, with eight non-inoculated replicates and eight FBR inoculated replicates per genotype. An aggressive *Fusarium oxysporum* isolate was used as inoculum. Symptoms were scored as wilting before harvest, infections of basal plates of the plants at harvest, and after four weeks storage. In this last evaluation, internally developed lesions were scored after slicing the basal plate. A molecular linkage map based on AFLP markers was developed for the *A. roylei* x *A. fistulosum* parent, with 111 markers allocated on the expected eight linkage groups. Resistance to FBR from *A. fistulosum* was dominantly expressed in the *A. roylei* x *A. fistulosum* parental hybrid and in the tri-hybrid population. FBR reduced the weight of A. cepa and susceptible tri-hybrid genotypes in comparison to non-inoculated controls. One QTL for FBR resistance from *A. roylei* was identified on a distal region of chromosome 2, and one QTL from *A. fistulosum* was identified on the long arm of chromosome 8. These two QTLs showed additive effect, and together accounted for 31 and 40% of the total variation for FBR incidence and severity at harvest; and 31 and 29% after storage respectively. Each QTL separately had significant effect on FBR but did not confer complete resistance, thus, more QTLs from *A. fistulosum* remain to be discovered. The Area under Disease Progress Curve (AUDPC) summed up for differences in timing of the disease regarding wilting during the season, harvest and post-harvest scores. Four QTLs for AUDPC were located, the two QTLs identified before and two additional ones on chromosomes 4 and 8 from *A. fistulosum*. QTLs for FBR resistance from *A. fistulosum* and *A. roylei* are a promising step towards the development of onion cultivars resistant to FBR.

[†] This chapter will be submitted as

Galván GA, Burger K, Keizer LCP, Hoekstra RF, Kik C, Scholten OE. The genetic basis of resistance to Fusarium basal rot in the tri-hybrid population *Allium cepa* x *(A. roylei* x *A. fistulosum*)

Introduction

Fusarium basal rot (FBR) is a cosmopolitan important soil-borne disease in onion (*A. cepa* L.) (Entwistle 1990). The major causal agent is *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen f. sp. *cepae* Hansen (Entwistle 1990). Genetic variation among *F. oxysporum* isolates pathogenic to onion was observed by AFLP markers, and isolates were grouped in two major clades (Chapter 2). *Fusarium proliferatum* (Mats.) Nirenb. was also found to cause FBR (Du Toit et al. 2003, Chapter 2).

FBR may cause plant death at seedling stage, growth reduction during the season due to root rot and infections of the basal plate of the bulb, and bulb rotting during storage. Although FBR may occur in a wide range of soil temperatures, the optimum is 25-27°C (Entwistle 1990). As a consequence, in temperate climates the largest incidence appears in summer and at the end of the season. In many regions FBR is mainly a post-harvest disease, which results in significant economic losses of up to 40-50% of the harvested product (De Visser 1999). FBR becomes severe under continuous onion mono-cropping. Rotation with non-host crops alleviates the spread and relevance of the disease, but rotation is not always possible. Chemical control is ineffective and, if used, it causes environmental pollution. It is only applied as seed coating.

Resistance to FBR is the more practical and environmentally friendly alternative. Most onion cultivars are susceptible, and the development of resistant onion cultivars is a permanent effort (reviewed by Cramer 2000, Shigyo and Kik 2008). Inheritance of host resistance to FBR was studied with the use of several breeding populations, and diverse genetic control systems were proposed (reviewed by Cramer 2000). In most cases, genetic variation for the response to FBR among onion cultivars or breeding lines was observed on a quantitative basis and resulted in partial levels of resistance (Gutierrez et al. 2005, 2006). In a short-day onion population, Cramer (2006) reported a realized heritability of 0.65 and 0.60 for FBR severity and incidence respectively. Until now no studies have been published describing the genetics of resistance to FBR by the identification of loci on a molecular linkage map.

As only intermediate levels of resistance have been found in *A. cepa*, resistance to FBR was investigated in related species in *Allium* section *Cepa* (sensu Friesen et al. 2006). High levels of resistance against *Fusarium* isolates pathogenic to onion were found in *A. fistulosum* L. (Holz and Knox-Davies 1974, Chapter 2), whereas intermediate levels of resistances were found in *A. roylei*

Stearn. These responses were observed against two isolates belonging to two different *F. oxysporum* clades, and a *F. proliferatum* isolate (Chapter 2). *Allium fistulosum* and *A. roylei* can thus be considered as potential sources of resistance to FBR in onion. In the past, direct introgression of traits from *A. fistulosum* into *A. cepa* had been hampered due to high levels of sterility of the progeny hybrids between these species (reviewed by Kik 2002). This interspecific barrier can be overcome by the use of *A. roylei* as bridge species between *A. cepa* and *A. fistulosum*. It was shown that with the obtained *A. cepa* x (*A. roylei* x *A. fistulosum*) tri-hybrid, the introgression of traits from *A. fistulosum* into the genetic basis of onion was possible, as well as the simultaneous exploitation of these two onion-related species (Khrustaleva and Kik 1998, 2000).

Molecular markers can facilitate the introgression of traits segregating in this tri-hybrid population, being of great help for breeders. A first AFLP linkage map for an *A. roylei* x *A. fistulosum* hybrid genotype was described by De Melo (2003). The aim of the current research was to study the inheritance of FBR resistance from *A. fistulosum* and *A. roylei* in a tri-hybrid *A. cepa* x (*A. roylei* x *A. fistulosum*) population, by scoring FBR symptoms and FBR effects on plant weight in comparison to a non-inoculated control treatment. QTLs linked to FBR resistance were located on a molecular linkage map of the *A. roylei* x *A. fistulosum* parent.

Materials and methods

Plant material

A tri-hybrid population was developed as described by Khrustaleva and Kik (1998). First, *A. roylei* (accession CGN 20520) was crossed with *A. fistulosum* (accession CGN 14763). One specific hybrid genotype derived from this cross (PRI 91021-08, hereafter referred to as RF-hybrid) was chosen as pollen donor in a cross with cytoplasmic male-sterile onion lines from the Rijnsburger group. Consequently, a population of *A. cepa* x (*A. roylei* x *A. fistulosum*) was built up (hereafter referred to as tri-hybrid population). Each tri-hybrid genotype has a set of chromosomes from *A. cepa*, and a set of chromosomes from *A. roylei*, or *A. fistulosum*, or recombinants between them (Khrustaleva and Kik, 1998).

An offspring of 97 tri-hybrid genotypes was used to develop a molecular linkage map of the RF-hybrid based on AFLP markers. This offspring consisted of two sets or sub-populations. One set was derived from a cross made in 1996 (42 genotypes) and used by De Melo (2003). The second set was obtained from

crosses made in 2003 and 2004 (55 genotypes). The new set was produced to extend the available population and, by this means, to improve the molecular linkage map and the analysis of FBR resistance. For both sub-populations, the same RF-hybrid genotype PRI 91021-8, vegetatively propagated, was used as pollen donor on different male-sterile onion plants. As a consequence, only the RF-hybrid is present as parental genotype across the whole tri-hybrid population. We assumed that *A. cepa* plants used in these crosses can be regarded as completely susceptible to FBR and therefore they did not contribute to quantitative variation for FBR resistance in the tri-hybrid population.

Screening for resistance to FBR

A greenhouse experiment was carried out to screen for FBR resistance. It comprised 83 tri-hybrid genotypes (28 from the cross made in 1996, and 55 from 2003-2004), the parental species and the RF-hybrid. In order to have clonal replications, each tri-hybrid genotype was introduced *in vitro* using sections of the basal plates as initial explants, as described by De Melo (2003). *In vitro* plantlets were clonally multiplied by successive divisions of the basal plates.

Plantlets grown for 3-4 weeks *in vitro*, with 2-3 leaves and well-developed roots, were transferred to trays containing a potting mixture (2:1 steamed peat-soil and sand), and placed in a greenhouse compartment for acclimatization. The trays were covered with a transparent lid which was progressively opened. After four weeks, eight to ten replications per genotype (individual plants) were transplanted to pots (3.3 litres, soil mixture as the trays) infested with *Fusarium*. Eight replications were kept as non-inoculated controls, in order to evaluate FBR effects on plant growth. *Fusarium* and control treatments were not randomized together to avoid cross-contamination. For each treatment plants were randomized over three blocks.

Fusarium oxysporum f. sp. *cepae* isolate 'EZA' (Chapter 2) was selected to be used for inoculations in the screening assay because of high aggressiveness in seedling tests. The isolate was maintained and multiplied on potato dextrose agar (PDA) in Petri dishes at 24°C. To generate an inoculated soil, four PDA discs of 5 mm in diameter from *Fusarium* colonies were placed on top of 250 ml jars containing an autoclaved mixture of peat soil and oat-flakes as growing medium (100 g oat-flakes per litre of peat soil). These jars were incubated for four weeks at 24°C. Then, the content of the jars was mixed up with the potting mixture in a 1:200 ratio to generate a *Fusarium* infested soil. This mixture was stored for two weeks at 27°C before transplanting. The final *Fusarium* concentration was estimated as $5 \cdot 104$ cfu$\cdot g^{-1}$ dry soil, by quantification of colonies on water-agar

after serial dilutions of two samples randomly taken from the infected soil. In addition, four and eight weeks after transplantation, a suspension of conidia ($5 \cdot 10^5$ conidia$\cdot$ml^{-1}, 20 ml$\cdot$pot^{-1}) was poured into each pot to ensure the development of the disease later in the season.

The initial temperature of the experiment was set at 21-18°C (day and night respectively) and gradually increased from the third till the seventh week to a final 28-20°C daily regime. Wilting plants were observed and weekly scored from the fifth week after transplanting. Severely wilted plants were harvested in advance, and evaluated as described below. FBR incidence for each genotype along the season was calculated as the proportion of wilted plants (confirmed as infected by *Fusarium* after incubation in humid chamber).

The experiment was harvested 13 weeks after transplanting. Table 6 lists variables used to score FBR symptoms, and to assess FBR effects on plant growth by comparison with the non-inoculated control. The levels of FBR were expressed as a severity index, on an ordinal scale from 0 to 3 (0: no symptoms; 1: slight infection with necrotic areas usually in lateral points; 2: intermediately infected basal plates; 3: severely infected or rotten plants and bulbs). Plant height, number of leaves, and number of stem-borne roots were recorded. Total plant weight was determined, as well as the partition in leaves and bulbs/false-stem. For non-bulbing genotypes, a portion 5 cm in length from the basal plate was taken as false-stem. After evaluation, bulbs and false-stems were stored during four weeks at 24°C in order to investigate further development of the disease. Then, FBR severity was scored as was done at harvest, and also by the observation of internally developed infections after slicing the basal plate (Gutierrez and Cramer 2005).

As *A. cepa* and some tri-hybrid genotypes showed wilting during the season, and in order to account for differences in the timing of FBR expression, the area under disease progress curve (AUDPC) was calculated by summing up the incidence of wilted plants along the weeks, plus FBR incidence at harvest and after four weeks storage.

Tri-hybrid genotypes segregated for their degree of bulbing. The relationship between bulbing ability and FBR resistance was analyzed because bulbing has implications for breeding purposes. Genotypes of the population were classified into four categories regarding the mean bulbing index ($BI = BulbDiameter/NeckDiameter$), as follows: null (BI = 1.0 to 1.6), low (1.6 to 2.4), medium (2.4 to 2.7), and high (> 2.7) degree of bulbing. The distribution of FBR resistant genotypes along bulbing classes was analyzed using a Chi-Square test.

Table 6. List of variables used to score FBR symptoms, and to assess the effect of FBR on plant growth.

Disease variables

- FBR incidence at harvest (cumulated proportion of symptomatic plants at harvest)
- FBR severity at harvest (scale 0-3)
- FBR incidence after storage (cumulated proportion of symptomatic plants)
- FBR severity after storage (scale 0-3)
- AUDPC (area under the cumulated progress curve for FBR incidence)

Plant variables

- Plant height (length from the basal disc till the top of the longest green leaf)
- Number of green leaves per plant
- Number of roots per plant
- Total fresh weight
- Leaves fresh weight
- Bulb fresh weight (for non bulbing genotypes, the bottom 5 cm of the false-stem)

Statistical analysis

FBR incidence at harvest, and four weeks after harvest, were considered as binomial variables and analyzed by a generalized linear model using Genstat 9th Ed. (Payne et al. 2006). The analysis of FBR severity concerned the fitting of a Proportional Odds Model, as described in Chapter 2. This implied that ordinal scores for FBR severity were modelled by reference to an underlying latent variable and threshold values associated with the ordinal scores (McCullagh and Nelder 1999). These parameters were estimated by maximum likelihood (Cox and Hinkley 1979). In order to improve the balance between classes in the number of observations, as required by the model, scores 2 and 3 (moderate and severe infections) were merged into one class. Thus, three classes were modelled (0, 1, 2+3). In addition, for the same purpose, tri-hybrid genotypes completely resistant were excluded of the analysis, as they only contribute with FBR scores = 0. An arbitrary score was assigned to these genotypes for QTL analysis, as the lowest modelled score (for genotypes having only one replicate slightly infected) minus twice the estimated standard deviation.

Table 7. Amplified fragment length polymorphism (AFLP) adapters and primers used in the ligation and pre-amplification steps, and their sequences.

Adapters	EcoRI adapters	5'-CTCGTAGACTGCGTACC-3' 3'- CTGACGCATGGTTAA-5'
	MseI adapters	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
	PstI adapters	5'-CTCGTAGACTGCGTACATGCA-3' 3'-TGTACGCAGTCTAC-5'
Universal primers	E00	5'- GACTGCGTACCAATTC-3'
	M00	5'- GATGAGTCCTGAGTAA-3'
	P00	5'- GACTGCGTACATGCAG-3'
Primer pairs in the Pre-Amplifications		
	E01 (E00+A) - M02 (M00+C) P01 (P00+A) - M01 (M00+A)	
	P01 (P00+A) - M02 (M00+C)	

AFLP mapping and QTL analysis

DNA was isolated from young leaves of each tri-hybrid genotype, the parental lines and the RF-hybrid, following the miniprep protocol described by Van Heusden et al. (2000a). AFLP® (Keygene B.V., The Netherlands) reactions were carried out according to Vos et al. (1995). Two restriction enzyme pairs were applied, namely *Eco*RI/*Mse*I and *Pst*I/*Mse*I. Because of the large genome size in *Allium* species, the pre-amplifications were done with three selective nucleotides (+1, +2) (Table 7), and the selective amplifications with seven nucleotides (+3, +4) for the *Eco*RI/*Mse*I enzyme pairs, and six (+3, +3) for the PstI/MseI combinations (van Heusden et al. 2000a). A total of 22 primer combinations were used in the selective amplifications (Table 8). AFLP fragments originating exclusively from *A. roylei* or *A. fistulosum* were scored using Quantar (Keygene B.V., The Netherlands). AFLP fragments were named as described by Van Heusden et al. (2000b). For instance, E38M52G-202F refers to restriction enzymes *Eco*I and *Mse*I, primers E38 and M52, 'G' identifies the additional 7th selective base, '202' is the estimated length of the fragment, and 'F' or 'R' means that the marker is specific for *A. fistulosum* or *A. roylei* respectively.

Table 8. Primer pairs used at the selective amplification, and number of amplified fragment length polymorphism (AFLP) markers from *A. roylei* and *A. fistulosum* located on the linkage map.

Primer pairs		**Markers in the linkage map**		
		A. roylei	***A. fistulosum***	**Total**
E35 (E00+ACA)	M52A (M00+CCCA)	0	1	1
	M52C (M00+CCCC)	1	2	3
	M52T (M00+CCCT)	1	0	1
E36 (E00+ACC)	M52A (M00+CCCA)	0	1	1
	M52C (M00+CCCC)	7	2	9
E37 (E00+ACG)	M52A (M00+CCCA)	8	4	12
	M52G (M00+CCCG)	0	1	1
E38 (E00+ACT)	M52G (M00+CCCG)	2	1	3
	M52T (M00+CCCT)	0	4	4
P31 (P00+AAA)	M33 (M00+AAG)	8	8	16
	M35 (M00+ACA)	5	5	10
P35 (P00+ACA)	M32 (M00+AAC)	6	6	12
	M33 (M00+AAG)	5	5	10
	M34 (M00+AAT)	1	1	2
	M35 (M00+ACA)	3	1	4
	M36 (M00+ACC)	3	0	3
	M47 (M00+CAA)	1	3	4
	M50 (M00+CAT)	2	2	4
P38 (P00+ACT)	M47 (M00+CAA)	1	0	1
	M48 (M00+CAC)	3	0	3
P43 (P00+ATA)	M36 (M00+ACC)	1	1	2
	M51 (M00+CCA)	1	4	5
Total	22	59	52	111
Average		2.7	2.4	5.0

A linkage map for the RF-hybrid was calculated using JoinMap® 3.0 (Van Ooijen and Voorrips, 2001). Population-type was set to haploid, and linkage groups were separated with a threshold LOD $\geq$ 4. Kosambi's mapping function was used to calculate map positions of the markers on the linkage groups. Linkage groups were assigned to chromosomes on the basis of AFLP markers in common with previous maps (Van Heusden et al. 2000a, De Melo 2003). Marker order was fixed for Chromosomes 5 and 8 according to Khrustaleva et al. (2005). Skewed segregation of a chromosomal region was defined when all markers in the region had a deviation in segregations towards the same parent (Chi-Square test, $p<0.05$).

Analysis of quantitative trait loci was done using MapQTL® 4.0 (Van Ooijen et al. 2002). Kruskal-Wallis test was used to determine the association between each individual marker in the map and the target traits. QTLs were identified by the multiple QTL mapping (MQM) procedure (Jansen, 1993; Jansen & Stam 1994), and were regarded significant at LOD threshold values with $p<0.05$. These threshold values were estimated for each trait on the basis of population type and 1000 times genome-wide permutations. Linkage maps and QTL figures were drawn in MapChart (Voorrips 2002).

Results

Screening for resistance to FBR

Fusarium oxysporum caused wilting of *A. cepa* plants before harvest, as well as for some particular tri-hybrid genotypes. The disease also caused early bulbing and leaf senescence of tri-hybrid susceptible genotypes in comparison to the non-inoculated controls. FBR symptoms were observed at harvest, and became more apparent after four-week storage. However, not all symptoms of necrosis in the basal plate scored at harvest were confirmed as FBR infections after storage. Therefore, the observation of internally developed infections after slicing the basal plate after a period of storage resulted in more accurate and reliable results.

Allium cepa was severely affected by FBR (Figure 4). Eight of ten inoculated plants were rotten, and five of them were harvested in advance after visible wilting. FBR was not observed for *A. fistulosum*, nor for the RF-hybrid. Both were completely resistant (Figure 4). *A. roylei* had no disease symptoms until harvest, but FBR infections developed in five out of eight replicates after four weeks storage. FBR incidence in *A. roylei* after storage did not differ significantly from *A. cepa*, although the development of the disease in time differed, as shown by the AUDPC (Figure 5).

In the tri-hybrid population, 28 of 83 screened genotypes remained resistant after storage, whereas 18 genotypes presented only slight infections (FBR severity scores 1) (Figure 6). The segregation ranged from this dominant resistant response similar to *A. fistulosum*, through intermediate levels, up to genotypes with FBR severity and incidence similar to the susceptible *A. cepa* parent (Figure 6). The mean positions estimated by the threshold model for FBR severity after storage fitted 41 genotypes in the resistant class, 10 genotypes in the intermediate class, and 4 genotypes in the susceptible class (55 genotypes analyzed in total, as 28 completely resistant genotypes were not included). FBR severity levels of 5 genotypes at harvest and 8 genotypes after storage were not distinguished from *A.*

cepa (Analysis of deviance, p>0.05). The tri-hybrid population segregated as well for the AUDPC (Figure 5), and this variable was skewed towards a resistance response.

For *A. cepa* and susceptible tri-hybrid genotypes, *Fusarium* caused a significant reduction in total fresh weight of the plants at harvest in comparison with the non-inoculated control (Table 9) (REML analysis, p<0.05). Both leaf dry weight and bulb/false-stem dry weights were reduced by FBR. Weight reduction in susceptible genotypes was associated with the presence of rotten plants. Fresh weight of *A. fistulosum, A. roylei*, the RF-hybrid, and resistant tri-hybrid genotypes was not affected by *Fusarium*. Among tri-hybrid genotypes as a whole, weight difference between inoculated and non-inoculated plants was positively correlated with FBR severity at harvest (r = 0.51), severity after storage (r = 0.42), and incidence after storage (r = 0.37) (Pearson correlation, n = 83, p<0.005).

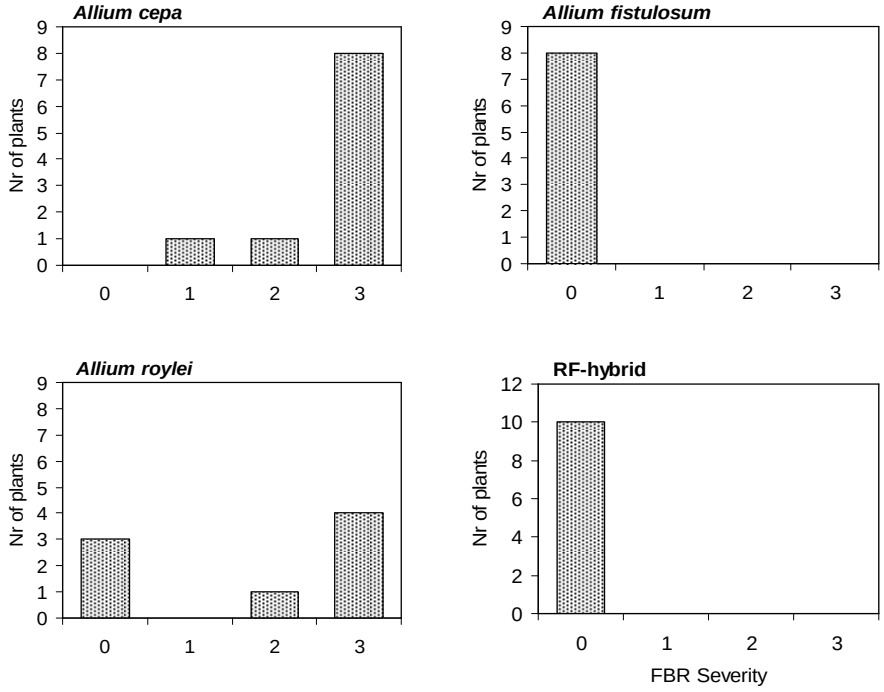


Figure 4. Distribution of scores into FBR severity classes for *Allium cepa, A. fistulosum, A. roylei* and the RF-hybrid. Scores accumulated after four weeks storage.

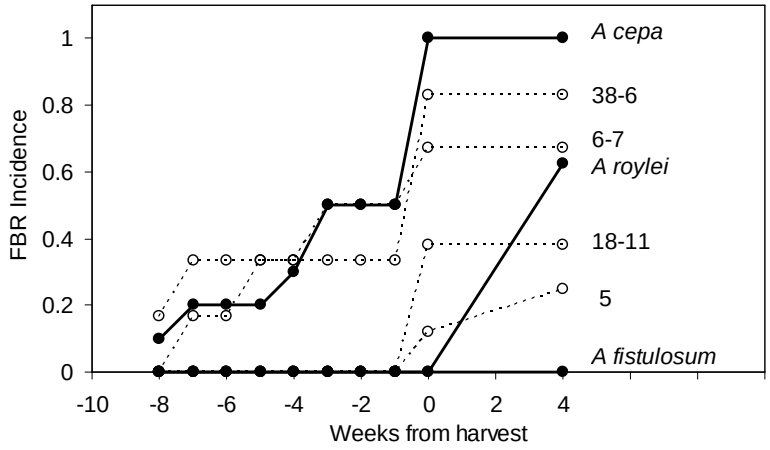


Figure 5. Evolution of Fusarium basal rot (FBR) incidence, as the cumulated proportion of wilting plants before harvest, incidence at harvest, and incidence after storage. Dashed curves are examples of tri-hybrid genotypes. The area under disease progress curve (AUDPC) for each genotype was calculated as the area below the curve.

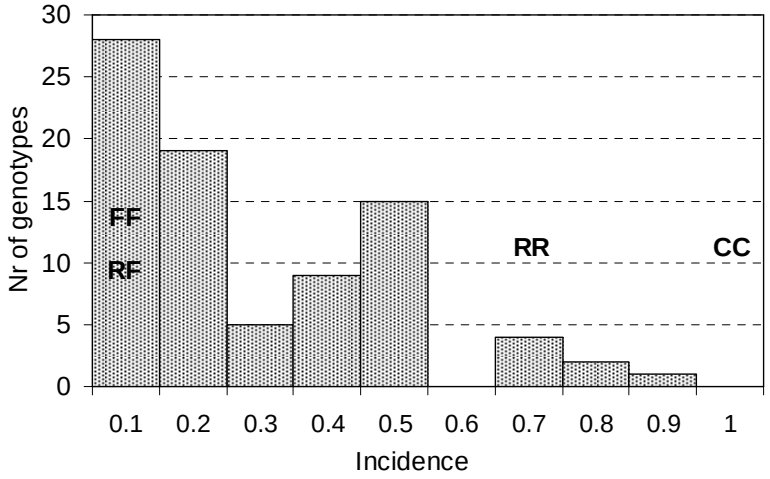


Figure 6. Distribution of 83 tri-hybrid genotypes in classes of FBR incidence after four weeks storage. The relative position of parental species is shown as CC: *Allium cepa*, FF: *A. fistulosum*, RR: *A. roylei*, RF: hybrid parent *A. roylei x A. fistulosum.*

Table 9. Effect of Fusarium basal rot on plant growth variables for the parental species, the RF-hybrid and the tri-hybrid *Allium* population ([a]).

Plant material	Plant fresh weight at harvest (g)		Bulb fresh weight at harvest (g) [c]		Bulb weight after storage [c]	
	Control	***Fusarium***	**Control**	***Fusarium***	**Control**	***Fusarium***
A. cepa	26.4 bc	13.6 d	21.1 a	13.2 b	19.3 a	9.4 b
A. roylei	23.0 bc	22.4 c	5.6 c	5.9 c	3.2 c	1.6 c
RF- hybrid [b]	30.4 ab	34.3 a	7.3 c	7.3 c	6.6 bc	4.5 bc
A. fistulosum	22.5 c	26.6 bc	5.0 c	6.2 c	3.1 c	3.1 c
Tri-hybrid population						
Mean	27.4	25.6	15.1	14.0	13.0	11.2
Minimum	4.6	4.3	4.2	4.1	3.7	2.2
Maximum	53.4	50.5	28.2	33.7	26.6	23.1

[a] REML analysis for the parental species, Means followed by the same letter do not differ statistically ($p<0.05$). For Bulb weight after storage, the interaction term (Accession x Fusarium) introduced after the main effects was nearly significant ($p=0.055$).
[b] Hybrid parental genotype *A. roylei x A. fistulosum* (PRI 91021-8).
[c] *A. roylei*, *A. fistulosum* and some genotypes of the tri-hybrid population do not form a bulb. The false stem area was considered to be the area in between the basal plante and 5 cm above this plate.

The relationship between FBR resistance with the number of roots per plant and with bulbing ability was investigated. No correlation was found between FBR disease indices and the number of stem-borne roots per plant (in the non-inoculated control). FBR decreased the number of roots at harvest for susceptible genotypes. Among the parental material, only *A. cepa* inoculated plants had less stem-borne roots at harvest than the non-inoculated control (data not shown, Mann-Whitney U test, $p=0.013$). FBR resistance and bulbing ability were also not correlated, and both genotypes without symptoms (FBR incidence = 0) and susceptible genotypes (not distinguished from *A. cepa*) were present in each bulbing class (Chi Square test, $\chi^2 = 8.16$, $p = 0.227$) (Figure 7). A QTL for bulbing ability was found on chromosome 5.

AFLP mapping and linkage analysis

A total of 359 polymorphic AFLP markers originating from either *A. roylei* or *A. fistulosum* were obtained by profiling tri-hybrid genotypes. Among these, 143 markers were mapped on 15 linkage groups (LOD $\geq$ 4). Markers from *A. roylei* that were earlier assigned to physical chromosomes by Van Heusden et al.

(2000b) and from *A. fistulosum* that were mapped to linkage groups by De Melo (2003) were used as reference to assign eight main linkage groups to chromosomes (LOD 4.4 to 5.4). The resulting linkage map for the RF-hybrid comprised 111 markers in eight linkage groups covering 886 cM (Figure 8). Marker positions on the chromosomes were calculated without forcing, and an overall mean marker Chi-Square contribution of 2.24 was obtained. Mean linkage group size was 110.7 cM, and mean marker interval was 8.1 cM. The map had four gaps larger than 18 cM in chromosomes 3, 5, and distal regions of chromosome 1 and 7. Skewed segregation in favour of *A. roylei* alleles was found on regions of chromosomes 1, 4, 6, 7 and 8, and in favour of *A. fistulosum* alleles on regions of chromosomes 3, 7 and 8 (Figure 8).

The remaining additional linkage groups (linkage groups 9 to 15) could not be associated to any chromosome because they only consisted of AFLP markers not reported before (Figure 9). These linkage groups were also studied in the QTL analysis.

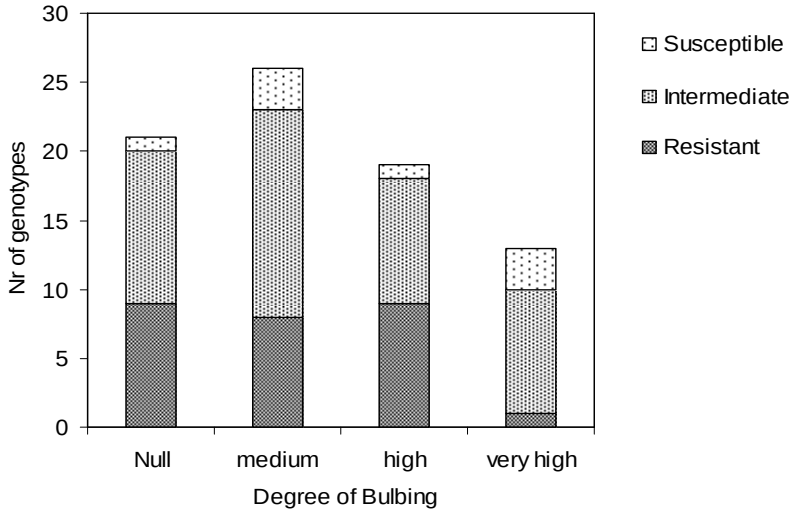


Figure 7. The distribution of tri-hybrid genotypes classified by the degree of bulbing and the level of FBR severity four weeks after storage, as follows: susceptible (no distinguished from *A. cepa*), intermediate (intermediate levels of FBR severity), and resistant (without FBR symptoms).

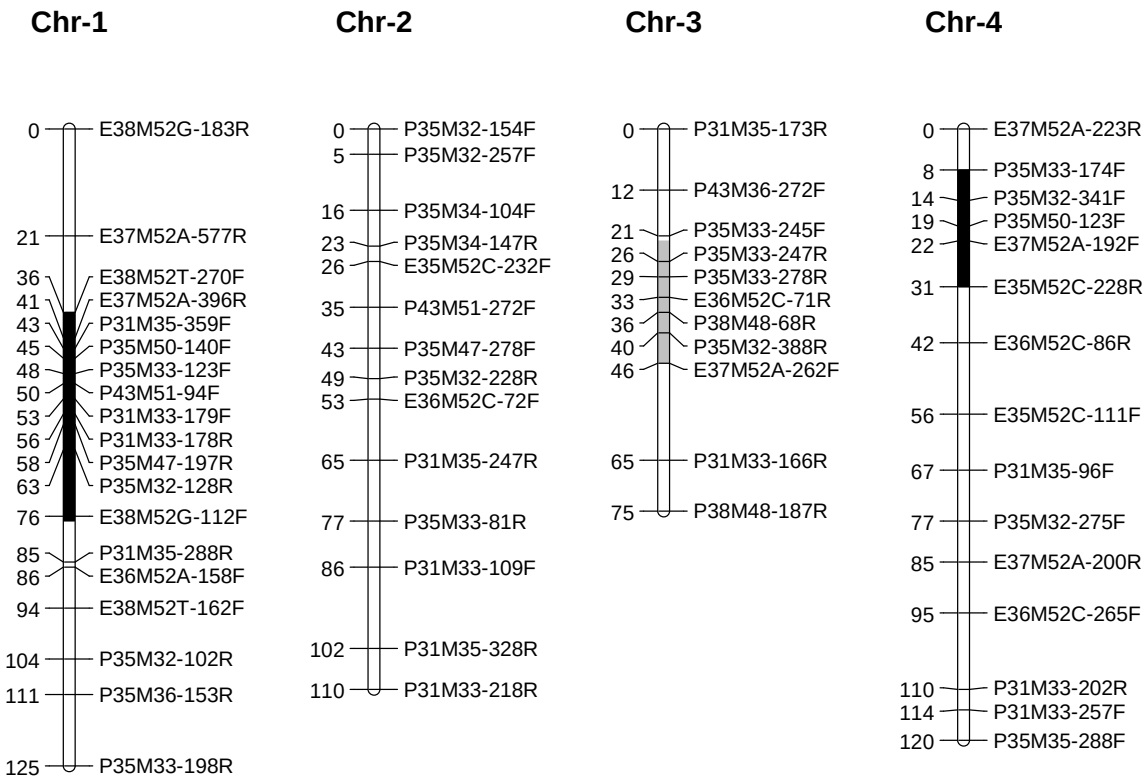


Figure 8. (see legend in the next page)

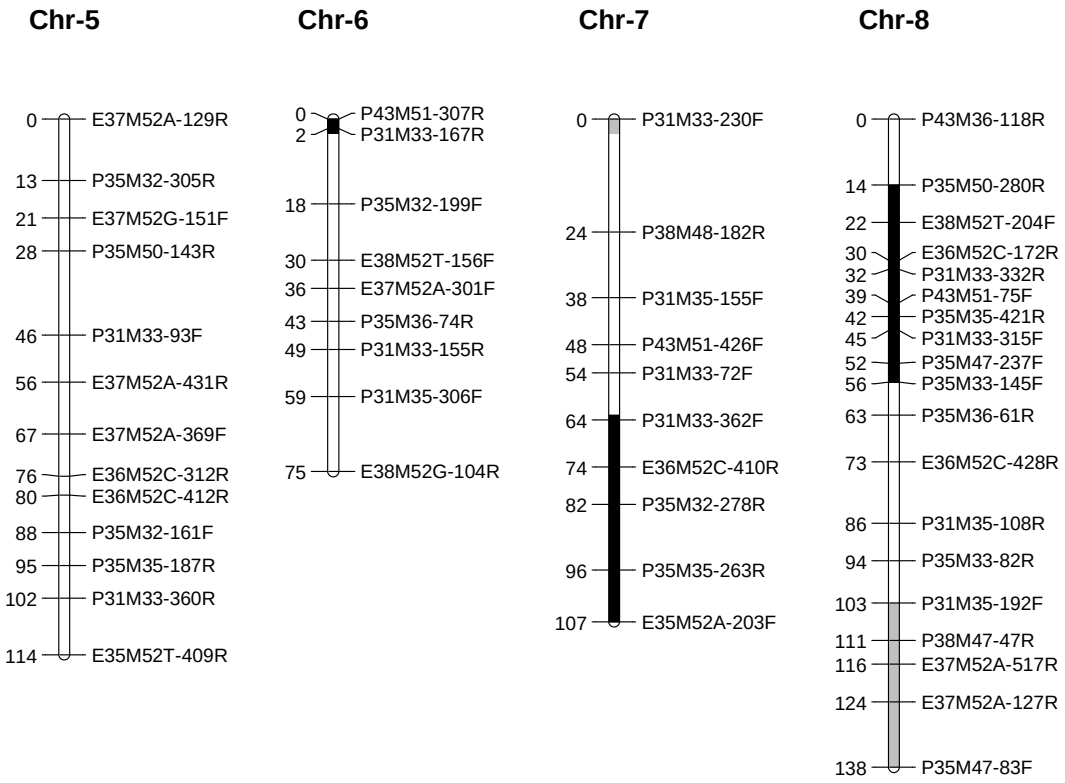


Figure 8. Molecular linkage map for the RF-hybrid (*A. roylei* x *A. fistulosum*) based on AFLP markers. Marker names ending with R are specific for *A. roylei*, and markers ending with F are specific for *A. fistulosum*. Filled bars indicate chromosomal regions with skewed segregation in favour of *A. roylei* (black) or in favour of *A. fistulosum* (gray).

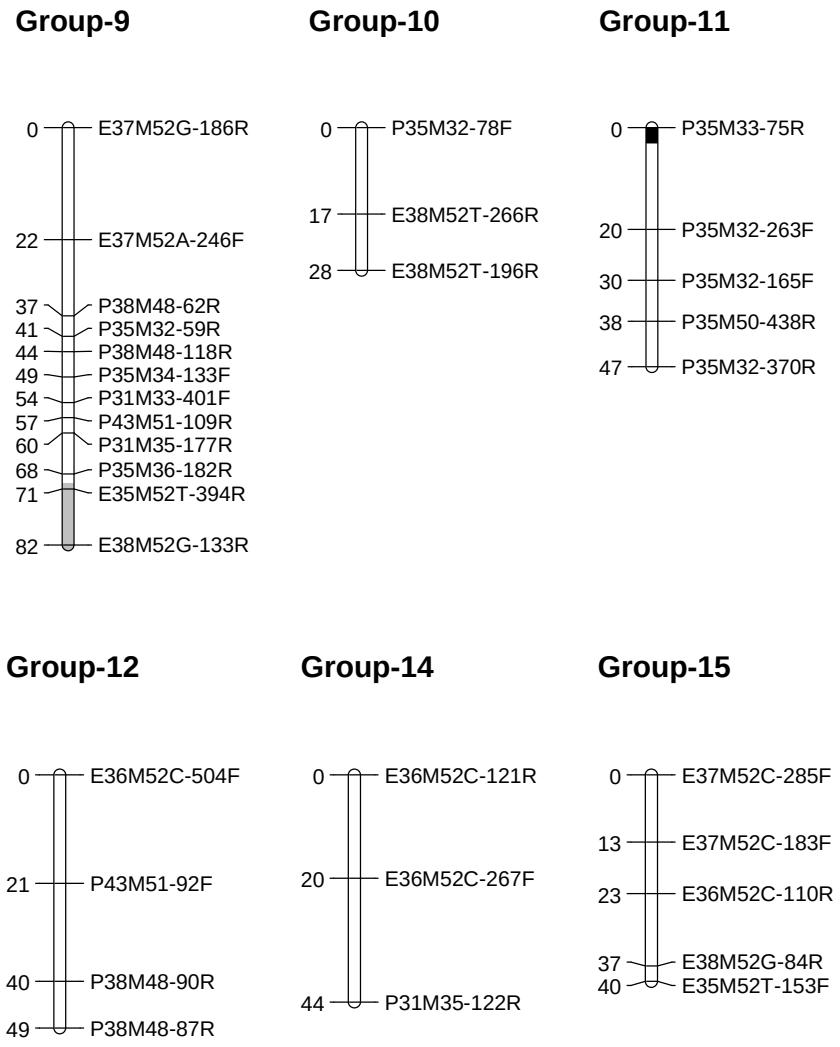


Figure 9. Additional linkage groups for the map of the RF-hybrid (*A. roylei* x *A. fistulosum*) based on AFLP markers. Marker names ending with R are specific for *A. roylei*, and markers ending with F are specific for *A. fistulosum*. Filled bars indicate skewed chromosomal regions in favour of *A. roylei* (black) or in favour of *A. fistulosum* (gray).

Table 10. Quantitative trait loci (QTLs) for Fusarium basal rot resistance, located on the linkage map of the RF-hybrid *(A. roylei* x *A. fistulosum)* by using multiple QTL mapping.

FBR traits	Best associated marker	Position	LOD [a]	Origin of alleles	% explained
FBR incidence [b]					
at harvest	P31M33-223R	Chr.2 – 110 cM	4.66 **	*A. roylei*	18.5
	P31M35-192F	Chr.8 – 103 cM	3.21 *	*A. fistulosum*	12.1
after storage	P31M33-223R	Chr.2 – 110 cM	4.66 **	*A. roylei*	18.6
	P31M35-192F	Chr.8 – 103 cM	2.93 *	*A. fistulosum*	12.3
FBR severity [c]					
at harvest	P31M33-223R	Chr.2 – 110 cM	5.50 **	*A. roylei*	21.7
	P31M35-192F	Chr.8 – 103 cM	4.59 **	*A. fistulosum*	17.9
after storage	P31M33-223R	Chr.2 – 110 cM	4.43 **	*A. roylei*	16.7
	P31M35-192F	Chr.8 – 103 cM	3.07 *	*A. fistulosum*	12.5
AUDPC [d]	P31M33-223R	Chr.2 – 110 cM	5.23 **	*A. roylei*	24.5
	P35M35-288F	Chr.4 – 120 cM	3.25 *	*A. fistulosum*	15.3
	P35M33-145F	Chr.8 – 57 cM	3.95 **	*A. fistulosum*	16.0
	P31M35-192F	Chr.8 – 103 cM	3.80 **	*A. fistulosum*	14.5

[a] Significance estimated by genome-wide permutation test, 1000 replicates (* $p < 0.05$; ** $p < 0.01$).
[b] Accumulated proportion of infected and rotten plants after storage.
[c] Severity disease index (0-3) for the presence of FBR infections in the basal plate of the plants, after threshold modelling.
[d] Area under disease progress curve, accounting for incidence of wilted plants weekly scored before harvest, FBR at harvest plus postharvest.

Mapping quantitative trait loci (QTLs)

QTLs for FBR resistance were identified in regions previously detected as significant by the Kruskal-Wallis test. A first QTL associated with *A. roylei* alleles was identified on a distal part of chromosome 2. This QTL had significant LOD scores for FBR severity and incidence at harvest, FBR severity and incidence after storage, and AUDPC (Table 9, Figure 10). A second QTL associated with *A. fistulosum* alleles was identified on the long arm of chromosome 8. This QTL had also significant LOD scores for variation in FBR severity and incidence at harvest, FBR severity and incidence after storage, and AUDPC (Table 9, Figure 11). These two QTLs together accounted for 30.6% and 30.9% of the total variation for FBR incidence at harvest and after four week

storage, as well as for 39.6 % and 29.2 % of the total variation for FBR severity at harvest and after storage respectively. When comparing the groups of genotypes carrying zero, one or two of these QTLs, significantly lower disease indices were observed for genotypes harbouring one QLT in comparison to genotypes without QTLs. Even lower disease indices were observed for the group of genotypes combining both QTLs for FBR incidence and severity at harvest, but genotypes harbouring one or two QTLs did not differ in disease indices after storage (Table 10).

For AUDPC, two additional QTLs from *A. fistulosum* were detected (Table 10). One was located on chromosome 4, and the other one was located also on the long arm of chromosome 8, sub-centromeric, 46.4 cM apart from the first QTL found in this region (Figure 11). The four detected QTLs explained 56 % of the total variation for AUDPC. An analysis of the effect of these QTLs by comparing groups of genotypes carrying zero to four QTLs, showed a significant effect of reduction in AUDPC due to the presence of anyone of the four QTL. Furthermore, a significant additional reduction was given by the combination of the QTL from *A. roylei* and one of the QTLs from *A. fistulosum*, but no significant additive effect was obtained between QTLs from *A. fistulosum* (data not shown).

Table 11. Median values for FBR incidence and severity at harvest, and after four weeks storage, for the groups of tri-hybrid genotypes having (+) or not (–) the QTLs from *A. roylei* (Chr. 2) and from *A. fistulosum* (Chr. 8).

Presence of QTLs		**Number of genotypes**	**FBR Incidence**		**FBR Severity**	
Chr.2 *A. roylei*	**Chr. 8 *A. fistulosum***		**at harvest**	**after storage**	**at harvest**	**after storage**
–	–	15	0.50 a	0.50 a	2.18 a	2.35 a
–	+	19	0.13 b	0.13 b	0.97 b	0.15 b
+	–	18	0.13 b	0.14 b	0.08 b	0.19 b
+	+	31	0 c	0 b	0 c	0 b

Groups of genotypes followed by different letters significantly differed (REML analysis, $p<0.05$). FBR severity values were obtained by threshold modelling of ordinal severity scores (0-3).

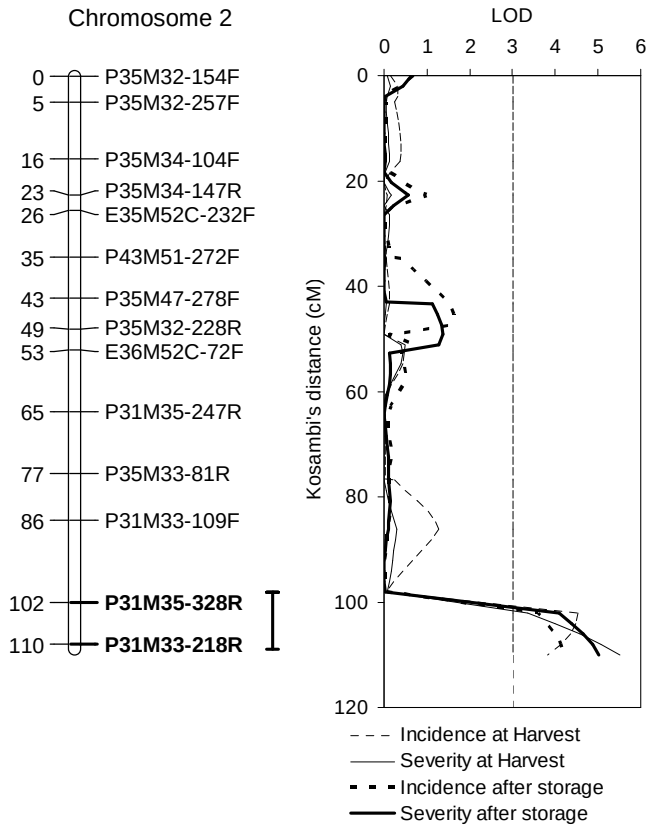


Figure 10. The position of QTLs for FBR resistance from *A. roylei* on Chromosome 2. The chart show the LOD values using multiple QTL mapping (MQM) for FBR incidence and severity at harvest, and after storage. The slashed line indicates the interval above the significant threshold value of the QTL for AUDPC.

Discussion

Screening for FBR resistance

A successful FBR screening assay was performed on adult plants, using the method as reported in Chapter 2. Plantlets grown *in vitro* developed normal basal plates (see Fig. 17 and 22), and allowed the occurrence of typical FBR symptoms. The susceptible *A. cepa* parent showed FBR incidence scores comparable to values found in infected fields (De Visser 1999, Cramer 2000, Gutierrez and Cramer 2005). FBR incidence and severity were lower at harvest than after four weeks storage. This phenomenon was particularly observed for *A. roylei* and for tri-hybrid genotypes with intermediate to high susceptibility.

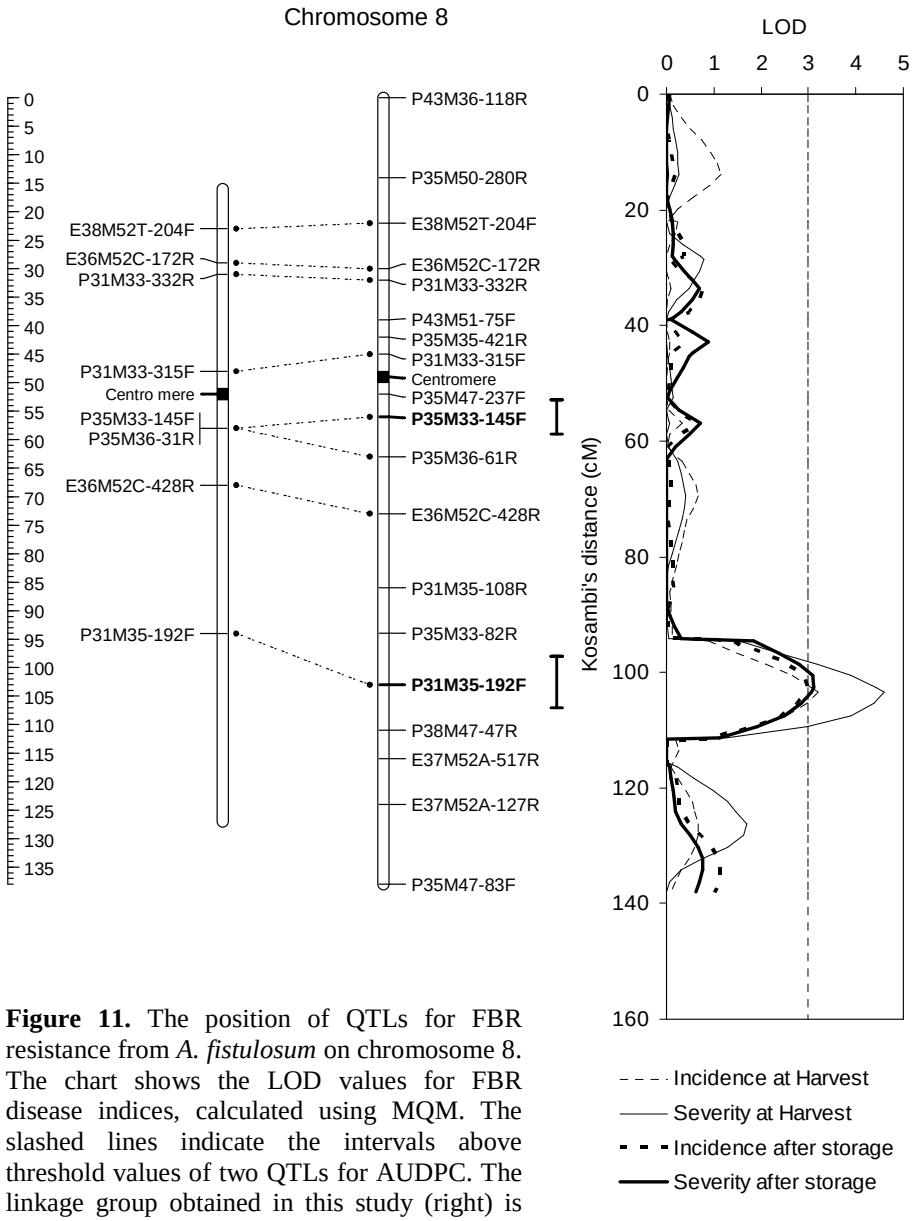


Figure 11. The position of QTLs for FBR resistance from *A. fistulosum* on chromosome 8. The chart shows the LOD values for FBR disease indices, calculated using MQM. The slashed lines indicate the intervals above threshold values of two QTLs for AUDPC. The linkage group obtained in this study (right) is compared with the map as in Khrustaleva et al. 2005 (left).

As a consequence, FBR scoring after a storage period and after slicing the basal plate to examine internal infections (proposed by Gutierrez and Cramer 2005) proved to be a useful tool to screen for FBR resistance. As a whole, the set up of this screening assay using experimental inoculation, *in vitro* propagated *Allium* plant material, and controlled raising temperature was efficient and may overcome year to year variations observed in field tests.

FBR influenced total plant fresh weight at harvest for *A. cepa* and susceptible tri-hybrid genotypes. This result is in agreement with previous screenings (Holz and Knox-Davies 1974, De Visser 1999, Cramer 2000). In general, the effects of FBR on plant weight and other plant growth variables were restricted to the development of visible symptoms in susceptible to partially resistant genotypes. Although the set up of the experiment was intended to use plant weight differences between inoculated and non-inoculated plants as an index for FBR resistance, experimental variation in plant weight of resistant genotypes demonstrated that in this experiment it could not be used as an index to estimate the effect of FBR. This variation may have been caused by variation in plant weight already present in the *in vitro* plant material at the moment of transplanting.

High levels of resistance found in *A. fistulosum* to a *F. oxysporum* isolate pathogenic to onion confirmed previous reports (Holz and Knox-Davies 1974, Chapter 2). Also the intermediate response in *A. roylei* confirmed previous results (Chapter 2). It is not possible to establish whether the conspicuous response of *A. roylei* (completely healthy until harvest, but highly susceptible to FBR after storage) is related to the inability of this species to be stored. Nevertheless, symptoms observed after storage developed from infections that began before harvest.

The *F. oxysporum* Clade 3 isolate used in this screening assay was chosen because in seedling tests it was highly virulent to onion cultivars. In previous screening assays, *A. fistulosum* proved to be resistant to two *F. oxysporum* isolates belonging to Clade 2 and Clade 3, and a *F. proliferatum* isolate, whereas *A. roylei* was partially resistant to isolates from these three taxa (Chapter 2). As various QTLs seem to be involved in achieving complete resistance, it is worthwhile to test the effects of specific QTL combinations regarding genetic variation among pathogenic *Fusarium* causing onion basal rot.

Molecular linkage map

Linkage groups were successfully assigned to chromosomes on the basis of markers in common with the maps previously developed for *A. roylei* (Van Heusden et al. 2000b) and the RF-hybrid (De Melo 2003). Our map covered in total 886 cM. The size of the map is larger than the expected 700-800 cM considering a chiasmata frequency of 14.2 for *A. cepa* x *A. roylei* cross (De Vries et al. 1992), and larger than previous maps (De Melo 2003, Van Heusden et al. 2000b). This may be the result of expanded regions due to scant marker information, represented as gaps larger than 18 cM in four chromosomes.

A fraction of 31 % of the markers remained unlinked, e.g., out of linkage groups with LOD ≥ 4 defined in JoinMap analysis. This result was comparable to 38 % unlinked markers reported for a subset of the same population by De Melo (2003), as well as 32 % for *A. cepa* and 20 % for *A. roylei* unlinked markers reported for an F2 from a cross between these two species (Van Heusden et al. 2000a). As discussed by De Melo (2003) and McCallum et al. (2006), poor linkage and poor effective mapping in *Allium* can be the result of complex banding patterns in electrophoresis assays, leading to defective marker scoring. That was the situation for *Eco*RI-*Mse*I primer combinations, which presented dense and faint banding patterns, even with the use of a seventh additional nucleotide in the specific amplification. Besides, skewed segregation in some chromosomal regions may also lead to deficiencies in mapping.

Distorted segregations are commonly found in inter-specific crosses (Chani et al. 2002, Jeuken et al. 2002, Mangum and Pleffey 2005, Finkers et al. 2007). In our research, 57% of the markers allocated in the map were skewed. De Melo (2003) reported 26% skewed markers, and Van Heusden et al. (2000a) 26% for *A. cepa*, and 51% for *A. roylei*. Skewed regions in this research were predominantly in favour of *A. roylei* (Figure 8), which is in agreement with De Melo (2003). Distorted segregations can be the result of differences in the reproductive ability (gamete and zygote formation), in fitness ability, and lethality (Rieseberg and Willis 2007). *A. roylei* is more closely related to *A. cepa* than *A. fistulosum* (Van Raamsdonk et al. 2003, Gurushidze et al. 2007). *Allium roylei* is used as the bridge species because of its better crossability with *A. cepa* (Van Raamsdonk et al. 2003, Kik 2002). Differences between karyotypes of *A. roylei* and *A. fistulosum* may lead to selection against particular *A. fistulosum* chromosomal regions. This difference may also explain the distorted segregations, because *A. roylei* may have genomic regions expanded in comparison to *A. fistulosum.*

Genetic mapping of FBR resistance

This is the first research that describes the mapping of QTLs for FBR resistance from *Allium* species related to onion. We detected one QTL from *A. roylei* and one from *A. fistulosum* for FBR incidence and FBR severity at harvest and after storage. Four QTLs were located for AUDPC, the two QTLs mentioned before, and two additional ones on chromosomes 4 and 8 from *A. fistulosum*.

Although *A. roylei* was only partially resistant to FBR, the QTL explained by this species was the most significant one. This QTL was located on a very distal part of chromosome 2, resembling the position of *Pd* gene from *A. roylei* responsible for resistance to downy mildew, mapped on a distal part of chromosome 3 (Van Heusden et al. 2000a, Scholten et al. 2007). The QTL from *A. fistulosum* was located on the long arm of chromosome 8, embedded in a region with high recombination rate (Khrustaleva et al. 2005). The frequent occurrence of chiasmata in this region enhances the possibilities for a successful introgression of this QTL into the onion genome. The estimated linkage distance with a second QTL found on this chromosome for AUDPC was in agreement with previous mapping (Khrustaleva et al. 2005) (Figure 11).

QTLs from *A. roylei* and *A. fistulosum* for FBR incidence and FBR severity explained 30-40% of the total variation. The remaining variation could be due to the combined result of minor genes for which no QTLs were detected. This hypothesis is supported by the fact that tri-hybrid genotypes without any QTL were not as susceptible as *A. cepa*. Other sources of unexplained variation are the occurrence of distorted segregations and experimental error (either in the screening assay or in the molecular linkage map).

Tri-hybrid genotypes harbouring either the QTL from *A. roylei* or the QTL from *A. fistulosum* had significantly lower values for FBR disease indices than genotypes without these QTLs. As genes from *A. roylei* and *A. fistulosum* in the tri-hybrid genotypes are in a heterozygous phase, this observation implies that dominant allele effects determine FBR resistance. Most genotypes with both QTLs showed complete resistance, indicating that additive effects occur. From a breeding point of view, simultaneous introgression of resistances from *A. roylei* and *A. fistulosum* seems possible and may be considered as a meaningful way forward.

Although *A. fistulosum* is highly resistant to FBR, and remained completely healthy in this screening, tri-hybrid genotypes carrying only the detected QTL

from *A. fistulosum,* as a group, were not as resistant as the *A. fistulosum* parent. Only 6 out of 19 individuals were completely free of symptoms. This finding supports that resistance from *A. fistulosum* may be polygenic, and more QTLs from *A. fistulosum* remain to be discovered. Two other detected QTLs from *A. fistulosum* for the AUDPC may point into this direction.

A larger population would allow the detection of more QTLs as well as more individuals into each QTL combination class. Furthermore, the analysis of epistatic effects between already identified QTLs could be improved by studying target populations that include also homozygous phases for each QTL (Chahal and Ghosal 2005). For this type of study, progenies should be obtained by selfing or inter-crossing tri-hybrid genotypes carrying the QTLs. Furthermore, the development of backcross generations between *A. cepa* and tri-hybrid resistant genotypes carrying one or more of the reported QTLs would result in additional crossing-over events between *A. roylei* and *A. fistulosum* genomes, as well as first crossing-over events with *A. cepa* (Khrustaleva and Kik 2000). In this way, the chromosomal regions involved in FBR resistance can be more precisely determined, and introgressed into the onion genome at the same time.

The number of stem-borne roots was not correlated with FBR incidence nor with FBR severity. In accordance, a QTL for the number of stem-borne roots from *A. fistulosum* was located on linkage group 9 (Chapter 5), and thus inherited independently of QTLs for FBR resistance. To explain variation among onion cultivars in response to FBR, Stadnik and Dhingra (1996) proposed that a larger number of roots may overcome Fusarium root rot and initial states of basal plate infections, by delaying or preventing wilting. However, this does not appear the case for FBR resistance in the tri-hybrid *Allium* population.

There was no relationship between FBR resistance and bulbing ability in the tri-hybrid population. This agrees with the finding of a QTL for bulbing ability on chromosome 5 (data not shown), which supports independent inheritance with QTLs for FBR resistance. This observation increases the feasibility of introgressing FBR resistance into the onion germplasm while maintaining bulbing ability.

In conclusion, this study has shown the usefulness of the simultaneous introgression of FBR resistance from *A. roylei* and *A. fistulosum*. The use of these resistant sources in commercial breeding programmes has become a realistic option, as QTLs for FBR resistance located in this research will speed up the breeding process to obtain resistant onion cultivars by marker assisted selection.

Chapter 4

Molecular diversity of arbuscular mycorrhizal fungi in onion roots from organic and conventional farming systems in the Netherlands[†]

Abstract

Diversity and colonization levels of naturally occurring arbuscular mycorrhizal fungi (AMF) in onion roots were studied to compare organic and conventional farming systems in the Netherlands. In 2004 twenty onion fields were sampled in a balanced survey between farming systems and between two regions, namely Zeeland and Flevoland. In 2005 nine conventional and ten organic fields were additionally surveyed in Flevoland. AMF phylotypes were identified by rDNA sequencing. All plants were colonized, with 60% for arbuscular colonization and 84% for hyphal colonization as grand means. In Zeeland, onion roots from organic fields had higher fractional colonization levels than those from conventional fields. Onion yields in conventional farming were positively correlated with colonization level. Overall, fourteen AMF phylotypes were identified. The number of phylotypes per field ranged from one to six. Two phylotypes associated with the *Glomus mosseae – coronatum* and the *G. caledonium – geosporum* species complexes were the most abundant, whereas other phylotypes were infrequently found. Organic and conventional farming systems had similar number of phylotypes per field and Shannon diversity indices. A few organic and conventional fields had larger number of phylotypes, including phylotypes associated with the genera *Glomus*-B, *Archaeospora* and *Paraglomus*. This suggests that farming systems as such did not influence AMF diversity, but rather specific environmental conditions or agricultural practices.

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Introduction

Onion (*Allium cepa* L.) has a sparse rooting system without root hairs which makes the crop dependent for water and nutrient acquisition on Arbuscular Mycorrhizal Fungi (AMF) (De Melo 2003, Stribley 1990). This dependency is especially true in case of cultivation under nutrient-poor soil conditions as is frequently the case in low-input and organic agriculture. AMF enlarge the soil volume from which nutrients can be taken up, via an extensive mycelium network, enabling host plants to access more resources (Finlay 2004). As a consequence, AMF enhance uptake of nutrients, particularly phosphorus (Hayman & Mosse 1971), and may allow for a reduction of the amount of fertilizers applied (Linderman & Davies 2004). Furthermore AMF can protect the plant against biotic (diseases) and abiotic (drought) stress, and improve soil aggregation (Gosling et al. 2006).

Research on *Allium* species and their interactions with AMF has a long history that dates back to 1884, when Mollberg described in roots of *Allium scorodoprasum* what we currently know as AMF (Koide & Mosse, 2004). *Allium* species, and in particular onion, are excellent models for mycorrhizal research because they have a simple rooting system, slow growth, and high response to AMF. The knowledge on *Allium*-AMF interactions benefitted greatly from the work of Mosse and co-workers, who presented detailed analyses of AMF functioning under field conditions (Hayman & Mosse 1971, Mosse & Hayman 1971, Mosse 1973, Owusu-Bennoah & Mosse 1979).

Onion, with a total annual acreage of 16000 - 19000 ha and an organically managed acreage of 600 ha, is an important crop in the Netherlands and a good model to monitor and compare the AMF status of agricultural soils. Numerous studies have shown that agricultural soils have low AMF species richness in comparison to natural ecosystems, such as woodlands and grasslands. The difference in AMF diversity is thought to be due to tilling-induced disruption of hyphal networks, rotation with non-mycorrhizal crop species, the occurrence of fallow periods, and the use of fertilizers and fungicides (Helgason et al. 1998, Daniell et al. 2001, Merryweather 2001, Jansa et al. 2002). However, agricultural soils can differ in species richness and composition of AMF because their management systems differ significantly. This is the case for example in low- and high-input farming systems (Ryan et al. 2000). In low-input and organic farming systems, synthetic fungicides and soluble phosphate fertilizers are limited or excluded. This may increase AMF inoculum potential and colonization levels

compared to conventional farming systems, as has been observed for wheat (Douds et al. 1993, Ryan et al. 1994) and clover-ryegrass pastures (Eason et al. 1999, Ryan et al. 2000). Furthermore, recent research showed that AMF biodiversity was higher in low-input systems compared to high-input systems (Oehl et al. 2003, 2004) although the relationship is not always straightforward (Hijri et al. 2006).

The contribution of AMF to crop-production increase in high-input agriculture is low, because phosphorus is amply available. In contrast, AMF might have a significant role in increasing crop production in low-input and organic agricultural systems. However this concept is far from being practically applied due to the lack of understanding of the functioning of AMF species (Scullion et al. 1998).

The present research aimed to study AMF species richness and composition in onion fields in the Netherlands, by comparing organic and conventional cultivation systems. In this way, we investigate if the adoption of organic practises on formerly conventionally managed farmlands leads to higher AMF diversity. Unlike previous studies, this research was carried out in a large number of sites. The primer sets developed by Redecker (2000) and Redecker et al. (2003) were used to identify AMF phylotypes that colonize onion plant roots, and therefore only the AMF assemblage of the target host species was analysed rather than the complete diversity in the soil.

Materials and Methods

Survey of onion fields and root colonization

Two traditional onion growing regions in the Netherlands were sampled, namely Zeeland in the southwest, and Flevoland (the Flevopolder and the Noordoostpolder) in the centre of the country. In Zeeland onion cultivation takes place already for centuries, whereas in Flevoland, on the land recently reclaimed from the sea, onion cultivation only takes place since the second half of the 20th century. In both regions the soils are classified as clay to loess-clay soils. Clay content ranged from 23 to 40% for the soils investigated in Zeeland, and 7 to 55% in Flevoland (Table 12). Seed-onions in the Netherlands are cultivated in rotation with other field crops. The soils are ploughed every year, either before or after the winter, and seedbeds are prepared consisting of fine soil aggregates. Sowing date is at the end of March and early April, and harvest takes place in the second half of August (early cultivars) and September (late cultivars). In both years,

samplings were done in the second half of June. Plants were in leaf development phase, with four to six expanded leaves.

Twenty onion fields were sampled in a balanced survey between cultivation systems and regions: five organic and five conventional fields in Zeeland, plus five organic and five conventional fields in Flevoland. Ten plants per field were randomly sampled. The surrounding soil was excavated in order to take out the rooting system as intact as possible. A new survey was done in June 2005, only in Flevoland, because the 2004 study suggested that management practises in this region could have an influence on AMF diversity. The 2005 study comprised ten organic and nine conventional onion fields. All of them were different from fields sampled in 2004, although in some cases they were located within the same farm. Organic farm fields followed ecological or biodynamic practices, and fulfilled the basic standards for organic production (available from IFOAM, 2007) as certified by SKAL (www.skal.com). Information on chemical characteristics of the soils (pH, organic matter, content of phosphorus and other nutrients) and onion yields were obtained from the farmers for 28 of the 39 sites, and the number of years under organic management queried for all 20 organic fields (Table 12).

Table 12. Average soil chemical parameters and soil history of onion fields surveyed in 2004 and 2005, by cultivation system and region in the Netherlands.

Cultivation system	Region	Number of fields	Years under organic cultivation [a]	Onion yield (ton/ha)	Soil properties				
					OM [b]	pH	Pw [c]	Ca [b]	K [d]
Organic		20	12.1	32	3.1	7.4	32	7.2	29
	Flevoland	15	13.8 (4–32)	33	3.1	7.4	32	6.5	30
	Zeeland	5	5.7 (1–12)	28	2.9	7.2	30	9.2	20
Conventional		19	–	70	3.0	7.3	44	5.1	26
	Flevoland	14	–	76	3.2	7.3	45	5.5	23
	Zeeland	5	–	59	2.5	7.5	42	2.8	33

[a] Average values. The range of values is indicated between brackets. [b] OM: organic matter (%). Ca: $CaCO_3$ (%). [c] mg $P_2O_5 \cdot liter^{-1}$ of dry soil. The difference in P content between cultivation systems was almost significant (REML analysis, p=0.071). [d] mg $K \cdot kg^{-1}$ soil.

AMF colonization was estimated only for samples collected in 2004. Staining of fungal structures was done using trypan blue, and colonization was quantified following the magnified intersections method (McGonigle et al. 1990). Hyphal (HC), arbuscular (AC) and vesicular colonization (VC) were quantified separately. Data analysis was carried out via ANOVA using Genstat 9.2 (Lawes Agricultural Trust, Rothamsted Exp. St., UK, 2006). The relationships between colonization parameters and environmental variables were studied using the Pearson correlation coefficient and linear regression analysis.

Molecular diversity analysis

AMF species colonizing onion roots were identified by sequencing the partial 18S-ITS1-5.8S-ITS2 rDNA region, as described by Redecker (2000), with minor modifications. DNA was isolated from one cm root pieces randomly taken from each onion sample. rDNA was amplified using a nested PCR approach. The primers NS5/ITS4i were used in the first step (Redecker et al. 2003) with an annealing temperature of 51°C. For the second amplification, PCR products were diluted 1:100. This second step was performed using the primers ACAU1660, ARCH1311, GLOM1310, and LETC1670 in combination with ITS4i in separate reactions. Primer GIGA5.8R was used only in combination with NS5. Primer sequences and protocols for PCR amplifications are available from Redecker (2000) and Redecker et al. (2003).

PCR products having the expected size were cloned by the pGEM-T vector system (Promega, Madison, USA). An aliquot of 8 µl of the successful clones were digested with restriction enzymes *Mbo*I, *Hinf*I and *Alu*I in 15 µl at 37°C for 6 hours. Restriction fragment patterns were run on a 3% gel made from RESponse agarose (Biozym group, Landgraaf, The Netherlands) and analyzed by the Phoretix 1D software (Nonlinear Dynamics, Durham, USA). A representative clone for each distinct restriction profile was sequenced by the dideoxynucleotide chain termination method using BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and an automatic sequencer (ABI PRISM 3700 DNA Analyser, Applied Biosystems). After removing chimerical results, a set of 65 sequences were obtained from the 5.8S-ITS2 rDNA region and when available the partial 18S region (each region 400-500 bps in length). Sequences are deposited in the European Molecular Biology Laboratory (EMBL and NCBI databases) under the Accession Numbers AM992799 to AM992864. These sequences were used for identification of AMF phylotypes after phylogeny analysis. In order to obtain that, sequences were submitted to the BLAST query tool (Altschul et al. 1997) and database www.ncbi.nih.gov/blast/ for an initial similarity analysis. Sequence alignment was performed manually. Finally, a

phylogenetic analysis was carried out by distance analysis using the neighbour joining method in PAUP 4b10 (Swofford 2003) with the Kimura two-parameter model and a gamma shape parameter of 0.5. Bootstrap analyses were done with 1000 replications. Sequences selected from public databases belonging to known AMF species were included in the phylogenetic analysis.

Phylotypes were determined from cladograms as clearly distinct monophyletic taxa which were also present in the respective maximum likelihood trees. AMF genera or morphospecies associated to each phylotype were assigned on the basis of the position of already known sequences from databases (Table 13). Phylotypes were associated either to a group of related species (e.g. *Glomus caledonium – geosporum*) or a genus (e.g. *Paraglomus*), and therefore the number of phylotypes is a conservative estimate for the number of morphospecies. Code names were assigned to phylotypes, as an acronym for the genus followed by a correlative number. *Glomus*-A and *Glomus*-B groups were distinguished according to Schwarzott et al. (2001).

AMF diversity was analyzed by integrating the data from 2004 and 2005, using the number of phylotypes found per onion field. In addition, Shannon-Weaver diversity indices (*H'*) were calculated based on the relative abundance of phylotypes per field, as $H_i' = -\sum [(n_i/N) \times Ln(n_i/N)]$, being n_i the number of observation for the *i* phylotype, whereas *N* is the total number of observations recorded (Mueller et al. 2004). Differences in AMF diversity indices between cultivation systems or between regions were tested by Residual Maximum Likelihood analysis (REML) using Genstat 9.2. Besides, the associations between AMF diversity with chemical soil parameters, and with the number of years under organic agriculture were studied by Pearson correlation coefficient and linear regression analysis.

Table 13. Arbuscular mycorrhizal colonization parameters by cultivation systems and regions in the Netherlands (Survey 2004).

Cultivation system	Region	Arbuscular colonization (AC) (%)	Hyphal colonization (HC) (%)	Vesicular colonization (%)	Ratio AC/HC
Organic	Flevoland	62 a	89 a	9.2	0.69 a
	Zeeland	65 a	85 a	6.5	0.76 a
Conventional	Flevoland	67 a	91 a	7.7	0.73 a
	Zeeland	46 b	72 b	3.6	0.62 b

Means within each column followed by the same letter do not differ statistically.
(Fischer LSD test, $p > 0.05$).

Correspondence Analysis (CA) was performed in CANOCO 4.53 (Ter Braak and Smilauer, 2004). CA allows to study the contribution of each sampled site (n = 39) to the total variation based on their AMF species composition, as well as the contribution from each phylotype (n = 14) based on their abundance along sampled sites (Jongman et al. 1995).

Results

AMF colonization in onions

In 2004 all sampled onion plants were colonized. The average colonization in both sampled regions and cultivation systems was 60% for arbuscular colonization (AC) and 84% for hyphal colonization (HC) as grand means (Table 13). The presence of vesicles was much lower, namely 7% on average. While neither region nor cultivation system was a significant source of variation, the interaction between regions and cultivation systems was significant for AC ($p = 0.004$) and HC ($p = 0.005$). This was due to the fact that conventional cultivation in Zeeland had a mean AC of 46%, a mean HC of and 72%, and both means differed significantly from the means of the other three combinations of region and cultivation system. Furthermore, onions grown in conventional fields in Zeeland had a significantly lower AC/HC ratio.

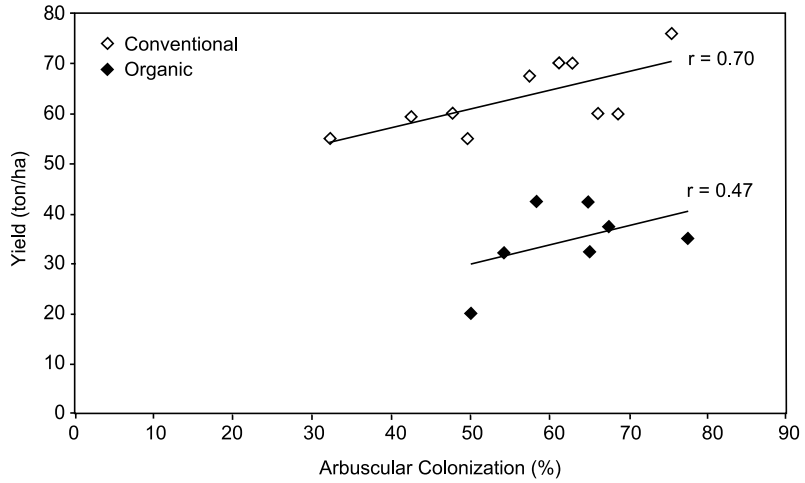


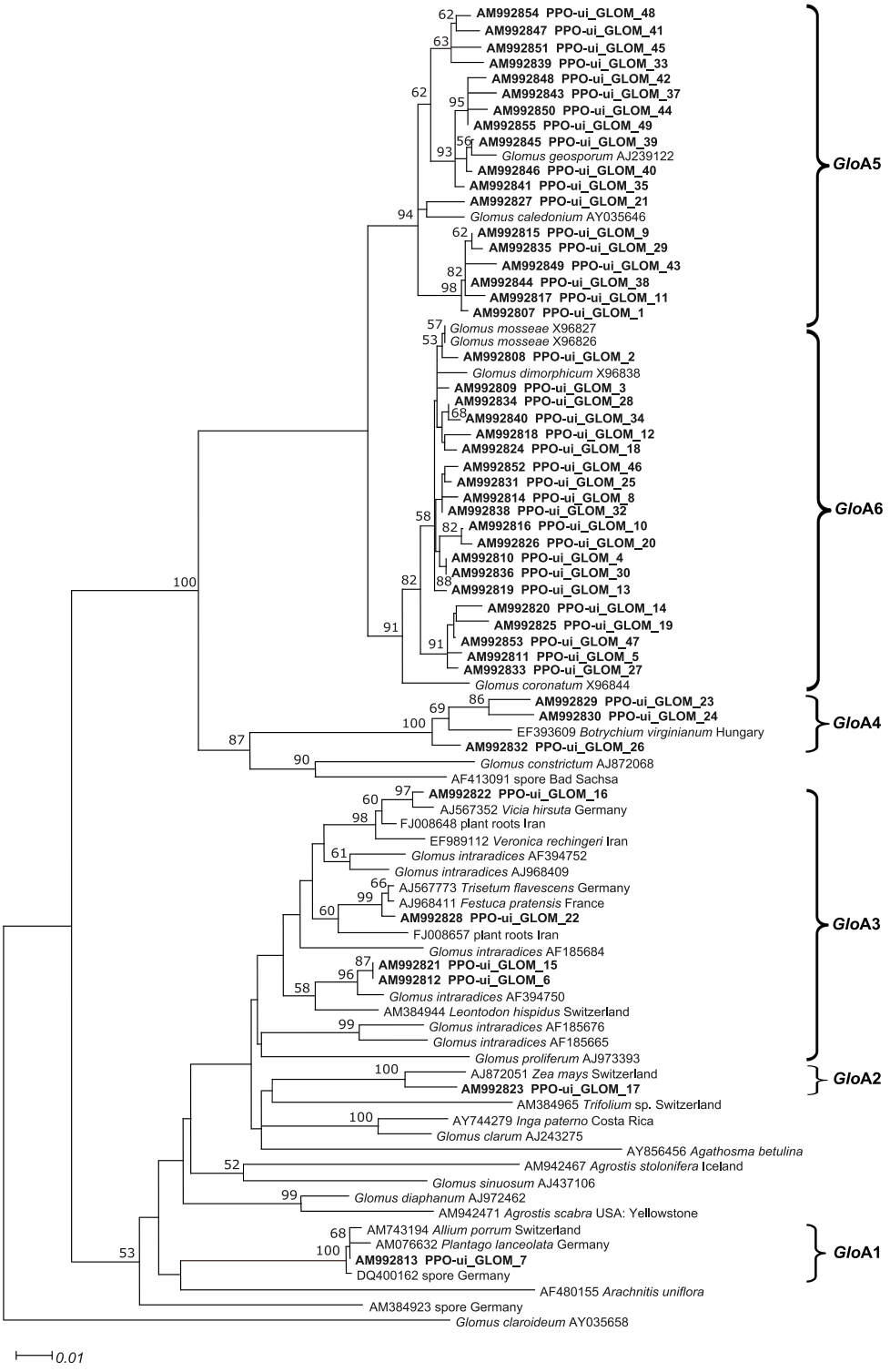
Figure 12. The correlation between arbuscular colonization (%) and onion yield (tons/ha) for conventional (n = 10) and organic (n = 7) management systems (survey 2004).

No correlation was found between average AC and HC and soil parameters on the one hand, and the number of years under organic cultivation on the other hand, except for the correlation between content of Calcium in the soil and AC ($r = 0.55$; $p < 0.05$). In addition, a significant correlation was found between AC and onion yield (OY) under conventional management ($r = 0.70$, $p < 0.05$; Figure 12), as well as between HC and OY ($r = 0.85$, $p < 0.05$). In case of conventional management ($n = 10$) the linear regression was estimated as $OY[ton \cdot ha^{-1}] = 0.38 \times AC\% + 42.1$. With smaller range of variation in AMF colonization level (AC, HC) and less data points available ($n = 7$), in case of organic management the regression was estimated as $OY[ton \cdot ha^{-1}] = 0.38 \times AC\% + 10.6$, though this regression was not significant.

AMF diversity in onion fields

Among the total plant samples, 56% in 2004 and 93% in 2005 yielded AMF amplification products and subsequent restriction profiles. The GIGA5.8R combined with NS5 did not yield any amplification product. After phylogenetic analysis, 65 rDNA obtained sequences were clustered in fourteen phylotypes, namely six *Glomus*-A, five *Glomus*-B, two *Archaeospora* and one *Paraglomus* phylotypes (Figures 13 and 14). Phylogenetic analysis of the ITS2-5.8S sequences of *Glomus*-A species were in agreement with the phylogenetic tree obtained for the sequences from the 18S region (Figure 15). An exception was the distinction between phylotypes *Glo*A5 and *Glo*A6, which were clearly distinct regarding the ITS2-5.8S region, whereas the 18S region clusters did not hold high bootstrap values.

Figure 13. (opposite page) Neighbour joining phylogenetic analysis of arbuscular mycorrhizal fungal 5.8S-ITS2 rDNA sequences obtained from onion root samples in the Netherlands for *Glomus*-A (as defined by Schwarzott et al. 2001) phylotypes, rooted to *Paraglomus brasilianum* sequence AJO12112. The scale represents substitutions per site. Sequences obtained in the present study are shown in boldface. Sequences from databases isolated from roots were labelled with the accession number, host plant, and country of origin, whereas those isolated from spores, with fungal species and accession number. Bootstrap values from 1000 replications larger than 50% are indicated above branches. Brackets to the right indicate phylotypes defined for the sequences obtained in this study.


AM992854 PPO-ui_GLOM_48
AM992847 PPO-ui_GLOM_41
AM992851 PPO-ui_GLOM_45
AM992839 PPO-ui_GLOM_33
AM992848 PPO-ui_GLOM_42
AM992843 PPO-ui_GLOM_37
AM992850 PPO-ui_GLOM_44
AM992855 PPO-ui_GLOM_49
AM992845 PPO-ui_GLOM_39
Glomus geosporum AJ239122
AM992846 PPO-ui_GLOM_40
AM992841 PPO-ui_GLOM_35
AM992827 PPO-ui_GLOM_21
Glomus caledonium AY035646
AM992815 PPO-ui_GLOM_9
AM992835 PPO-ui_GLOM_29
AM992849 PPO-ui_GLOM_43
AM992844 PPO-ui_GLOM_38
AM992817 PPO-ui_GLOM_11
AM992807 PPO-ui_GLOM_1
Glomus mosseae X96827
Glomus mosseae X96826
AM992808 PPO-ui_GLOM_2
Glomus dimorphicum X96838
AM992809 PPO-ui_GLOM_3
AM992834 PPO-ui_GLOM_28
AM992840 PPO-ui_GLOM_34
AM992818 PPO-ui_GLOM_12
AM992824 PPO-ui_GLOM_18
AM992852 PPO-ui_GLOM_46
AM992831 PPO-ui_GLOM_25
AM992814 PPO-ui_GLOM_8
AM992838 PPO-ui_GLOM_32
AM992816 PPO-ui_GLOM_10
AM992826 PPO-ui_GLOM_20
AM992810 PPO-ui_GLOM_4
AM992836 PPO-ui_GLOM_30
AM992819 PPO-ui_GLOM_13
AM992820 PPO-ui_GLOM_14
AM992825 PPO-ui_GLOM_19
AM992853 PPO-ui_GLOM_47
AM992811 PPO-ui_GLOM_5
AM992833 PPO-ui_GLOM_27
Glomus coronatum X96844
AM992829 PPO-ui_GLOM_23
AM992830 PPO-ui_GLOM_24
EF393609 Botrychium virginianum Hungary
AM992832 PPO-ui_GLOM_26
Glomus constrictum AJ872068
AF413091 spore Bad Sachsa
AM992822 PPO-ui_GLOM_16
AJ567352 Vicia hirsuta Germany
FJ008648 plant roots Iran
EF989112 Veronica rechingeri Iran
Glomus intraradices AF394752
Glomus intraradices AJ968409
AJ567773 Trisetum flavescens Germany
AJ968411 Festuca pratensis France
AM992828 PPO-ui_GLOM_22
FJ008657 plant roots Iran
Glomus intraradices AF185684
AM992821 PPO-ui_GLOM_15
AM992812 PPO-ui_GLOM_6
Glomus intraradices AF394750
AM384944 Leontodon hispidus Switzerland
Glomus intraradices AF185676
Glomus intraradices AF185665
Glomus proliferum AJ973393
AJ872051 Zea mays Switzerland
AM992823 PPO-ui_GLOM_17
AM384965 Trifolium sp. Switzerland
AY744279 Inga paterno Costa Rica
Glomus clarum AJ243275
AY856456 Agathosma betulina
AM942467 Agrostis stolonifera Iceland
Glomus sinuosum AJ437106
Glomus diaphanum AJ972462
AM942471 Agrostis scabra USA: Yellowstone
AM743194 Allium porrum Switzerland
AM076632 Plantago lanceolata Germany
AM992813 PPO-ui_GLOM_7
DQ400162 spore Germany
AF480155 Arachnitis uniflora
AM384923 spore Germany
Glomus claroideum AY035658
GloA5
GloA6
GloA4
GloA3
GloA2
GloA1
0.01

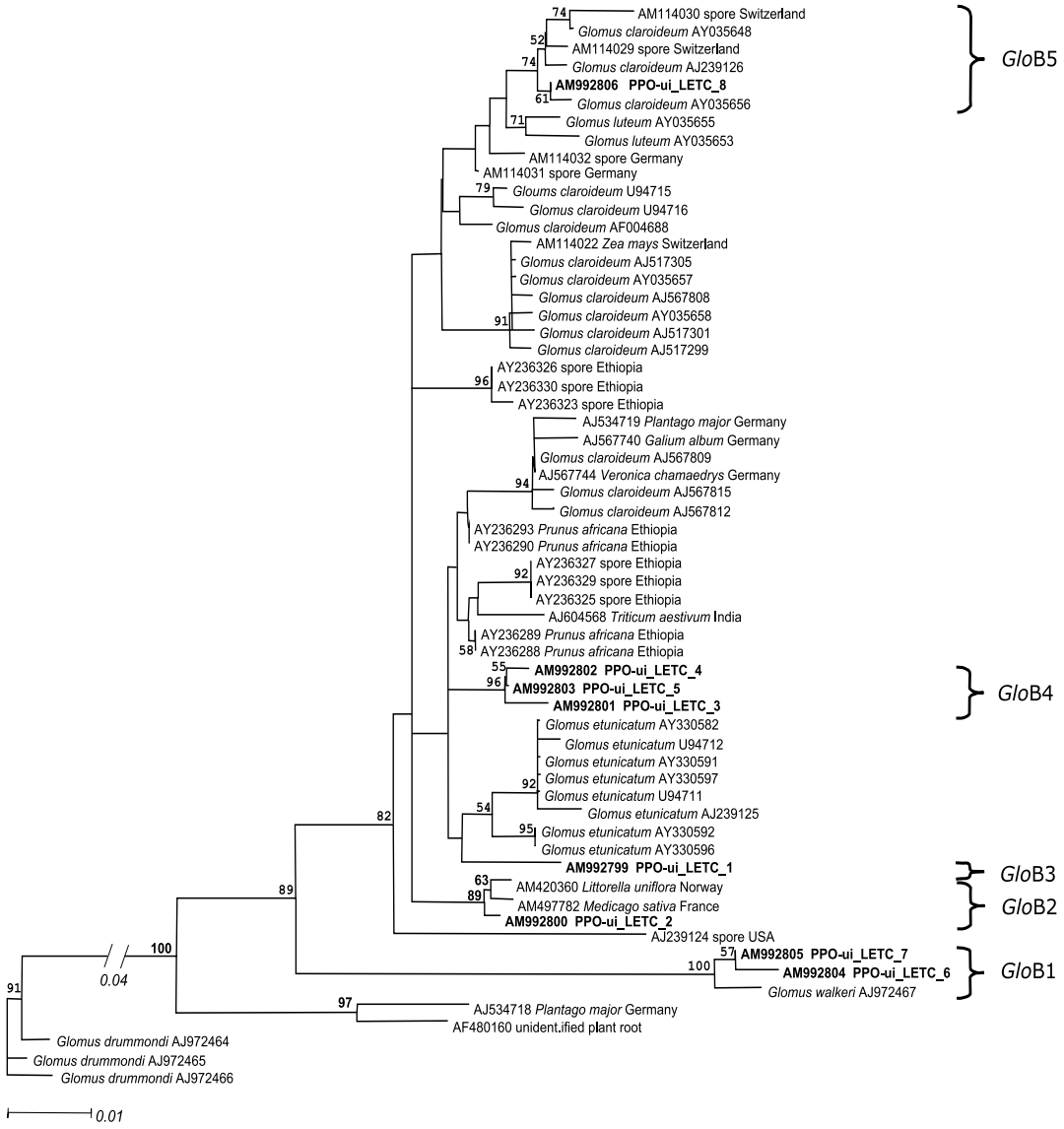


Figure 14. Neighbour Joining phylogenetic analysis of arbuscular mycorrhizal fungal 5.8S-ITS2 rDNA sequences obtained from onion root samples in the Netherlands for *Glomus*-B (as defined by Schwarzott et al. 2001) phylotypes, rooted to the *G. drummondii* sequence AJ972466. The scale represents substitutions per site. Sequences obtained in the present study are shown in boldface. Sequences from databases isolated from roots were labelled with the accession number, host plant, and country of origin, whereas those isolated from spores, with fungal species and accession number. Bootstrap values from 1000 replications larger than 50% are indicated above branches. Brackets to the right indicate phylotypes defined for the sequences obtained in this study.

Table 14. Number of fields containing each AMF phylotype, by cultivation system and region in the Netherlands.

Phylotypes [a]	Cultivation systems		Regions		Total nr of fields
	Organic	Conven-tional	Flevoland	Zeeland	
GloA1 (*Glomus*-A)	1		1		1
GloA2 (*Glomus*-A)	1			1	1
GloA3 (*G. intraradices*)	3	1	2	2	4
GloA4 (*Glomus*-A)	2		2		2
GloA5 (*G. caledonium-geosporum)*	16	15	22	9	31
GloA6 (*G. mosseae-coronatum)*	19	19	28	10	38
GloB1 (*G. walkeri*)		2	2		2
GloB2 (*Glomus*-B)		1		1	1
GloB3 (*Glomus*-B)	1			1	1
GloB4 (*Glomus*-B)	2		2		2
GloB5 (*G. claroideum*)		1	1		1
Par1 (*Paraglomus* sp.)	5		5		5
Arch1 (*Archaeospora* sp.)	1	4	5		5
Arch2 (*Archaeospora* sp.)	2		2		2
Total number of surveyed fields	20	19	29	10	39

[a] Distinct monophyletic taxa after neighbour joining analysis of rDNA sequencies. Whenever known, species names associated with a phylotype are indicated. *Glomus*-A and B, as defined by Schwarzott et al. (2001).

The other phylotypes were present in a much lower frequency, three of them being exclusively found in conventional fields, and seven only found in organic fields (Table 15). Some phylotypes were only found in 2004, such as *Glo*A1, *Glo*A2 and *Glo*A3, whereas others were found only in 2005, such as Arch1 (Table 16). Noticeably, onion fields of two organic farms harboured phylotypes *Par*1 (*Paraglomus*) and *Arch*2 (*Archaeospora*) in 2004, and the next year, sampling again on different fields of the same farms resulted in the finding of the same phylotypes.

The number of AMF phylotypes per field ranged from one to six. The highest diversity in phylotypes was found in two organic and two conventional fields. Organic and conventional management systems did not differ in the number of phylotypes per field (REML analysis, $p = 0.48$), nor did the regions ($p = 0.99$). In

Table 15. Number of fields which harbour specific AMF phylotypes expressed per surveyed year, cultivation system and region in the Netherlands.

Phylotypes	Survey 2004				Survey 2005	
	Flevoland		Zeeland		Flevoland	
	OF	CF	OF	CF	OF	CF
GloA1 (*Glomus*-A)	1					
GloA2 (*Glomus*-A)			1			
GloA3 (*G. intraradices*)	2		1	1		
GloA4 (*Glomus*-A)	2					
GloA5 (*G. caledonium-geosporum)*	1	2	5	5	10	9
GloA6 (*G. mosseae-coronatum)*	4	5	5	5	10	9
GloB1 (*G. walkeri*)						2
GloB2 (*Glomus*-B)			1			
GloB3 (*Glomus*-B)				1		
GloB4 (*Glomus*-B)						1
GloB5 (*G. etunicatum*)	1				1	
Par1 (*Paraglomus* sp.)	1				4	
Arch1 (*Archaeospora* sp.)					1	4
Arch2 (*Archaeospora* sp.)	1				1	
Total number of surveyed fields	5	5	5	5	10	9

OF: Organic farms, CF: Conventional farms.

Figure 15 (opposite page). Neighbour Joining phylogenetic analysis of 18S rDNA sequences of arbuscular mycorrhizal fungi obtained from onion root samples in the Netherlands, for Glomus-A (as defined by Schwarzott et al. 2001), Archaeospora and Paraglomus phylotypes, rooted to the Paraglomus occultum sequence AJ006799. The scale represents substitutions per site. Sequences obtained in the present study are shown in boldface. Sequences from databases isolated from roots were labelled with the accession number, host plant, and country of origin, whereas those isolated from spores, with fungal species and accession number. Bootstrap values from 1000 replications larger than 50% are indicated above branches. Brackets to the right indicate phylotypes defined for the sequences obtained in this study.

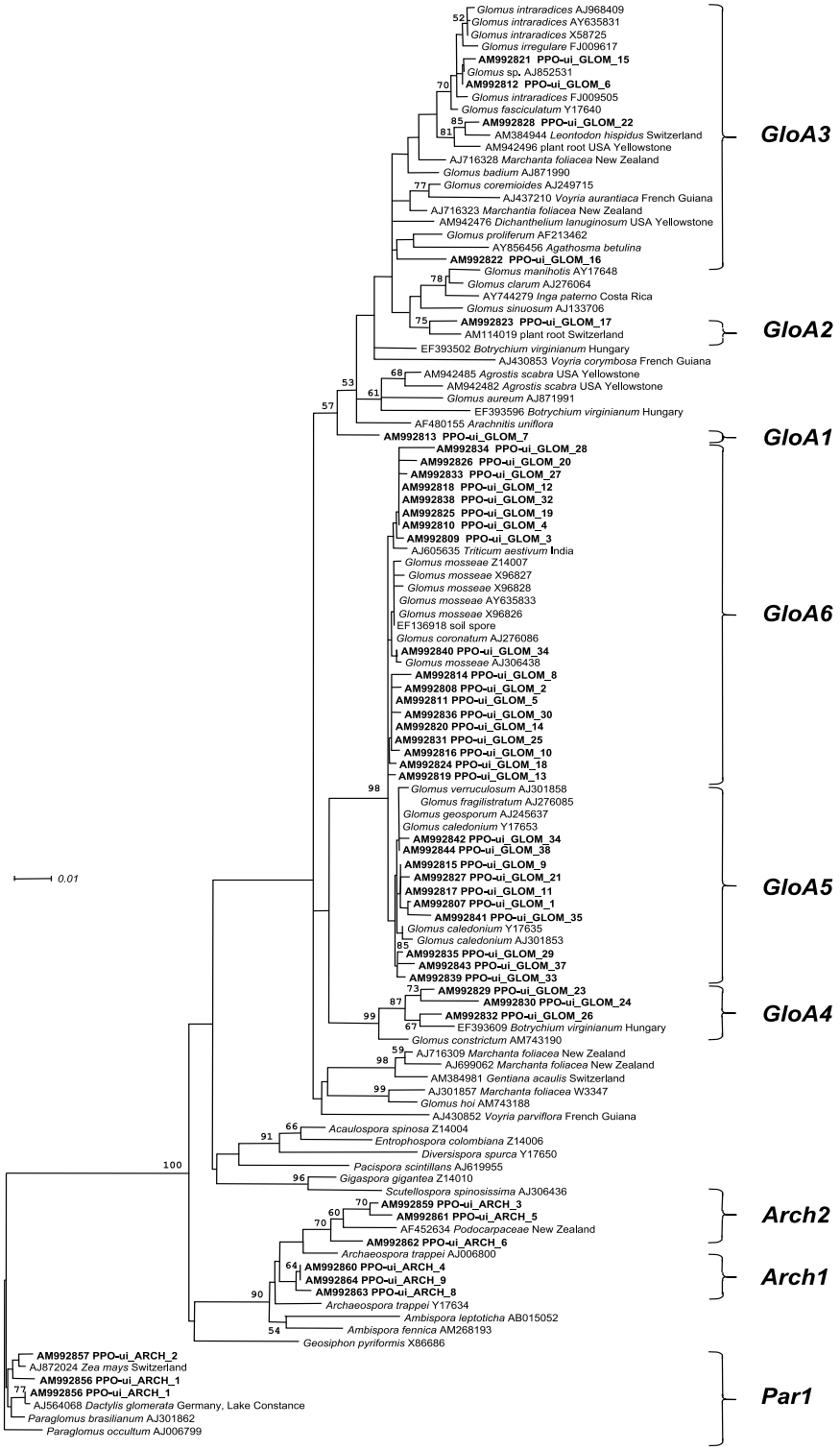

Glomus intraradices AJ968409
Glomus intraradices AY635831
Glomus intraradices X58725
Glomus irregulare FJ009617
AM992821 PPO-ui_GLOM_15
Glomus sp. AJ852531
AM992812 PPO-ui_GLOM_6
Glomus intraradices FJ009505
Glomus fasciculatum Y17640
AM992828 PPO-ui_GLOM_22
AM384944 Leontodon hispidus Switzerland
AM942496 plant root USA Yellowstone
AJ716328 Marchanta foliacea New Zealand
Glomus badium AJ871990
Glomus coremioides AJ249715
AJ437210 Voyria aurantiaca French Guiana
AJ716323 Marchantia foliacea New Zealand
AM942476 Dichanthelium lanuginosum USA Yellowstone
Glomus proliferum AF213462
AY856456 Agathosma betulina
AM992822 PPO-ui_GLOM_16
Glomus manihotis AY17648
Glomus clarum AJ276064
AY744279 Inga paterno Costa Rica
Glomus sinuosum AJ133706
AM992823 PPO-ui_GLOM_17
AM114019 plant root Switzerland
EF393502 Botrychium virginianum Hungary
AJ430853 Voyria corymbosa French Guiana
AM942485 Agrostis scabra USA Yellowstone
AM942482 Agrostis scabra USA Yellowstone
Glomus aureum AJ871991
EF393596 Botrychium virginianum Hungary
AF480155 Arachnitis uniflora
AM992813 PPO-ui_GLOM_7
AM992834 PPO-ui_GLOM_28
AM992826 PPO-ui_GLOM_20
AM992833 PPO-ui_GLOM_27
AM992818 PPO-ui_GLOM_12
AM992838 PPO-ui_GLOM_32
AM992825 PPO-ui_GLOM_19
AM992810 PPO-ui_GLOM_4
AM992809 PPO-ui_GLOM_3
AJ605635 Triticum aestivum India
Glomus mosseae Z14007
Glomus mosseae X96827
Glomus mosseae X96828
Glomus mosseae AY635833
Glomus mosseae X96826
EF136918 soil spore
Glomus coronatum AJ276086
AM992840 PPO-ui_GLOM_34
Glomus mosseae AJ306438
AM992814 PPO-ui_GLOM_8
AM992808 PPO-ui_GLOM_2
AM992811 PPO-ui_GLOM_5
AM992836 PPO-ui_GLOM_30
AM992820 PPO-ui_GLOM_14
AM992831 PPO-ui_GLOM_25
AM992816 PPO-ui_GLOM_10
AM992824 PPO-ui_GLOM_18
AM992819 PPO-ui_GLOM_13
Glomus verruculosum AJ301858
Glomus fragilistratum AJ276085
Glomus geosporum AJ245637
Glomus caledonium Y17653
AM992842 PPO-ui_GLOM_34
AM992844 PPO-ui_GLOM_38
AM992815 PPO-ui_GLOM_9
AM992827 PPO-ui_GLOM_21
AM992817 PPO-ui_GLOM_11
AM992807 PPO-ui_GLOM_1
AM992841 PPO-ui_GLOM_35
Glomus caledonium Y17635
Glomus caledonium AJ301853
AM992835 PPO-ui_GLOM_29
AM992843 PPO-ui_GLOM_37
AM992839 PPO-ui_GLOM_33
AM992829 PPO-ui_GLOM_23
AM992830 PPO-ui_GLOM_24
AM992832 PPO-ui_GLOM_26
EF393609 Botrychium virginianum Hungary
Glomus constrictum AM743190
AJ716309 Marchanta foliacea New Zealand
AJ699062 Marchanta foliacea New Zealand
AM384981 Gentiana acaulis Switzerland
AJ301857 Marchanta foliacea W3347
Glomus hoi AM743188
AJ430852 Voyria parviflora French Guiana
Acaulospora spinosa Z14004
Entrophospora colombiana Z14006
Diversispora spurca Y17650
Pacispora scintillans AJ619955
Gigaspora gigantea Z14010
Scutellospora spinosissima AJ306436
AM992859 PPO-ui_ARCH_3
AM992861 PPO-ui_ARCH_5
AF452634 Podocarpaceae New Zealand
AM992862 PPO-ui_ARCH_6
Archaeospora trappei AJ006800
AM992860 PPO-ui_ARCH_4
AM992864 PPO-ui_ARCH_9
AM992863 PPO-ui_ARCH_8
Archaeospora trappei Y17634
Ambispora leptoticha AB015052
Ambispora fennica AM268193
Geosiphon pyriformis X86686
AM992857 PPO-ui_ARCH_2
AJ872024 Zea mays Switzerland
AM992856 PPO-ui_ARCH_1
AM992856 PPO-ui_ARCH_1
AJ564068 Dactylis glomerata Germany, Lake Constance
Paraglomus brasilianum AJ301862
Paraglomus occultum AJ006799
0.01
GloA3
GloA2
GloA1
GloA6
GloA5
GloA4
Arch2
Arch1
Par1

Table 16. Presence (1) or absence (0) of AMF phylotypes in each onion field expressed per surveyed year, cultivation system and region in The Netherlands. The data for each field is arranged in a column.

Phylotypes	Survey 2004				Survey 2005			
	Zeeland		Flevoand		Flevoland			
	Organic	Conv.	Organic	Conv.	Organic		Conventional	
GloA1 (*Glomus*-A)	00000	00000	00001	00000	00000	00000	00000	0000
GloA2 (*Glomus*-A)	00100	00000	00000	00000	00000	00000	00000	0000
GloA3 (*G. intraradices*)	01000	10000	10001	00000	00000	00000	00000	0000
GloA4 (*Glomus*-A)	00000	00000	00101	00000	00000	00000	00000	0000
GloA5 (*G. cal.-geosp.)*	11111	11111	00001	00110	11111	11111	11111	1111
GloA6 (*G. moss-coron.)*	11111	11111	01111	11111	11111	11111	11111	1111
GloB1 (*G. walkeri*)	00000	00000	00000	00000	00000	00000	00010	0100
GloB2 (*Glomus*-B)	00010	00000	00000	00000	00000	00000	00000	0000
GloB3 (*Glomus*-B)	00000	10000	00000	00000	00000	00000	00000	0000
GloB4 (*Glomus*-B)	00000	00000	00000	00000	00000	00000	00000	0100
GloB5 (*G. etunicatum*)	00000	00000	10000	00000	00010	00000	00000	0000
Par1 (*Paraglomus* sp.)	00000	00000	00100	00000	10100	01100	00000	0000
Arch1 (*Archaeosp.* sp.)	00000	00000	00000	00000	00010	00000	11001	0100
Arch2 (*Archaeosp.* sp.)	00000	00000	00001	00000	00001	00000	00000	0000
Nr. of phylotypes per field	23332	42222	21316	11221	32343	23322	33233	2522

the same way, the Shannon-Weaver index calculated on the basis of the relative abundance of phylotypes per field (Table 17) did not significantly differ for the management systems studied, and also not for the four management systems – region combinations (REML analysis).

The number of years a field was under organic management was neither correlated with the number of phylotypes per field, nor with the Shannon-Weaver index. Onion yields in organic or conventional farming were also not correlated significantly with AMF diversity indices (data not shown).

The contribution of phylotypes and sampled fields to the total variation in diversity was analyzed via Correspondence Analysis (CA) (data matrix shown in Table 16). Figure 16 presents the biplot for the first and second CA axes, which explained 31.4% of the total variance (first axis: 16.8%, second axis 14.6%, eigenvalues 0.54 and 0.47 respectively; total inertia 3.24). Phylotypes *Glo*A6 (*G. mosseae* ─ *coronatum* complex) and *Glo*A5 (*G. caledonium* ─ *geosporum* complex) took a central position in the CA space, as these phylotypes did not contribute much to the variation between sampled sites. Other less frequent phylotypes, which took more extreme positions in the CA space, contributed more to the variation between fields. The larger proportion of sampled sites, 13 conventional and 8 organic ones, took a central position in the chart, close to the most abundant phylotypes (*Glo*A5 and *Glo*A6), and irrespective of cultivation system and sampled region (Figure 16). As a result, only a few other organic and conventional sites had larger AMF diversity, differing in community composition among them (Figure 16).

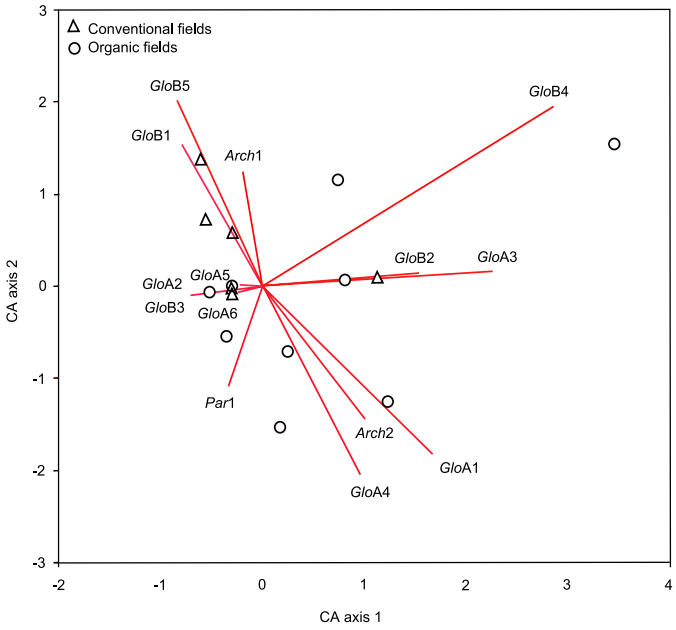


Figure 16. Biplot for the first and second axis in the Correspondence Analysis for the number of AMF phylotypes in conventional onion fields (open triangles) and organic fields (open circles) in the Netherlands in 2004 and 2005. Some field positions overlap (same AMF species composition). AMF phylotypes are presented as vectors.

Table 17. Means and their standard errors (± S.E.) of the number of phylotypes per field, and the Shannon-Weaver indices based on the relative abundance of phylotypes per field, for the cultivation systems and regions in 2004 and 2005.

Diversity Indices	Cultivation system	Survey 2004		Survey 2005
		Zeeland	Flevoland	Flevoland
Nr of phylotypes	Conventional	2.4 ± 0.9	1.4 ± 0.5	2.8 ± 1.0
	Organic	2.6 ± 0.5	2.6 ± 2.1	2.7 ± 0.7
Shannon index	Conventional	0.70 ± 0.31	0.25 ± 0.35	0.89 ± 0.30
	Organic	0.66 ± 0.22	0.66 ± 0.69	0.88 ± 0.20

Discussion

Molecular Diversity

Only a few studies have addressed the question if and to what extent AMF species richness and composition differ between organic and conventional farming systems (Oehl et al. 2003, 2004, Hijri et al. 2006). To contribute to this field of knowledge, the present research was carried out involving a larger number of sites compared to previous studies, and different environmental conditions like a temperate sea climate.

AMF diversity was estimated via the number of different phylotypes per field, as no reliable molecular method has been developed to determine all different AMF morphospecies yet. This estimator can be seen as a lower limit for diversity estimation, as each phylotype may contain different AMF morphospecies. Phylotypes were defined as monophyletic groups in a conservative way. Since the taxonomic position of rDNA sequences from root samples is unknown, and rDNA sequence variation within a given morphospecies or even within a single spore exists (Jansa et al. 2002), AMF diversity may be overestimated if smaller monophyletic groups are assumed as phylotypes. Furthermore, we used the Shannon-Weaver index calculated on the basis of the number of phylotypes per field.

A total of fourteen AMF phylotypes were detected in onion roots, with one to six phylotypes per field. The total number of phylotypes and number of phylotypes per field were well in line with those obtained by Hijri et al. (2006) who studied AMF diversity in arable soils using the same primer set for AMF identification and a similar sampling effort. The most abundant phylotypes *Glo*A6

(associated with *G. mosseae — coronatum* species complex) and *Glo*A5 (*G. caledonium — geosporum* species complex) could be respectively phylotypes GLOM-A3 and GLOM-A4 in Hijri et al. (2006) and Appoloni et al. (2008). Phylotype *Glo*A3 was associated with *G. intraradices* and corresponds with GLOM-A1 in Hijri et al. (2006) and Sýkorová et al. (2007). Phylotype GloA2 comprised the sequence AJ872051, which is representative of GLOM-A2 (Hijri et al. 2006).

Phylotype GloA4 clustered with a sequence from naturally occurring *Botrychium virginianum* L. (Kovacs et al. 2007) and a *Glomus constrictum* sequence (Hijri et al. 2006). Phylotype Arch2 corresponds with phylotype ARCH-1 defined by Hijri et al. (2006) and ARCH-4 of Sýkorová et al. (2007), as these share the same *Archaeospora trappei* sequence and a sequence from a species of *Podocarpaceae*. Our *Paraglomus* sequences clustered in phylotype *Par*1 that corresponds to phylotype PARA-1 in Appoloni et al. (2008), as both clustered with the same sequence from *Zea mays*, except for our sequence PPO-ui_ARCH_7 which showed similarity to AJ564068 from *Dactylis glomerata* (Wirsel 2004).

Predominance of some *Glomus*-A phylotypes in AMF communities was in agreement with previous reports for agricultural lands. For example, analyzing SSU rDNA fragments, Helgason et al. (1998) found predominance of *G. mosseae* or closely related species, whereas Daniell et al. (2001) found predominantly *G. caledonium — G. geosporum* sequences. The dominance of *Glomus*-A species was also reported in studies based on the morphology of spores, *G. mosseae* being the most observed AMF species (Oehl et al. 2003, Cheng and Baumgartner 2004, Sjöberg et al. 2004, Wang et al. 2008). In other studies, however, *G. intraradices* was the dominant AMF species (Hijri et al. 2006, Mathimaran et al. 2005). The dominance of few *Glomus*-A species is probably a consequence of the strong selection pressure imposed by agricultural practices leading to the predominance of fast root-colonizing species (Oehl et al. 2004) and species able to tolerate, among others, the repeated disruption of external hyphal networks, periods with without mycorrhizal host plants, and the application of fertilizers and fungicides (Gosling et al. 2006).

In contrast, other AMF phylotypes were found at very low frequencies, which make it difficult to establish associations between farming systems with specific phylotypes or community composition. Whilst some *Glomus*-B, *Paraglomus* and *Archaeospora* phylotypes were occasionally found in some Dutch farm fields, neither *Acaulospora* nor *Scutellospora* rDNA sequences were detected at all. In previous studies, the presence of *Glomus*-B, *Archaeospora, Acaulospora,*

Scutellospora and *Paraglomus* species in arable lands was low in comparison to grasslands, or they were absent (Oehl et al. 2004, Hijri et al.2006).

We found that AMF diversity in organically grown onions did not differ from conventional ones, with a considerable overlap between farming systems in the canonical analysis. This result is in contrast with Oehl et al. (2003) who reported that AMF diversity in organic fields (13 – 18 AMF species) took an intermediate position between conventional fields (8 – 10) and grasslands (20 – 25). However, Oehl and co-workers analysed the morphology and number of AMF spores in soil samples and trap cultures, and therefore they analyzed a broader part of the AMF species richness than the present research. Hijri et al. (2006) used an approach similar to our study, relying on the same primer set for AMF identification. Among four management systems established on the same loess soil (the DOK experiment), higher AMF biodiversity in organically managed plots than in conventional ones was observed, though not significantly. This trend is not in agreement with our results. Regional characteristics and specificities in AMF community composition may explain this difference. Furthermore, these authors compared a few fields at one location, whereas our study had a much broader set up, as we compared in total 20 organic and 19 conventionally managed fields belonging to commercial farms on clay and loess-clay soils.

Among the studied farmlands in the Netherlands, only a few had larger AMF diversity indices. This is in line with Hijri et al (2006) who found, after studying two fields apart from the DOK experiment, that AMF diversity varied also within management systems depending upon the specific agricultural history. It was noteworthy, in the present study, that AMF diversity was larger in fields belonging to two farms sampled both years, in which onions were grown each year on different pieces of land within the farm. These observations suggest that farming system as such did not influence AMF diversity, but rather specific management practices or environmental conditions may contribute to the maintenance of more diverse AMF community in some farmlands. For instance, the continuous cultivation of mycorrhizal host crops in the rotations, or the use of green cover crops instead of fallow periods might favour the presence of more AMF species in the long term (Gosling et al. 2006, Hijri et al. 2006).

Onion yields were not correlated with AMF diversity indices. Nevertheless, the practical role of AMF diversity in agricultural systems should be further studied. First indications coming up from experimental setups in which mixed AMF species were applied as inocula point to the complexity arising from multiple fungi-host interactions. For instance, Van der Heijden et al. (2006) reported variable benefit in biomass increase from mixed inocula, whereas Jansa

et al. (2008) reported only improved phosphorus acquisition in comparison to the same AMF species acting separately.

Colonization in onion roots

Onion roots in Dutch agricultural fields had high AMF colonization levels in comparison to levels reported for naturally occurring AMF in other crops and environments (Ryan et al. 2000, Mäder et al. 2000). Although obtained at a single sampling date within a single growing season, our results revealed the abundance of AMF in agricultural soils in the Netherlands, including reclaimed lands.

As conventional farming in Zeeland had significantly lower AMF colonization levels compared to the other three management systems — region combinations (Table 13), the adoption of organic farming seemed to increase AMF colonization levels quickly. Indeed, the organic fields sampled in Zeeland were only one to twelve years taken out of conventional management. In contrast, mean colonization levels in Flevoland were high regardless of the cultivation system, and not correlated with the number of years under organic management.

Furthermore, we found that colonization parameters were neither correlated with the concentration of readily available phosphorus in the soil, even when a trend towards a higher P content for conventional fields was observed (Table 12). Several authors reported larger AMF colonization for organically managed fields compared to conventionally managed fields. Amongst others, Ryan et al. (1994, 2000) found higher colonization levels in organically grown wheat and pastures than conventionally managed ones, whereas Mäder et al. (2000, 2002) reported higher colonization in organic winter wheat, vetch-rye and grass-clover when comparing organic and conventional soil management systems. Similarly, Oehl et al. (2003) found higher colonization levels in plants grown on organic soils compared to conventional ones. In these studies, differences in colonization levels were explained mainly by the lower P concentration in organically managed soils, which was not clearly the case for the onion fields in the present research.

A striking finding of our survey was the significant correlation between colonization parameters of naturally occurring AMF and onion yields in conventionally managed onion farmlands, under moderate to high concentrations of readily available phosphorus (Table 12, Figure 12). Organically managed fields followed the same trend as conventional ones, but the correlation coefficient was considerably lower and not significant for AC. The slope of both linear regression lines was 0.38 indicating that onions benefitted similarly from AMF in both management systems. Alternatively, it could be speculated that AMF colonization and onion yields benefit simultaneously from yet unidentified environmental

conditions. Anyhow, in order to practically exploit the fact that 10% increase in AMF colonization may represent an average yield increase of 3.8 tons·ha^{-1}, it would be necessary to better understand farm management practices or environmental factors leading to higher vitality of indigenous AMF.

Concluding remarks

This study did not demonstrate differences in biodiversity of AMF colonizing onion roots grown under organic and conventional cultivation, and this result raises at least two important questions. Firstly, is the lack of differences in AMF community composition between cultivation systems due to the fact that nutrient levels (especially phosphorus) are moderate to high in organic fields? If this is the case, it is uncertain that a further continuation under organic cultivation (with a concomitant decrease in soil nutrient levels) would increase mycorrhizal fungal diversity over a longer temporal scale.

Secondly, the positive correlation between mycorrhizal colonization and onion yield established for conventional fields suggests a mycorrhizal benefit even at high nutrient availability. It is therefore tempting to speculate that onion, with its depauperate root system, depends on mycorrhiza even at high nutrient levels, thereby preventing the selection for reduced functioning of the symbiosis under agricultural intensification postulated by Johnson (1991) and Kiers et al. (2002). Breeding onions with improved rooting system while retaining a diversity of mycorrhizal benefits would be an urgent further research step. Otherwise, onion cultivation under nutrient-poor conditions might result in yields that are too low to be economically attractive. In this context the research of De Melo (2003) on the genetic basis of traits describing the *Allium* rooting system can be seen as a promising first step.

Chapter 5

Genetic analysis of the interaction between *Allium* species and arbuscular mycorrhizal fungi [†]

Abstract

The response of three species of *Allium* (*A. cepa*, *A. roylei*, *A. fistulosum*), the hybrid *A. fistulosum x A. roylei*, and a large number of genotypes of the tri-hybrid *A. cepa x (A. roylei x A. fistulosum)* to the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* was studied in order to analyze the genetic basis for this trait in the tri-hybrid population. The experiment was executed in 2006 and 2007.

Plant response to and benefit from the mycorrhizal symbiosis was expressed as Mycorrhizal Responsiveness (MR), Mycorrhizal Benefit (MB), and Mycorrhizal Breeding Value (MV).

Results in both years were significantly correlated. Plant biomass in the non-mycorrhizal condition was significantly correlated with that of mycorrhizal plants. MR was significantly negatively correlated with biomass of non-mycorrhizal plants and was hence unsuitable as a breeding criterion. MB and MV were significantly correlated with plant biomass of mycorrhizal plants, whereas MB and MV showed lower (and partly not significant) correlations with biomass of non-mycorrhizal plants.

QTLs were located on a linkage map of the *A. roylei x A. fistulosum* parental genotype. Both QTLs from *A. roylei* and *A. fistulosum* contributed to mycorrhizal response. Two QTLs associated with *A. roylei* alleles were detected on chromosomes 2 and 3 for MB, MV, and plant weight of AM plants. A QTL associated with *A. fistulosum* alleles was detected on linkage group 9 for MV (but not MB), plant weight of AM and NM plants, and the number of stem-borne roots. Coincident QTLs regions for plant weight, MB and MV indicate that selection for plant weight may also select for enhanced MB and MV. Moreover, it suggests that modern onion breeding did not select against the response to AMF. Positive correlation between number of roots and large response to mycorrhiza for *Allium* species opens prospects to combine these traits in the development of more robust onion cultivars.

[†] This chapter will be submitted as

Galván GA, Kuyper TW, Burger K, Keizer LCP, Hoekstra RF, Kik C, Scholten OE. Genetic analysis of the interaction between *Allium* species and arbuscular mycorrhizal fungi

Introduction

In order to obtain high yields of onion (*Allium cepa* L.), large amounts of fertilizers are used in high-input cropping systems (Bosch-Serra and Currah 2002). These fertilizers are costly, and as the crop takes up only a minor part of these inputs (Greenwood et al. 1982), negative impacts on the environment can be expected. In organic and low-input cropping systems a balance is sought between yield and goals that minimize impacts on the environment (Rossing et al. 2007). As a consequence synthetic fertilizers are applied at lower rates in low-input farming systems than in conventional ones, or are even completely excluded as in organic systems. Thus, crop yield and economic results may be lower. A Dutch survey showed that onion yields in organic cultivation were 54% lower than those in conventional cultivation systems (Chapter 4). Both different practices (e.g. planting distance) and differences in soil nutrient supply cause this yield gap. In the study by Galván et al. (Chapter 4), the average phosphorus concentration (P_w) in organic soils was 27% lower than in conventional soils. Onion has a sparse and shallow rooting system, consisting mainly of stem-borne roots that rarely branch and lack root hairs (Portas 1973, Greenwood et al. 1982). Because P diffuses very slowly through the soil solution, plants with poor rooting systems cannot maintain an adequate P uptake and therefore yields are hampered (Mengel and Kirkby 2001).

Various plant breeding strategies can contribute to maintain high yields in organic and low-input agricultural systems by improving P uptake and P use. One strategy is the development of cultivars with improved rooting systems (Lynch 2007). De Melo (2003) proposed to use *Allium fistulosum* L. as a donor species because it has a larger and denser rooting system than onion. Although progeny plants of *A. cepa x A. fistulosum* are partially sterile, gene transfer from *A. fistulosum* to *A. cepa* is possible by the use of *A. roylei* as a bridge species (Khrustaleva and Kik 1998, 2000). De Melo (2003) investigated the inheritance of root traits in an *A. cepa x* (*A. roylei x A. fistulosum)* population. QTLs for traits of the rooting system were found. His results indicate that breeding for an improved rooting system in onion is possible (De Melo 2003).

A second breeding strategy may be the selection for enhanced response to the symbiosis with arbuscular mycorrhizal fungi (AMF). These fungi naturally occur in soils. In this symbiosis, the fungus gets carbohydrates from the plant, whereas the plant improves its nutrient acquisition, particularly P, among other

benefits (Stribley 1990). Improved nutrient uptake depends on the build up of a mycelial network beyond the depletion zone (George 1995, Mengel and Kirkby 2001). As a result, plant biomass of mycorrhizal plants is larger than that of non-mycorrhizal plants (reviewed by Lekberg and Koide 2005).

The response to mycorrhiza is dependent on P availability in the soil. Under high P-levels, smaller benefits from mycorrhiza were observed than in P-deficient soils (Hayman and Mosse 1971). As a consequence, contributions from the AMF symbiosis are particularly expected in organic and low-input agricultural systems (Ryan and Graham 2002, Gosling et al. 2006).

Differences in the response to mycorrhiza have been observed in various crops, for example, between wheat cultivars (Hetrick et al. 1993), maize inbred lines (Kaeppler et al. 2000), and onion cultivars (Powell et al. 1982). Intraspecific differences in response to mycorrhiza indicate a genetic basis for the plant – AMF interaction. Therefore, breeding for an enhanced response to mycorrhiza was proposed by several authors (Parke and Kaeppler 2000, Plenchette et al. 2005, Gosling et al. 2006, Sawers et al. 2008). These authors hypothesized that cultivars that benefit more from this symbiosis would contribute to a more sustainable agriculture as amounts of fertilizers could be reduced (Ryan and Graham 2002). Genomic regions quantitatively linked to the response to AMF were identified for wheat (Hetrick et al. 1995) and maize (Kaeppler et al. 2000). Up to now, no practical application of that knowledge has been reported.

The existence of various plant strategies to enhance P-uptake raises the question to what extent they are additive or have to be traded off. For rice Gao et al. (2007) reported that the ability of non-mycorrhizal rice to acquire nutrients was negatively correlated with the mycorrhizal responsiveness. For bean Lynch (2007) showed that the effect of root hairs (as part of an improved rooting system) and of AMF was additive. Because a high response to AMF was also described for *A. fistulosum* (Tawaraya et al. 2001), the question arises whether this species could also be used to improve the response to mycorrhiza in onion.

The aim of the present study was to investigate the genetic basis that underlies the interaction between relatives of onion and AMF, more specifically the response of these plants to AMF. Quantitative trait loci (QTLs) involved in that interaction were mapped on an AFLP linkage map. Other traits such as plant biomass, rooting system and bulbing ability were also linked to this AFLP linkage map.

Materials and Methods

Plant material

Allium cepa (Rijnsburger group), *A. roylei* Stearn (PRI-1270), and *A. fistulosum* (PRI-2002-232) were tested for the response to mycorrhiza. The hybrid *A. fistulosum* x *A. roylei* (RF-hybrid) and the tri-hybrid population *A. cepa x (A. roylei x A. fistulosum)* were developed as described by Khrustaleva and Kik (1998). Each tri-hybrid genotype has a set of chromosomes from *A. cepa*, and a set of chromosomes from *A. roylei,* or *A. fistulosum,* or recombinants between them.

An offspring of 96 tri-hybrid genotypes was used to develop a linkage map of the RF-hybrid. The parental species, the RF-hybrid, and each genotype of the tri-hybrid were clonally multiplied *in vitro* in order to have replicates for the experiments. Sections of the basal plates were used as initial explants, as described by De Melo (2003), and clonally multiplied by successive divisions of the basal plates.

Evaluation of the response to AMF

The evaluation of the parental species, the RF-hybrid, and the tri-hybrid population was executed in 2006 and 2007. The tri-hybrid population comprised 77 genotypes in 2006 and 83 in 2007, of which 68 genotypes were evaluated in both years. Plantlets grown *in vitro* for 3-4 weeks with 2-3 leaves and well-developed roots were transferred to trays and covered with a transparent lid that was further opened daily. The trays contained a potting mixture (steamed peat-soil and sand, 2:1 ratio). After four weeks, plants were transferred to individual pots 2.1 (in 2006) and 3.3 (in 2007) litres in size. The pots contained a mixture of gamma-irradiated clay soil, sand and perlite (6:1:1 ratio). The clay soil was collected from an organic farm land.

Glomus intraradices Schenck and Smith inoculum was kindly provided by Dr. Y. Kapulnik (Volcani Center, Israel), as spores in dry vermiculite (Alkan et al. 2003). A full spoon of inoculum (ca. 1.7 g) was added to each pot in a hole made for transplanting. The same inoculum previously autoclaved (60 minutes, two times) was added in a similar way to the pots belonging to the non-mycorrhizal control. A soil wash was added to both the AM and NM treatment.

Six replicates (individual plants) of each species, the RF-hybrid and tri-hybrid genotypes were inoculated with *G. intraradices* and six replicates were kept as non-mycorrhizal control. Genotypes were randomized within six blocks of AM and NM treatment each, and placed on separate tables. Experiments took place in a compartment with controlled temperature and relative humidity set at

22-18°C (day-night). Nitrogen as diluted $Ca(NO_3)_2$ was applied seven weeks after transplanting, at a rate of 160 mg $N \cdot kg^{-1}$ soil. Plants were harvested 14 weeks after transplanting. Total above-ground fresh and dry weights were evaluated, as well as partitioning in leaf weight and bulb/false stem (lower 5 cm) weight. The number of leaves, false stems and plant height (length from basal plate to the longest green leaf tip) were recorded. The number of stem-borne roots was also recorded. Assessing below-ground biomass was not possible, because detachments at harvest prevented accurate measurements.

Indices for the benefit from mycorrhiza

Response to AMF was assessed by calculating mycorrhizal responsiveness as proposed by Baon et al. (1993): $MR = (AM - NM)/NM$, the weight difference between mycorrhizal and non-mycorrhizal plants divided by the weight of non-mycorrhizal plants. Because of the problematic nature of that dimensionless parameter (see Results and Discussion) two further indices for plant benefit from mycorrhiza were used: (1) Mycorrhizal Benefit (MB), the difference in total weight between AM and NM treatments: $MB = AM - NM$ This parameter indicates the biomass increment due to the presence of AMF at a given P level. (2) Mycorrhizal Breeding Value (MV), the average plant weight of AM and NM treatments: $MV = (AM + NM)/2$. This index takes into account the response to mycorrhiza and the ability of a genotype to grow in the absence of AMF (see Figure 17 as an illustrative example). MB and MV were calculated for plant total dry weight and for other plant variables.

To evaluate mycorrhiza and genotype effects Residual Maximum Likelihood (REML) analysis was used in Genstat 9.2 (Lawes Agricultural Trust, Rothamsted Exp. St., UK, 2006). Significant differences were established with Fischer's-protected LSD. Residuals were checked for normality. All variables except plant height were square-root transformed because of their large residuals. The relationships between indices for the response to mycorrhiza and plant traits were studied by Pearson correlation analysis. As tri-hybrid genotypes segregated for bulbing ability (see Figure 22), the relationship with this trait was also studied. In order to do that, genotypes were classified in four categories regarding the average bulbing index ($BI = BulbDiameter/NeckDiameter$), as follows: null (BI = 1.0 to 1.6), low (1.6–2.4), medium (2.4–2.7), and high (>2.7) degree of bulbing. MB, MV and plant biomass for genotypes belonging to these bulbing classes were compared by REML analysis. Significant differences between species (incl. the RF-hybrid) and differences between bulbing categories of the tri-hybrid were established with Fischer's-protected LSD. Residuals were checked for normality.

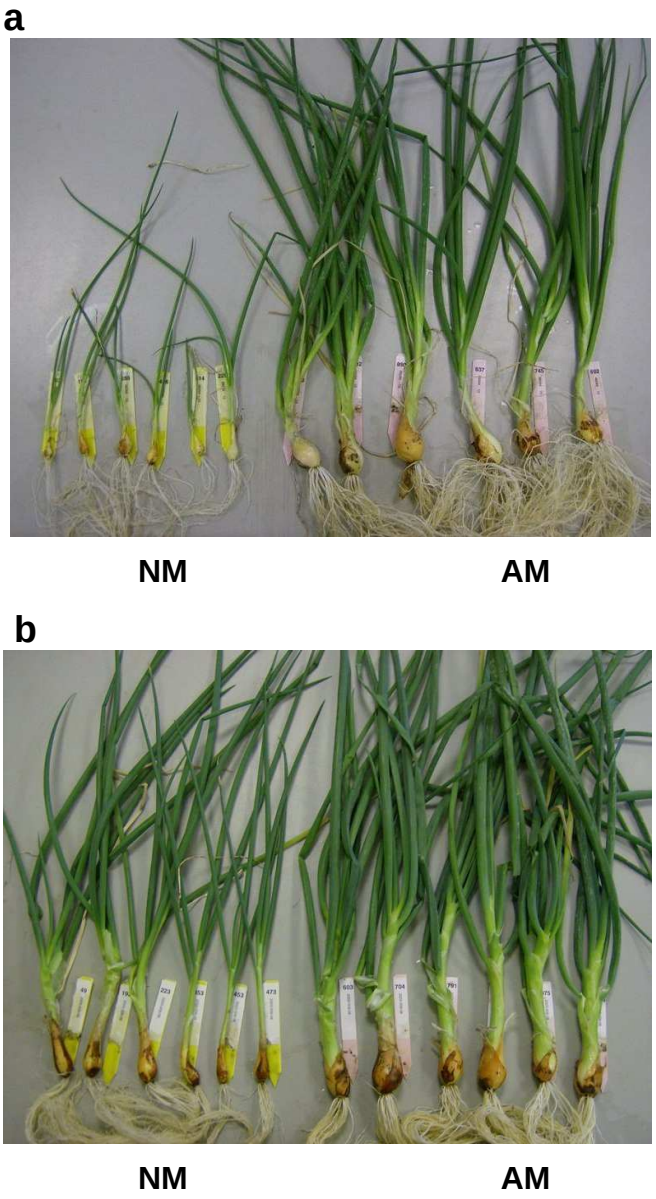


Figure 17. Two examples of tri-hybrid genotypes with contrasting response to the inoculation with *G. intraradices*, in the non-inoculated (NM) and inoculated (AM) treatments after harvesting experiment 2007. **(a)** Genotype unable to grow in a P-deficient soil without mycorrhiza, having large benefit (MB) from the symbiosis but moderate mycorrhizal breeding value (MV). **(b)** Genotype less dependent on AMF, having smaller benefit (MB) from the symbiosis but larger breeding value (MV).

Early colonization in selected genotypes

In addition, early colonization of a number of tri-hybrid genotypes was evaluated. Eight (from 77) tri-hybrid genotypes with a range of mycorrhizal responses were selected after the experiment in 2006. Parental species and the RF-hybrid were also included. Plant material was propagated, transplanted to individual pots and inoculated with *G. intraradices* as described before. Six replicates per genotype were randomly arranged in three blocks in a greenhouse compartment. Three replicates were evaluated three weeks after inoculation, and the remaining three replicates, seven weeks after inoculation. Root colonization was estimated for each individual plant independently, applying the magnified intersection method, after staining with trypan blue. For each sample, 100 observation points were evaluated (McGonigle et al. 1990). Differences in colonization between genotypes were compared after REML analysis. Possible relationship between mycorrhizal benefit (MB) in 2006 and fractional colonization was calculated.

AFLP linkage analysis and QTL mapping

DNA of each tri-hybrid genotype, the parental lines and the RF-hybrid was isolated from young leaves, following the miniprep protocol described by Van Heusden et al. (2000). AFLP® (Keygene B.V., The Netherlands) reactions were carried out according to Vos et al. (1995). Two pairs of restriction enzymes were used, namely *Eco*RI/*Mse*I and *Pst*I/*Mse*I. Because of the large genome size in *Allium* species, pre-amplifications were done with three selective nucleotides (+1, +2) and the selective amplifications with seven nucleotides (+3, +4) for the *Eco*RI/*Mse*I enzyme pairs, and six (+3, +3) for the PstI/MseI pairs (Van Heusden et al. 2000). A total of 22 primer pairs were used in the selective amplifications (Table 18). AFLP fragments originating exclusively from *A. roylei* or *A. fistulosum* were scored using Quantar (Keygene B.V., The Netherlands). Markers were named as described by van Heusden et al. (2000). For instance, E38M52G-202F refers to restriction enzymes *Eco*I and *Mse*I, primers E38 and M52, 'G' identifies the additional 7th base, '202' is the estimated length of the fragment, and 'F' or 'R' means that the marker is specific for *A. fistulosum* or *A. roylei* respectively.

A linkage map for the RF-hybrid was calculated using JoinMap 3.0 (Van Ooijen and Voorrips 2001). Population-type was set to haploid, and linkage groups were separated with a threshold LOD $\geq$ 4. Kosambi's mapping function was used to calculate map positions of the markers. Linkage groups were assigned

Table 18. Amplified fragment length polymorphism (AFLP) adapters and primers used in the ligation and amplification steps, and their sequences.

Adapters	EcoRI adapters	5'-CTCGTAGACTGCGTACC-3'
		3'- CTGACGCATGGTTAA-5'
	MseI adapters	5'-GACGATGAGTCCTGAG-3'
		3'-TACTCAGGACTCAT-5'
	PstI adapters	5'-CTCGTAGACTGCGTACATGCA-3'
		3'-TGTACGCAGTCTAC-5'
Universal primers	E00	5'- GACTGCGTACCAATTC-3'
	M00	5'- GATGAGTCCTGAGTAA-3'
	P00	5'- GACTGCGTACATGCAG-3'
Primer pairs in the pre-amplification		
	E01 (E00+A) - M02 (M00+C)	
	P01 (P00+A) - M01 (M00+A)	
	P01 (P00+A) - M02 (M00+C)	
Primer pairs in the selective amplification		
	E35 (E00+ACA)	M52A (M00+CCCA)
		M52C (M00+CCCC)
		M52T (M00+CCCT)
	E36 (E00+ACC)	M52C (M00+CCCC)
	E37 (E00+ACG)	M52A (M00+CCCA)
		M52C (M00+CCCC)
		M52G (M00+CCCG)
	E38 (E00+ACT)	M52G (M00+CCCG)
		M52T (M00+CCCT)
	P31 (P00+AAA)	M33 (M00+AAG)
		M35 (M00+ACA)
	P35 (P00+ACA)	M32 (M00+AAC)
		M33 (M00+AAG)
		M34 (M00+AAT)
		M35 (M00+ACA)
		M36 (M00+ACC)
		M47 (M00+CAA)
		M50 (M00+CAT)
	P38 (P00+ACT)	M47 (M00+CAA)
		M48 (M00+CAC)
	P43 (P00+ATA)	M36 (M00+ACC)
		M51 (M00+CCA)

to chromosomes on the basis of AFLP markers in common with previous maps (Van Heusden et al. 2000, de Melo 2003). Marker order was fixed for Chromosomes 5 and 8 according to Khrustaleva et al. (2005).

Quantitative trait loci (QTLs) analysis was performed using MapQTL® 4.0 (Van Ooijen et al. 2002). Kruskal-Wallis test was applied to determine the association between each individual marker in the map and the target traits. QTLs were identified by the multiple QTL mapping (MQM) procedure (Jansen 1993, Jansen and Stam 1994), and were regarded significant at LOD threshold value with $p<0.05$. This threshold value was estimated for each trait on the basis of population type and 1000 times genome-wide permutations. Linkage maps and QTL figures were drawn in MapChart (Voorrips 2002).

Results

Response to mycorrhiza in *Allium*

Non-mycorrhizal plants (NM) remained free of mycorrhiza. In 2006 and 2007, total dry weight of AM plants was larger than that of NM plants for the parental species and the RF-hybrid (REML analysis, $p<0.05$). *Allium roylei* had lowest biomass, whereas the differences between *A. cepa*, *A. fistulosum* and the RF-hybrid were not significant (Table 19). For most tri-hybrid genotypes the weight of AM plants was also significantly larger than that of NM plants. Five genotypes (out of 77) in 2006 and 3 genotypes (out of 83) in 2007 did not have significantly more biomass in the mycorrhizal condition. Maximum biomass of the tri-hybrid (both when AM and NM) was larger than that of the best performing parent, while minimum biomass was lower than that of *A. roylei*, the parent with lowest biomass (transgressive segregation) (Table 19).

Differences between AM and NM treatments were larger in 2007 than in 2006 (Figure 18). Mean total dry weight of AM plants was in 2006 2.5 times and in 2007 4 times higher than that of NM plants (Table 19). Performance of mycorrhizal plants in 2006 and 2007 was significantly correlated ($r = 0.76$, $n = 68$; $p<0.001$). Performance of non-mycorrhizal plants in both years was also significantly correlated ($r = 0.38$, $n = 68$, $p < 0.01$) (Figure 19).

Mycorrhizal responsiveness of tri-hybrid genotypes was negatively correlated with biomass of NM plants (Figure 20). Because this negative relationship implies that selection for higher responsiveness results in selection for smaller plants in the non-mycorrhizal condition, we used two further parameters to express the benefit that plants obtain from the symbiosis, MB and MV.

Correlations between biomass in the AM and NM condition, MB and MV for 2006 and 2007 are shown in Figures 19 and 20. Growth of NM and AM plants

was positively correlated in both years. MB and MV were very significantly correlated with biomass of AM plants, while the correlations between MB and MV with biomass of NM plants were lower and not always significant. MB and MV were also significantly correlated in both years (dta not shown).

Allium cepa had the lowest number of stem-borne roots, and *A. fistulosum* the highest (Table 19). The RF-hybrid had more roots than *A. fistulosum* in 2006, but less in 2007. Among tri-hybrid genotypes, the average number of stem-borne roots showed transgressive segregation (Table 19). The number of roots in the AM treatment was positively correlated with total dry weight (r = 0.59 and 0.69), MB (r = 0.45 and 0.65), and MV (r = 0.62 and 0.64) (values respectively for experiments 2006 and 2007, $p<0.001$ in all cases).

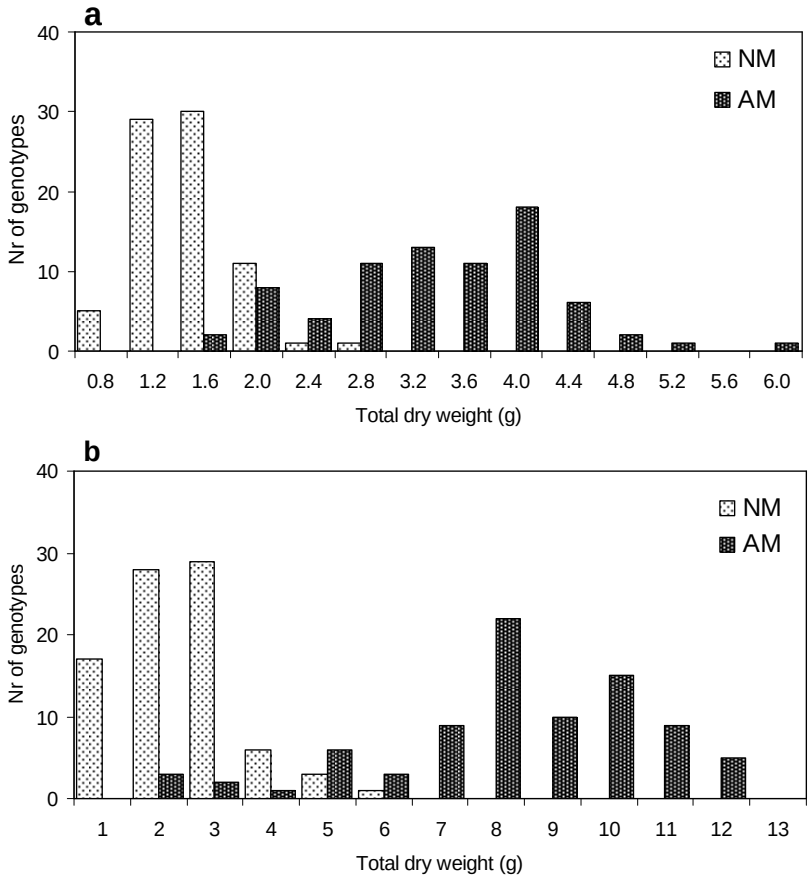


Figure 18. Frequency distributions of total plant dry weight of the tri-hybrid genotypes in the non-inoculated (NM) and inoculated with *G. intraradices* (AM) treatments, for **(a)** experiment 2006, and **(b)** experiment 2007. Note that different scales were used for total dry weight.

Table 19. Total dry weight, number of stem-borne roots per plant of non-mycorrhizal (NM) and mycorrhizal plants (AM), and indices for the response to mycorrhiza of the parental material and the tri-hybrid genotypes in Experiments 2006 and 2007.

Plant Material	Total dry weight (g)				Nr of stem-borne roots per plant			
	NM [a]	AM [a]	MB [b]	MV [c]	NM [a]	AM [a]	MB [b]	MV [c]
Experiment 2006								
Allium cepa	1.24 bc	2.79 a	1.55	2.01	8.6 f	11.1 ef	2.5	9.9
Allium fistulosum	0.70 c	1.95 ab	1.25	1.33	19.9 cd	31.4 b	11.5	25.7
Allium roylei	0.54 d	1.58 b	1.04	1.06	11.3 ef	16.3 de	5.0	13.8
RF-hybrid	1.20 bc	2.88 a	1.68	2.04	27.2 bc	43.7 a	16.5	35.5
Tri-hybrid genotypes (n = 77)								
• Mean	1.29	3.17	1.88	2.23	15.3	25.4	10.2	20.3
• Minimum	0.45	1.30	0.23	1.19	6.3	7.5	1.2	6.9
• Maximum	2.58	5.67	3.89	3.73	33.3	35.7	2.5	34.5
Experiment 2007								
Allium cepa	2.00 c	6.16 a	4.15	4.09	3.7 e	8.2 de	4.5	5.9
Allium fistulosum	1.49 c	6.22 a	4.73	3.85	30.1 bc	74.7 a	44.6	52.4
Allium roylei	0.68 d	3.45 b	2.77	2.06	27.0 bc	19.2 cd	7.2	15.5
RF-hybrid	1.53 c	5.63 a	4.10	3.58	11.9 de	43.5 b	16.6	35.3
Tri-hybrid genotypes (n = 83)								
• Mean	1.97	7.71	5.81	4.88	21.1	38.9	17.8	30.0
• Minimum	0.37	1.18	0.68	1.01	3.0	2.4	- 0.6	2.7
• Maximum	5.34	11.44	8.89	7.83	39.6	66.3	26.7	53.0

[a] Within each experiment, treatment–genotype combinations followed by the same letter do not differ ($p<0.05$). REML analysis followed by Fischer-protected LSD-test, for square root transformed data. [b] Mycorrhizal Benefit, as MB = (AM – NM). [c] Mycorrhizal Breeding Value, as MV = (AM + NM)/2.

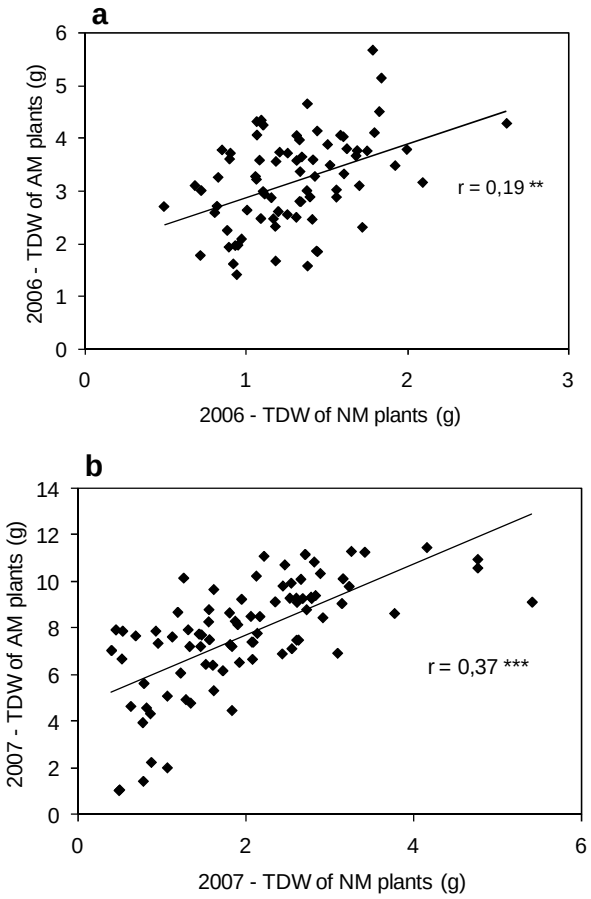


Figure 19. Genetic variation in the tri-hybrid *Allium* population. Relationship between total dry weight (TDW) of non-mycorrhizal (NM) and mycorrhizal (AM) plants in experiments **(a)** 2006 and **(b)** 2007. Pearson correlations (** $p<0.01$; *** $p<0.001$).

Plant height, number of leaves and leaf dry weight were lowest in *A. cepa*, but bulb dry weight was highest for *A. cepa*. MB and MV showed the same pattern (Figure 21). Tri-hybrid genotypes were classified in four bulbing classes (Figure 22). There were no significant differences in bulb / false-stem weight between the four classes, but genotypes with high degree of bulbing had significantly lower AM plant weight, leaf biomass and MB than the other bulbing classes (Table 20). In 2006, no differences between bulbing levels were found for MV, whereas in 2007, MV was also lower for genotypes with high degree of bulbing. Within the class of genotypes with high bulbing, two genotypes in 2006 (17%) and five genotypes in 2007 (33%) presented early bulbing and leaf senescence before harvesting.

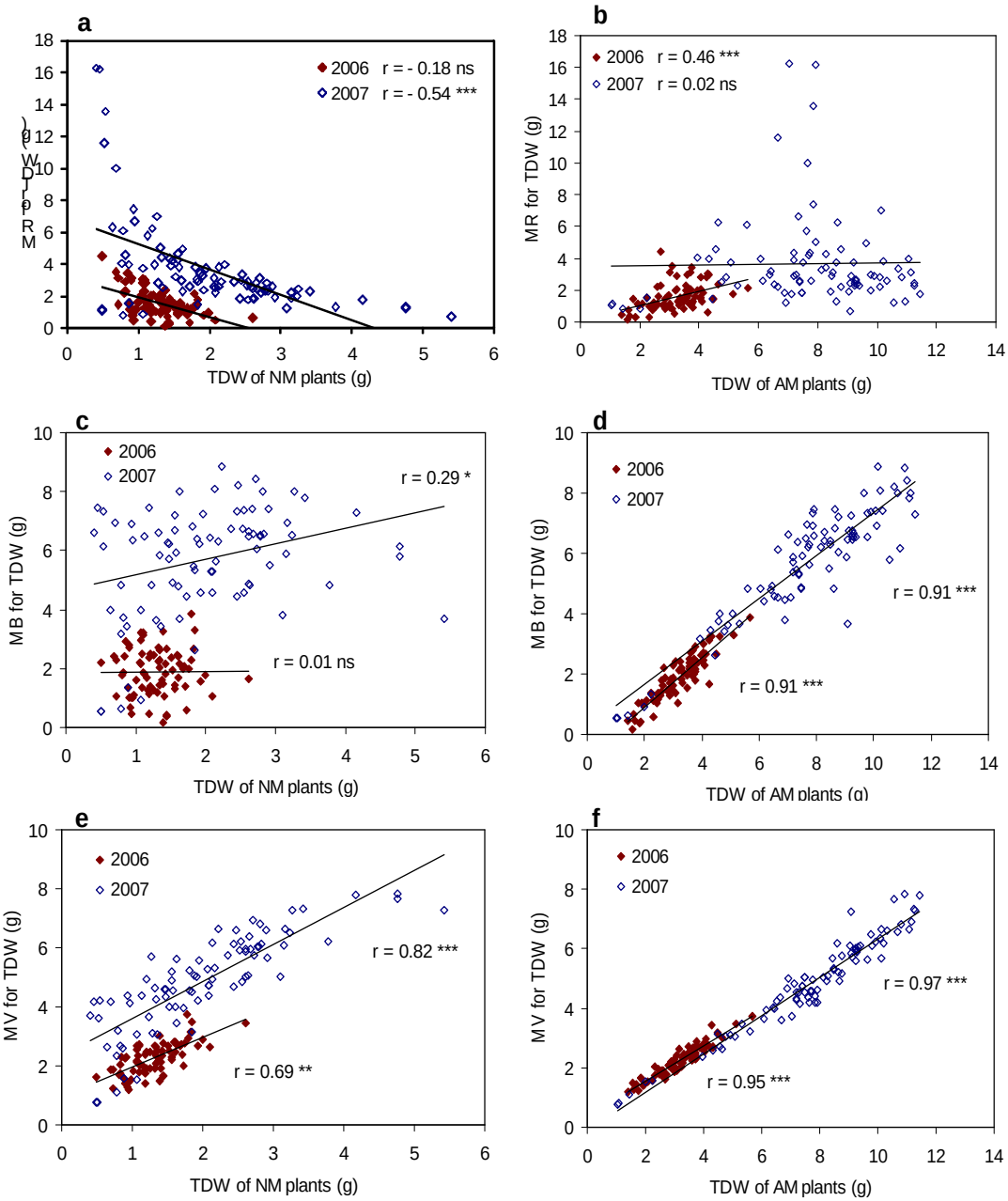


Figure 20. Genetic variation in the tri-hybrid *Allium* population. **(a)** Mycorrhizal responsiveness (MR) as a function of total dry weight (TDW) of non-mycorrhizal (NM) plants in experiments 2006 and 2007; **(b)** Idem, for mycorrhizal (AM) plants; **(c)** Mycorrhizal benefit (MB) as a function of TDW of NM plants in 2006 and 2007; **(d)** idem for AM plants; **(e)** mycorrhizal breeding value (MV) as a function of TDW of NM plants in 2006 and 2007; **(f)** idem for AM plants. Pearson correlations (ns: p>0.05; * p<0.05; ** p<0.01; *** p<0.001).

Early colonization in selected genotypes

Hyphal colonization and arbuscular colonization were significantly correlated (data not shown) and therefore only data on arbuscular colonization are presented. Tri-hybrid genotypes differed in fractional colonization, with values ranging from 33 to 73% after three weeks and seven weeks (REML analysis, $p<0.05$). Tri-hybrid genotypes and species that showed larger MB (for total dry weight and bulb dry weight) in 2006 showed higher levels of colonization after seven weeks (Figure 23) but not after three weeks (data not shown).

AFLP mapping and QTL analysis

A total of 359 polymorphic AFLP markers originating from either *A. roylei* or *A. fistulosum* were analysed. Among these, 143 markers were mapped on 15 linkage groups (LOD $\geq$ 4). Eight main groups (LOD 4 to 5.4) were assigned to chromosomes on the basis of markers mapped earlier (Van Heusden et al. 2000, De Melo 2003). The resulting linkage map for the RF-hybrid contained 111 markers in eight linkage groups covering 886 cM. Marker positions on the chromosomes were calculated without forcing, and an overall mean marker Chi-Square contribution of 2.24 was obtained. Mean linkage group size was 110.7 cM, and mean marker interval was 8.1 cM. The map had four gaps larger than 18 cM in chromosomes 3, 5, and distal regions of chromosomes 1 and 7.

The remaining additional linkage groups (linkage groups 9 to 15) could not be associated to any chromosome because they only consisted of AFLP markers not reported before. These linkage groups were included in the QTL analysis. QTLs for 'traits' that indicate response to or benefit from mycorrhiza were detected on chromosomes 2, 3, and the linkage group 9 (Table 21, Figure 24). A first QTL was located on the central part of chromosome 2 and associated with *A. roylei* alleles. This QTL was significant for MB in 2006 and 2007, and MV in 2006. The estimated position for MB differed between experiments. This chromosomal region coincided with QTLs for total dry weight, leaf dry weight and plant height of AM plants in 2006. A second QTL located on a distal part of chromosome 2, also associated with *A. roylei* alleles, was significant for MB, MV and AM plant weight but only in 2007.

A third QTL was detected on a distal part of chromosome 3, associated with a specific *A. roylei* marker (Table 21, Figure 24). This QTL was significant for MB in 2007 and MV in 2006. Its location coincided with that of QTLs for total dry weight of AM and NM plants in 2006, and AM plant weight in 2007. In addition, this region coincided with that of QTLs for plant height and leaf dry weight in both years (Table 21). QTLs detected for MB explained 29.3% and 50.1% of the total variation in 2006 and 2007 respectively (Table 21).

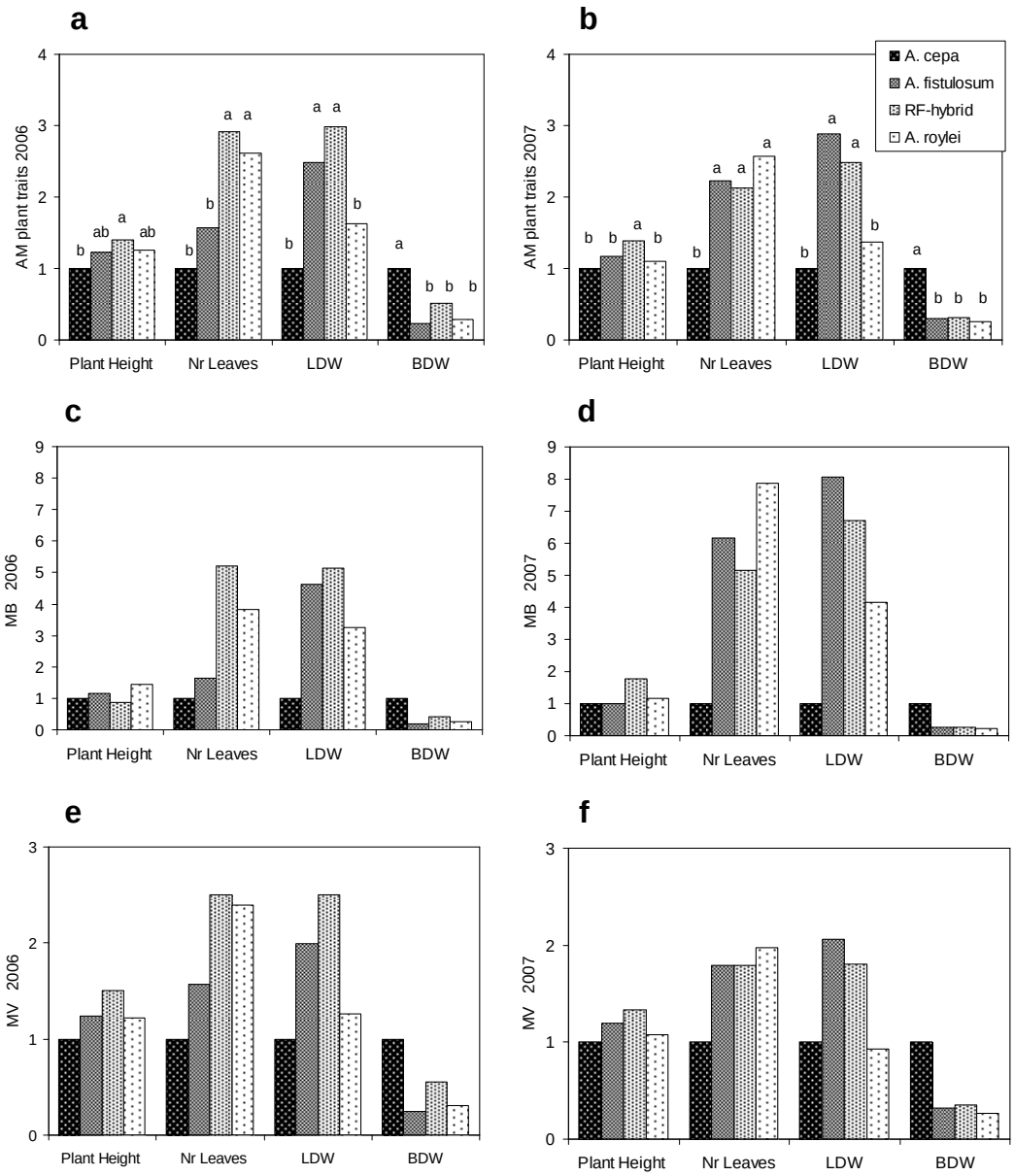


Figure 21. (a, b) Plant height, number of leaves, dry weight of the leaves (LDW), and dry weight of the bulb / false-stem (BDW) for *A. cepa*, *A. fistulosum*, *A. roylei* and the RF-hybrid inoculated with *G. intraradices.* Measurements at harvest in experiment 2006 and 2007. Columns with the same letter on top within each experiment and trait do not differ (REML analysis, $p<0.05$). **(c, d)** Mycorrhizal Benefit. **(e, f)** Mycorrhizal breeding Value. All scales are relatives to *A. cepa* (= 1).

Table 20. Plant traits and indices for the response to AMF of tri-hybrid genotypes classified with regard to the level of bulbing.

Traits	Levels of Bulbing				Wald statistic
	Poor	Low	Medium	High	F probability
Experiment 2006					
Nr of observations	18	22	18	12	
AM total dry weight (g)	3.52 a	3.43 a	3.08 ab	2.72 b	0.040
AM leaf dry weight (g)	1.82 a	1.58 ab	1.38 b	0.87 c	<0.001
AM bulb dry weight (g)	1.65	1.80	1.76	1.85	NS 0.483
Nr of Leaves	7.3	7.6	7.2	6.1	NS 0.208
Nr of roots	24.4	27.2	25.6	24.7	NS 0.498
MB (g)	2.37 a	2.0 ab	1.7 bc	1.4 c	0.003
MV (g)	2.33	2.41	2.24	2.01	NS 0.192
Experiment 2007					
Nr of observations	22	26	19	15	
AM total dry weight (g)	7.93 a	8.64 a	8.04 a	5.34 b	<0.001
AM leaf dry weight (g)	5.38 a	5.37 ab	4.78 b	2.62 c	<0.001
AM bulb dry weight (g)	2.55 b	3.25 a	3.28 a	2.78 b	0.025
Nr of Leaves	10.6 a	9.9 a	10.0 a	6.5 b	<0.001
Nr of roots	38.5	41.8	40.4	31.1	NS 0.064
MB (g)	6.00 a	6.12 a	6.18 a	3.86 b	<0.001
MV (g)	4.93 a	5.58 a	4.95 a	3.41 b	<0.001

Means followed by the same letter for each trait (each row) do not differ ($p < 0.05$). REML analysis followed by Fischer-protected LSD-test, for square root transformed data.

A QTL for MV (but not for MB) was located on linkage group 9, and associated with *A. fistulosum* alleles (Table 21, Figure 24). This region also harboured QTLs for total dry weight of AM and NM plants in both years, as well as for bulb dry weight of AM plants in both years. A QTL for the number of stem-borne roots per plant was detected also on linkage group 9, which accounted for 19.1 and 17.9 % of the total variation for this trait in 2006 and 2007 respectively.

Figure 22. Examples of tri-hybrid genotypes classified according to the degree of bulbing (see the text for explanation).

QTLs for MV on chromosomes 2, 3 and the linkage group 9 accounted for 56% of the total variation in experiment 2006 and 49% in 2007. These three regions accounted also for 40% and 39% of the variation among genotypes in total dry weight of AM plants in 2006 and 2007 respectively. Other QTLs detected for MB and MV were only significant for one of the years, and contributed to a lesser extent in explaining total variation (Table 21).

Discussion

Genetic analysis of the response to mycorrhiza in *Allium*

This research identified regions linked to quantitative 'traits' involved in response to or benefit from mycorrhiza in an *A. cepa x (A. roylei x A. fistulosum)* population. We put trait between inverted commas, because in a strict sense properties like MR, MB or MV are not plant traits. Three QTLs were identified for MB or MV located on the central part of chromosome 2, a distal part of chromosome 3 and linkage group 9. These QTLs were identified both in experiments 2006 and 2007.

These three chromosomal regions also harboured QTLs for total dry weight of AM plants. Considering the highly significant correlations between biomass of AM plants, MB and MV (Table 19; Figure 20) this result is not surprising. Even more so, the large effect of AMF on plant biomass (AM plants had 2.5 times higher biomass than NM plants in 2006 and 4 times higher in 2007) makes this correlation rather like autocorrelation. Linkage group 9 also coincided with a

QTL for total dry weight of NM plants. This result is consistent with the correlation between growth of AM and NM plants. Apparently, plants that have the ability to take up more P in a P-deficient soil, retain that benefit in the mycorrhizal condition. Large NM values were obtained when *A. fistulosum* alleles are present in this region, a result that corresponds with the finding that this species performed better than *A. roylei* in the NM treatment (Tables 19 and 21).

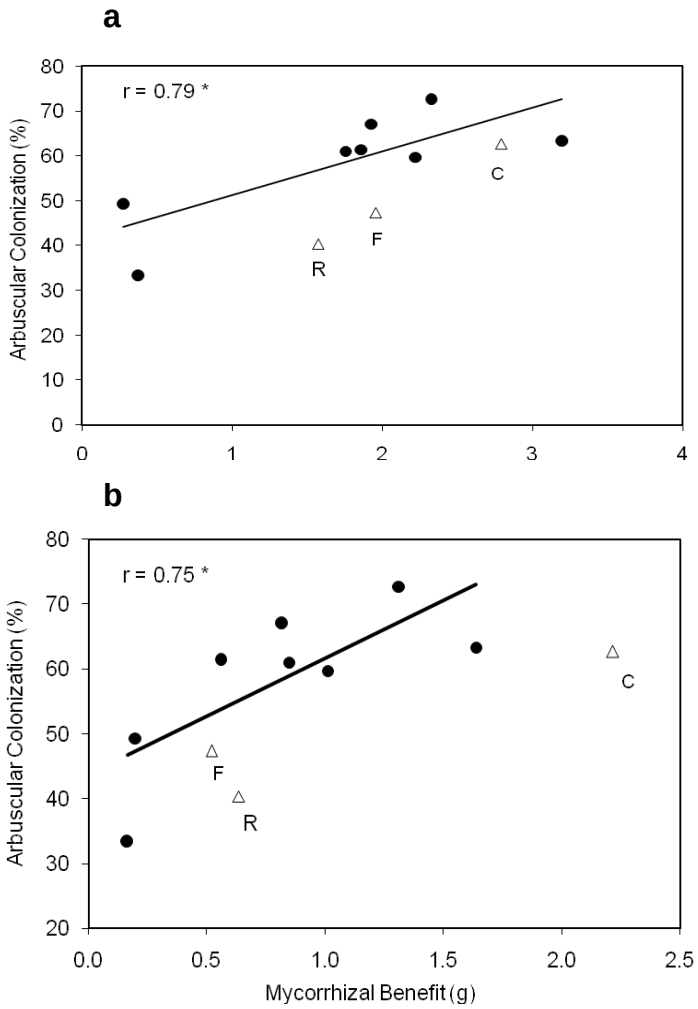


Figure 23. Arbuscular colonization (%) seven weeks after inoculation, as a function of Mycorrhizal Benefit in experiment 2006 (MB) calculated for **(a)** total dry weight (TDW) and **(b)** bulb/false-stem dry weights (BDW), for a set of eight tri-hybrid genotypes and the parental species. C: *Allium cepa*, F: *A. fistulosum*; R: *A. roylei* (Pearson correlations, n=8, * $p<0.05$, ** $p<0.01$).

Table 21. QTLs for the indices for the response to mycorrhiza and plant traits in experiments 2006 and 2007.

Traits [a]	Peak marker	Linkage Group	LOD [b]	Mean of the locus from		Expl. Var. (%)
				roylei	*fistulosum*	
Indices for the benefit from mycorrhiza (based on total plant dry weight)						
MB 2006	P35M47-278F	Chrom-2	3.84 **	2.31	1.64	15.8
	E37M52A-301F	Chrom-6	4.08 **	1.54	2.24	13.5
MB 2007	P31M35-247R	Chrom-2	3.70 *	6.47	4.95	18.6
	P31M35-328R	Chrom-2	3.86 **	6.34	4.92	16.3
	P38M48-187R	Chrom-3	3.60 *	6.32	4.94	15.2
MV 2006	P35M47-278F	Chrom-2	3.59 *	2.49	2.02	19.6
	P38M48-187R	Chrom-3	3.13 *	2.40	1.98	15.7
	E36M52C-86R	Chrom-4	3.00 *	2.38	2.01	12.9
	P35M34-133F	Group-9	4.53 **	2.00	2.48	21.0
MV 2007	P31M35-328R	Chrom-2	4.00 **	5.21	4.04	13.2
	P31M33-401F	Group-9	4.98 **	4.01	5.66	24.7
Total plant dry weight [c]						
AM 2006	P35M47-278F	Chrom-2	3.44 *	3.59	2.85	18.8
	E38M52T-156F	Chrom-6	3.00 *	2.85	3.50	14.5
	P35M32-59R	Group-9	3.52 *	2.83	3.51	15.3
	P38M48-187R	Chrom-3	3.57 *	3.36	2.75	0.40
AM 2007	P31M35-328R	Chrom-2	4.89 **	8.68	6.70	17.6
	P38M48-187R	Chrom-3	3.57 *	8.52	6.85	12.6
	P31M33-401F	Group-9	4.29 **	6.72	8.91	21.8
NM 2006	P38M48-187R	Chrom-3	3.64 *	1.37	1.07	15.3
	P38M48-62R	Group-9	3.69 *	1.07	1.37	16.0
NM 2007	E35M52T-394R	Group-9	4.50 **	1.31	2.33	23.5

[a] MB: mycorrhizal benefit, as (AM–NM); MV: mycorrhizal breeding value, as (AM+NM)/2. AM: total dry weight in the treatment inoculated with *G. intraradices;* NM: total dry weight in the non-inoculated treatment.

[b] Calculated using multiple QTL mapping (MQM) in MapQTL 5.0. Significance: (**) $p < 0.01$, (*) $p < 0.05$, (ns) no significant.

(cont.)

Table 21 (*cont.*)

Traits [a]	Peak marker	Linkage Group	LOD [b]	Mean of the locus from		Exp. Var. (%)
				roylei	*fistulosum*	
Other plant traits of AM plants						
Height 2006	P35M32-228R	Chrom-2	5.47 **	63.5	51.2	28.7
	P38M48-187R	Chrom-3	3.49 *	60.7	68.6	19.3
Height 2007	P38M48-187R	Chrom-3	5.85 **	81.2	1.21	22.4
LDW 2006 [C]	P35M47-278F	Chrom-2	4.83 **	1.80	1.11	20.8
	P38M48-187R	Chrom-3	3.25 **	1.68	1.82	15.8
	P31M33-167R	Chrom-6	3.04 *	1.24	4.01	12.4
LDW 2007 [C]	P38M48-187R	Chrom-3	2.86 *	5.40	5.27	12.8
	P31M35-306F	Chrom-6	3.91 **	4.14	1.37	18..8
BDW 2006 [C]	P38M48-62R	Group-9	5.70 **	1.23	1.81	28.0
BDW 2007 [C]	P31M33-401F	Group-9	5.02 **	1.56	27.5	24.6
Nr Roots 2006	P38M48-118R	Group-9	3.79 **	22.1	45.6	19.1
Nr Roots 2007	P31M33-401F	Group-9	3.74 **	34.8	45.6	17.9

[a] AM: total dry weight in the treatment inoculated with *G. intraradices;* NM: total dry weight in the non-inoculated treatment.

[b] Calculated using multiple QTL mapping (MQM) in MapQTL 5.0. Significance: (**) $p < 0.01$, (*) $p < 0.05$, (ns) no significant.

QTLs for both MB and MV were found on chromosomes 2 and 3, but some QTLs were only recovered in one of the two years that the experiment took place. Despite the highly significant correlations between growth of AM plants in both years, and a somewhat lower but still significant correlation between both years for NM plants, experimental variation resulted in such differences between experiments in detected QTLs. Such variation should be reason for a cautionary note regarding the genetic basis for such traits.

A counterintuitive result is that these QTLs can be ascribed to *A. roylei* alleles, because this species had the lowest weight of AM and NM plants and thus the lowest values for MB and MV. A positive contribution by the *A. roylei* background (in combination with *A. cepa* and *A. fistulosum* genes) may be the result of non-additive effects. Transgressive segregation observed for tri-hybrid genotypes, with values for MB, MV, and total dry weight of AM plants exceeding both the minimum and maximum of the parental values is consistent with such a hypothesis (Table 19).

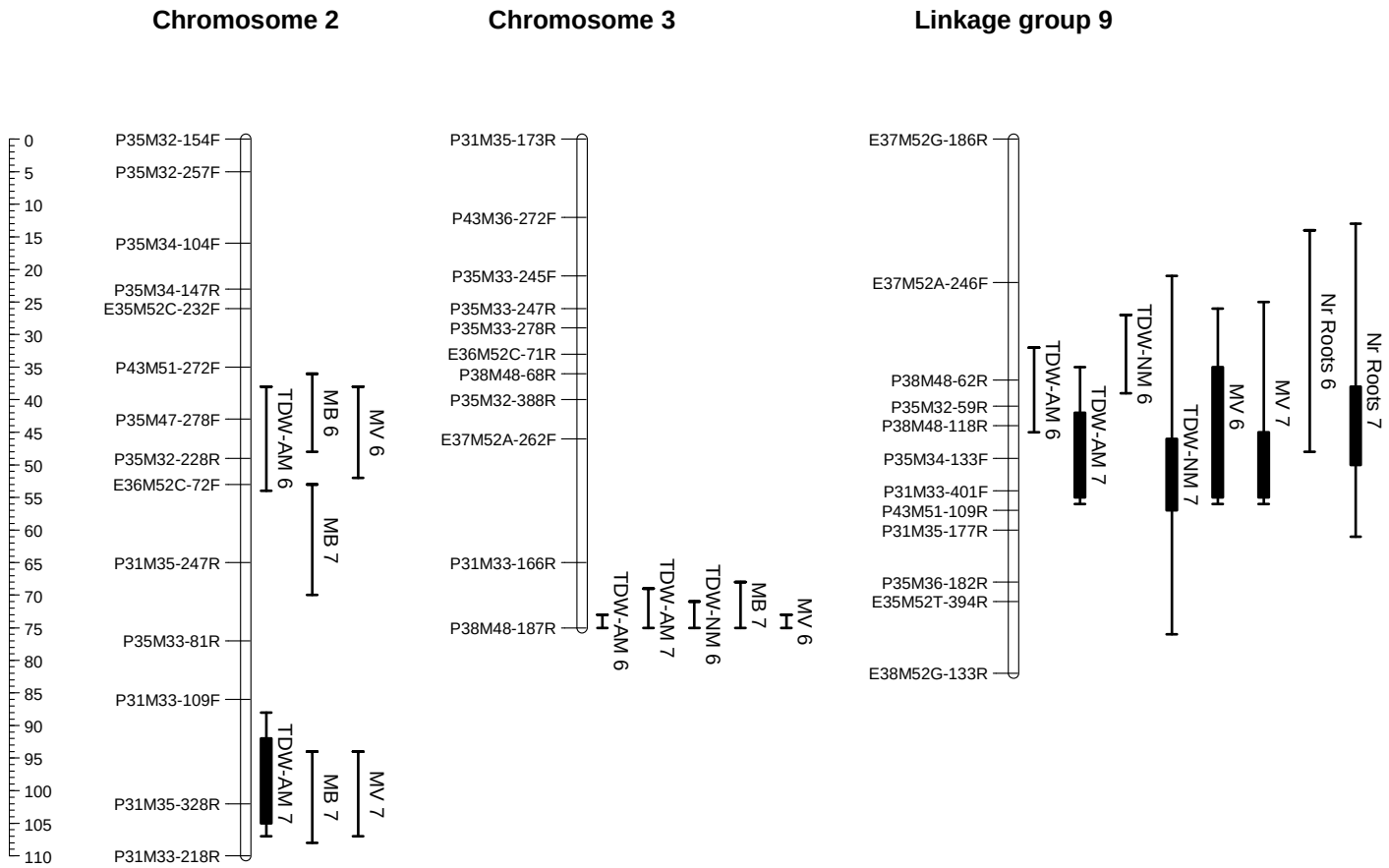


Figure 24. QTLs on the linkage map of the RF-hybrid for the benefit from mycorrhiza (MB), mycorrhizal breeding value (MV), total dry weight (TDW) and the number of stem-borne roots of the plants inoculated with *Glomus intraradices* (AM) and non-mycorrhizal (NM) in experiments 2006 and 2007. Lines with dashed ends show the LOD region above threshold value ($p<0.05$) and solid bars represents 1 LOD interval from the maximum LOD score if fitted within the significant region. The ruler indicates Kosambi's distances in cM.

Transgressive segregation for the number of stem-borne roots in the tri-hybrid population was less evident, as *A. fistulosum* had high numbers of roots in both years. One QTL for the number of stem-borne roots of AM plants was found on the linkage group 9, associated with *A. fistulosum* alleles. This QTL for the number of stem-borne roots was likely the same as QTLs for MV, and total dry weight of AM and NM plants. Again, this observation is consistent with the positive correlation between these traits and number of stem-borne roots. Because *A. roylei* also formed a relatively large number of stem-borne roots (the species was much smaller than *A. cepa*, but formed more roots), the variation for this trait may be limited. Further analysis in a backcross with *A. cepa* may allow larger variation for this trait and the identification of more QTLs for the number of roots. De Melo (2003) did not find significant QTLs for this trait, a difference that may be caused by the smaller set of genotypes that he analyzed. A QTL from *A. fistulosum* for the presence of lateral roots was located on chromosome 1 (LOD 6.0), and a QTL for the length of fine roots was located on chromosome 6 (LOD 4.2) (De Melo 2003), two traits that contribute to a denser and more efficient rooting system (Lynch 2007).

The comparison of tri-hybrid genotypes in the classes with different degrees of bulbing showed that MB, MV and plant weight were lower for the group with high degree of bulbing. The lowest MB and MV values were observed for genotypes that presented early bulbing and senescence of the leaves. It would seem, therefore, that selection for bulbing would select against maximisation of plant benefit by AMF. However, several cautionary notes are in order. First, the trade off between bulbing and MB/MV was only apparent for genotypes with the highest degree of bulbing, not for genotypes with medium degree of bulbing. Second, the lower biomass of bulbing genotypes was most apparent in those genotypes that also showed early leaf senescence. Further research into the underlying mechanisms that cause early bulbing and early leaf senescence is required. Finally, there were no differences in bulb / false stem weight between the four classes – so the link between bulb shape and bulb biomass remains unclear. Considering high nutrient demand (for N and P) for investment in the production of new leaves, and much lower nutrient demand for bulbs (which essentially store carbohydrates), a differential role of AMF in enhancing leaf and bulb development needs further study. Back-crosses of the tri-hybrid with *A. cepa* are needed to further investigate whether leaf biomass is traded off against bulbing.

Fractional colonization in a subset of eight tri-hybrid genotypes were in the same range as reported earlier for several *Allium* species (Powell et al. 1982,

Tawaraya et al. 2001). Two genotypes showed low colonization rates as well as low MB values (Figure 23). Differences in colonization are consistent with genetic differences in the establishment of the symbiosis. The correlation between MB and fractional colonization after seven weeks, although based on results of independent experiments could suggest that differences in early colonisation translate into differential growth performance. Alternatively, differential growth performance (and hence C-availability) of *Allium* genotypes could determine fungal performance. Positive relationships between colonization and bulb weight were also observed by Powell et al. (1982) for onion cultivars. Results for *Allium* differ from results reported for wheat (Hetrick et al. 1993) and maize (Kaeppler et al. 2000), where no correlation between colonization and plant weight was found. Further research is needed to establish the importance of early colonization in *Allium*.

Allium–AMF interactions

Allium species and tri-hybrid *Allium* genotypes benefitted to a large extent from the inoculation with AMF. These results agree with studies by Hayman and Mosse (1971) who found that the weight of AM plants increased up to 18 times that of NM plants. Similarly, plant growth of 27 *A. fistulosum* cultivars was enhanced by AMF, and increased up to 20 times compared to the NM control in an extremely responsive cultivar (Tawaraya et al. 2001). Among cultivated species, onion and leek (*A. porrum* L.) are regarded as species highly responsive to AMF (Miller et al. 1986, Plenchette et al. 1983). Such very high responsiveness to mycorrhiza of *Allium* species implies that these plants are unable to complete their life cycle in the absence of AMF, due to insufficient P uptake and hence insufficient growth. This inability was discussed before for onion (Charron et al. 2001), leek (Sasa et al. 1987) and for *A. fistulosum* (Tawaraya et al. 2001). Our results on the tri-hybrid genotypes confirmed this conclusion, even though a small number of genotypes showed only a non-significant biomass increase in the mycorrhizal condition (Table 20, Figure 17). This inability to grow in the absence of AMF has a major drawback when investigating the genetic basis for the plant response to mycorrhiza because MB and MV are then almost completely determined by the growth of the mycorrhizal plant. Consequently, selection for MB or MV would imply selection for the largest plant, not selection for traits that AMF specifically enhance.

The response to mycorrhiza has been expressed as mycorrhizal responsiveness (MR), a dimensionless ratio indicating the weight difference between mycorrhizal and non-mycorrhizal plants as percentage of that of the non-

mycorrhizal plant (Baon et al. 1993, Janos 2007). However, use of ratios with variable denominators may give misleading conclusions due to indirect effects (Righetti et al. 2007). In practice, a larger responsiveness can result from larger AM plants (numerator) or from smaller NM plants (denominator). In this study, MR was negatively correlated with weight of non-mycorrhizal plants (Figure 19). The same negative relationship was reported for wheat (Hetrick et al. 1995), *A. fistulosum* (Tawaraya et al. 2001) and maize (Kaeppler et al. 2000). In fact, as argued by Kuyper et al. (in prep.), a negative correlation would likely occur in (almost) all circumstances. For that reason, the search for chromosomal regions or QTLs that are linked to MR in order to breed for higher MR, would result in plants that produce less biomass when NM. Thus, responsiveness is not an indication of better plant growth, and is rather a misleading index when selecting plant genotypes for high benefit from mycorrhiza.

Therefore, the use of MR was rejected and two alternative parameters, MB and MV, were introduced. The concept of MB as used here is slightly different from the term as used by Janos (2007). Janos proposed to draw response curves along a P-gradient for AM and NM plants, and then to derive the maximum weight difference. Disadvantages of that method are that determining response curves over a P-gradient for a large number of genotypes is prohibitively time-consuming and expensive; and that mycorrhizal benefit of different genotypes may occur at different P-levels, thereby hampering comparability. Because the tri-hybrid genotypes showed a large response to AMF, MB was significantly correlated with biomass of mycorrhizal plants (Figure 20). Selection for high MB then equals selection for genotypes that perform best under 'normal' (i.e., mycorrhizal) conditions. However, for plants that show a lower response to AMF, the correlation between MB and plant performance in the mycorrhizal condition is not straightforward and in some cases MB may also involve selection for small plants in the non-mycorrhizal condition (Kuyper et al. in prep.). Such genotypes are unlikely to provide any mycorrhizal beneficial effects in combination with plant material with a high-yielding background in advanced breeding generations (Sawers et al. 2008).

To avoid problems with MR and MB, a complementary index was developed: Mycorrhizal breeding Value (MV). This index could be relevant for plant breeders and growers as a high value generally indicates that genotypes are able to perform well in a range of environmental conditions (Figure 17). Selection for yield stability under conditions where mycorrhizal inoculum potential is variable (or even absent) could be achieved by selecting for high MV. To be more specific, if colonization by native AMF is limited, onion cultivars are preferred

that still produce acceptable yields under such conditions. For the tri-hybrid genotypes, MV correlated well with biomass of mycorrhizal plants, and MB and MV were also significantly correlated. However, for plants with a lower response to AMF, MV may be superior as a criterion for genotype selection.

In the case of onion breeding through introgression of genetic material from *A. roylei* and *A. fistulosum*, breeding for higher MB and MV implies a selection for plant biomass in both AM and NM conditions. Or stated differently, selection for plant weight under 'normal' conditions will automatically imply a positive selection for response to or benefit from mycorrhiza. One could therefore hypothesise that past selection for high-yielding onion genotypes, the ultimate aim of plant breeding, may have selected onions with a higher response to mycorrhiza. That hypothesis is the opposite from the hypothesis by Hetrick et al. (1993, 1995) and Zhu et al. (2001) who, on the basis of a comparison of old and modern wheat cultivars, concluded that modern plant breeding reduced the response to mycorrhiza (lower MR and MB values for modern than old cultivars). A possible explanation for these contradictory outcomes is that modern wheat breeding, by focusing on yield stability, probably improved the ability of genotypes to grow in the absence of AMF or under conditions where there is strong AMF-inoculum limitation. For onion and its relatives, which are so dependent on AMF that they cannot complete their life cycle without AMF, breeding has apparently never succeeded in enhancing the plant's intrinsic capability to acquire sufficient P. In any case, onion breeders aiming to select for high response to AMF may simply achieve this by selecting for large plant weight under 'normal' (i.e., mycorrhizal) conditions.

Concluding remarks

The latter conclusion raises a final question: is there room for a contribution from *A. fistulosum* or *A. roylei* to improve the response to mycorrhiza in *A. cepa*? Both species have a much better developed rooting system than *A. cepa*. A better rooting system may lead to a lower response to AMF in other plant families (Schultz et al. 2001), but this was not the case in *Allium.* The absence of a trade off between an improved rooting system and a decreased response to mycorrhiza implies that *A. fistulosum* (and *A. roylei*) can be used to improve the rooting system of *A. cepa* while maintaining the response to mycorrhiza. Introgression of *A. fistulosum* genes into *A. cepa* could therefore contribute to improved performance in environments where mycorrhizal inoculum is limited, due to an improved rooting system, while maintaining its response to mycorrhiza.

An interesting result was the transgressive segregation observed for tri-hybrid genotypes. It seems that the combination of three genomes expands the genetic variation for plant growth and the response to mycorrhiza. Further research is needed to address whether this variation can be exploited in onion breeding, especially regarding the translation of this potential biomass improvement into the development of a larger bulb. In this regard, it is promising that at least some genotypes with high degree of bulbing also possessed large MB, MV and total plant weight values. Therefore, either by the large response to mycorrhiza, the large rooting system, or the transgressive segregation, new opportunities arise from *Allium* introgressions towards the development of robust onion cultivars.

Chapter 6

General Discussion

Introduction

Designing cultivars capable to overcome numerous biotic and abiotic stresses has been a significant achievement of plant breeding in order to respond to the increasing demand for food. At the same time, this contribution meets societal demands for the use of less chemical inputs and minimum impacts on the environment (Lynch 2007, Fageria et al. 2008). Most of the genes used to develop improved varieties originated from the primary gene pool of crops. When genetic variation within crop species is not sufficient, wild relatives of crops may be a source of genes to introgress resistance or tolerance, as well as to broaden the adaptability of cultivated species to other environments. However, with the exception of a few crops, and especially for simple-inherited traits, those wild relatives are promising but at the same time hardly exploited resources (Hajjar and Hodgkin 2007).

Cultivated *Allium* species and particularly onion (*A. cepa*) are no exception in this regard. Breeding activities in onion have largely exploited the intraspecific genetic variation (Shigyo and Kik 2006). Kik (2002) noted that relatives of onion can also be sources to enrich its gene pool for economically important traits. Recent and ongoing developments of breeding tools, such as molecular markers and genomic *in situ* hybridization (GISH), have increased the possibilities to exploit these relatives (Kik 2002, Scholten et al. 2007). For the further exploitation of the *Allium* germplasm, collecting activities next to characterization, evaluation and documentation of its genetic resources are urgently needed (Kik 2008).

The research reported in this thesis aimed to analyse the value of *A. fistulosum* and *A. roylei* as sources of variation for resistance to Fusarium basal rot (FBR) and for response to arbuscular mycorrhizal fungi (AMF). This research fits within the broader aim of designing cultivars for agricultural systems that are less dependent on external inputs. The genetic basis for the two aforementioned traits was studied, as well as relationships with other traits. For the response to AMF, new insights were obtained, and new indices were proposed from a plant breeding perspective. The findings of this study and their implications are discussed in this chapter, and new lines of research are indicated.

Potential contributions from *A. fistulosum* and *A. roylei* to onion breeding using a gene pool approach

The mapping approach that I employed is based on the use of a so-called gene pool approach (Hermsen 1992). Firstly, hybrid populations are made between wild relatives of a cultivated crop, before a cross and subsequent backcrosses are made with the cultivated crop species as the recurrent parent. The tri-hybrid population employed in the present research originates from a cross between onion and an interspecific hybrid between *A. roylei* and *A. fistulosum* (RF-hybrid; Khrustaleva and Kik, 2000). This approach was also used because introgressions from *A. fistulosum* via backcrossing into onion are very problematic due to nuclear-cytoplasmatic imbalances (Mangum and Peffley 2005).

The mapping approach is based on genotypes in which only one chromosome of the homologues is a recombinant one (Khrustaleva et al. 2005). Strictly speaking, the obtained linkage map is based on the recombination that occurs during the formation of gametes of the RF-hybrid, its haplotypic phase. This approach was successfully applied in this study and the study of De Melo (2003) to find associations between phenotypic variation and genomic regions in the tri-hybrid population, and consequently, to identify QTLs for various traits.

Figure 25 gives an overview of the available information on the linkage map of the interspecific hybrid *A. roylei* x *A. fistulosum*. Mapping was possible by AFLP profiling and phenotyping of progeny plants of the tri-hybrid cross *A. cepa* x *(A. roylei* x *A. fistulosum)*. One QTL for FBR resistance from *A. fistulosum* was located on chromosome 8, and one QTL from *A. roylei* was located on chromosome 2 (Chapter 3). Regarding the benefit from mycorrhiza, two QTLs for mycorrhizal benefit (MB) and mycorrhizal breeding value (MV) from *A. roylei* were identified on chromosomes 2 and 3, and a QTL for MV from *A. fistulosum* was located on linkage group 9 (Chapter 5). In addition, QTLs for plant weight, bulbing ability, the number of stem-borne and lateral roots, and the relative root length of fine roots were also detected (Figure 25). The resistance gene for *Peronospora destructor* from *A. roylei* (*Pd*) is located on chromosome 3 (Van Heusden et al. 2000, Scholten et al. 2007). This gene was mapped in the progeny between onion and *A. roylei*, but it has not yet been located on the RF-hybrid map.

As a whole, resistance to FBR, *P. destructor*, *Botrytis squamosa* (De Vries et al. 1992a), enhanced MB, MV, plant weight, and large rooting system are traits present in the tri-hybrid *Allium* population thereby offerring interesting possibilities concerning the development of broadly adapted onion cultivars.

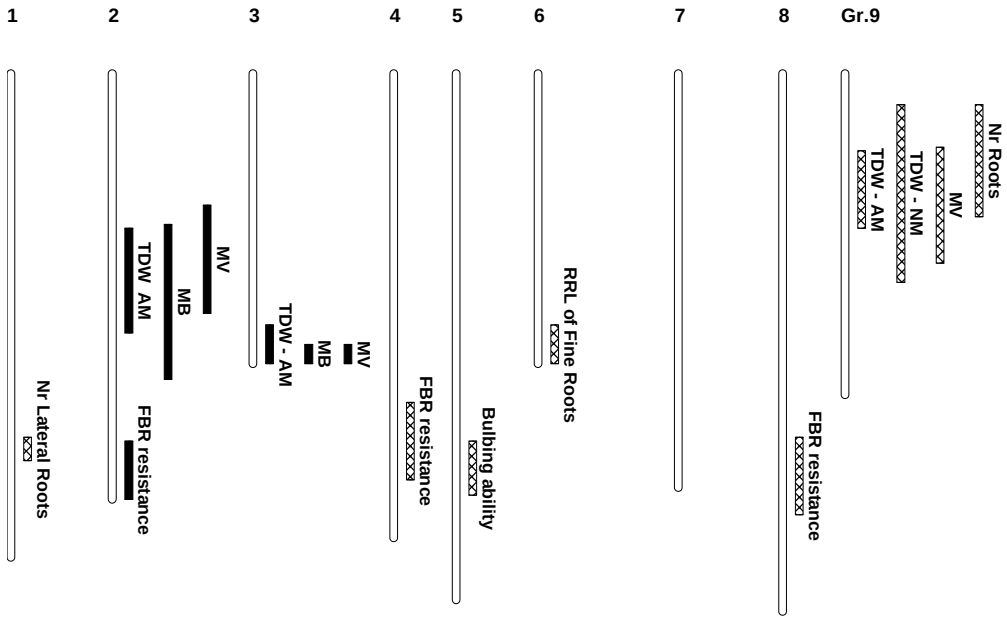


Figure 25. The position of detected QTLs from *A. roylei* (solid bars) and *A. fistulosum* (hatched bars) on the linkage map of the *A. roylei* x *A. fistulosum* parental hybrid. QTLs on chromosome 1 and 6 for rooting traits were reported by De Melo (2003).

These traits could be of special interest (but not exclusively) for low-input and organic agricultural systems.

As some traits may be controlled by alleles from either *A. fistulosum* or *A. roylei*, like FBR resistance and the number of stem-borne roots, an advantage of the tri-hybrid *Allium* population is the gene-pool approach: the possibilities for simultaneous introgression of alleles from both species (Kik 2002). As an example, additive action of QTLs for FBR from *A. fistulosum* and *A. roylei* observed in this research can be exploited in a pyramiding strategy with little effort.

A potential constraint in the use of the tri-hybrid population is the low to moderate seed setting of the cross between onion and the RF-hybrid. This is due to species incongruencies (sensu Hogenboom 1984), as the genetic make-up of the three species differs from each other. Imbalance between the *A. cepa* and *A. fistulosum* genomes leads to irregular formation of tetrads and migration of

chromatides during micro-sporogenesis. This constraint is not easily circumvented, but in practice it was shown that its negative effects are reduced in successive generations (Khrustaleva and Kik 2000). Limited pollen fertility of some tri-hybrid genotypes can be due to incongruence of the aforementioned species, and/or can result from the absence of restorer genes, as CMS-T male sterile onion plants were used as female parent. As it is known that *A. roylei* can restore CMS-T male-sterility (De Vries and Wiestsma 1992), this constraint will not be a large problem, and pollen fertile genotypes in the tri-hybrid population can be selected for.

In vitro maintenance and propagation of tri-hybrid genotypes has permitted repeated screening in this research. However, genotypes of the population differed in the ability to be multiplied *in vitro,* thus for few of them this technique did not provide the number of replicates required. This constraint was mainly observed for genotypes that resembled the *A. cepa* parent in morphology: easy bulbing, and developing few lateral stems. In addition, large morphological differences between genotypes (vigour, tillering, bulbing ability) has potential usefulness for breeding, but may interfere with some phenotypic evaluations.

The hybrid vigour (transgressive segregation) of some tri-hybrid genotypes observed in this research is a marked outcome from the gene pool approach. Yield increases by broadening the genetic basis of cultivated species, as observed in various crop species complexes, is explained by the limited genetic background used during domestication, or during adaptation of cultivated species to specific regions. For example, the mean of two inter-specific *Solanum* progenies between potato and wild relatives exceeded the yield of potato control cultivars by 161 and 128% (Buso et al. 2002). Inter-specific genotypes have shown positive transgressive segregation for yield also in the gene pool of rice (Xiao et al. 1996) and tomato (Tanksley and McCouch 1997). In the tri-hybrid *Allium* population, positive epistatic interactions arising from the combination of the three genomes may lead to the observed response. The usefulness for onion breeding, however, depends on the actual translation of this vigour to the harvestable organ, the bulb. In other words, whether the increased plant weight observed for some tri-hybrid genotypes can be preserved after selection for bulbing in the next generations.

Tri-hybrid genotypes segregated for the degree of bulbing (Chapter 5). As explained before, the karyotype of progeny plants of the cross *A. cepa* x *(A. roylei x A. fistulosum)* consists of a complete set of *A. cepa* chromosomes, and a set of recombinant chromosomes between *A. fistulosum – A. roylei*. Whereas *A. cepa* forms a bulb, the two other species do not. Therefore, segregation in bulbing ability among tri-hybrid genotypes is not likely to be caused by recombination in

A. fistulosum – A. roylei chromosomes, but rather by suppression of the bulbing ability conferred by *A. cepa* chromosomes. In this regard, a QTL for bulbing ability was located on chromosome 5 associated with *A. fistulosum* alleles (Figure 25). When *A. roylei* alleles are present in this region, suppression of bulbing ability probably takes place.

The bulb of *A. cepa* consists of a storage organ formed by translocation of carbohydrates to the base of the sheaths. Genetic analysis of carbohydrate accumulation resulting in the formation of the bulb concluded the presence of QTLs and/or genes on chromosomes 3, 5, 6 and 8 (Galmarini et al. 2001, Martin et al. 2005, McCallum et al. 2006, Masuzaki et al. 2007, Yaguchi et al. 2008). For instance, a QTL identified by Galmarini et al. (2001) was assigned to chromosome 5 (Martin et al. 2005), and proved to be a phloem sucrose transporter gene (Masusaki et al. 2007). This gene in *A. cepa* may be in the position of the QTL for bulbing ability located in the present study. More research is needed to elucidate whether both QTLs are located on the same position, and whether they can be even alleles of the same locus with a dominant effect of *A. roylei*.

Masusaki et al. (2007) studied bulbing ability in *A. fistulosum* plants harbouring additional chromosomes from shallot, *A. cepa* var. *aggregatum* (e.g., FF+nA). Genotypes harbouring at least five *A. cepa* chromosomes formed a bulb, but did not when chromosome 2 from *A. cepa* was present, which seemed to negatively influence bulbing (Masusaki et al. 2007). Genotypes harbouring a complete set of *A. cepa* chromosomes (a triploid FFA) formed a bulb (Masusaki et al. 2007). It seems therefore that the *A. fistulosum* genome does not suppress the action of genes controlling bulb formation in *A. cepa*. Regarding *A. roylei*, an *A. cepa x (A. cepa x A. roylei)* population consisted of a considerable proportion of onion-like plants (Kik 2002). Furthermore, programmes aiming to introgress the *Pd* gene from *A. roylei* were successfully completed without compromising bulb formation, resulting in the release of commercial cultivars (Scholten et al. 2007). Therefore, I expect that traits from *A. fistulosum* and *A. roylei* can be introgressed into onion without negative effects on onion bulbing. The lack of bulbing observed in tri-hybrid genotypes will likely be removed in the next backcross generations, a process that will depend upon the possible linkage between target traits and loci controlling bulbing in onion.

In order to exploit genes from *A. fistulosum* and *A. roylei*, introgression strategies from tri-hybrid genotypes may differ depending upon the genetic basis of the target trait (Figure 26). A trait controlled by a single gene is suitable for a simple back-cross to *A. cepa*. This is completed in 5-6 generations or less when

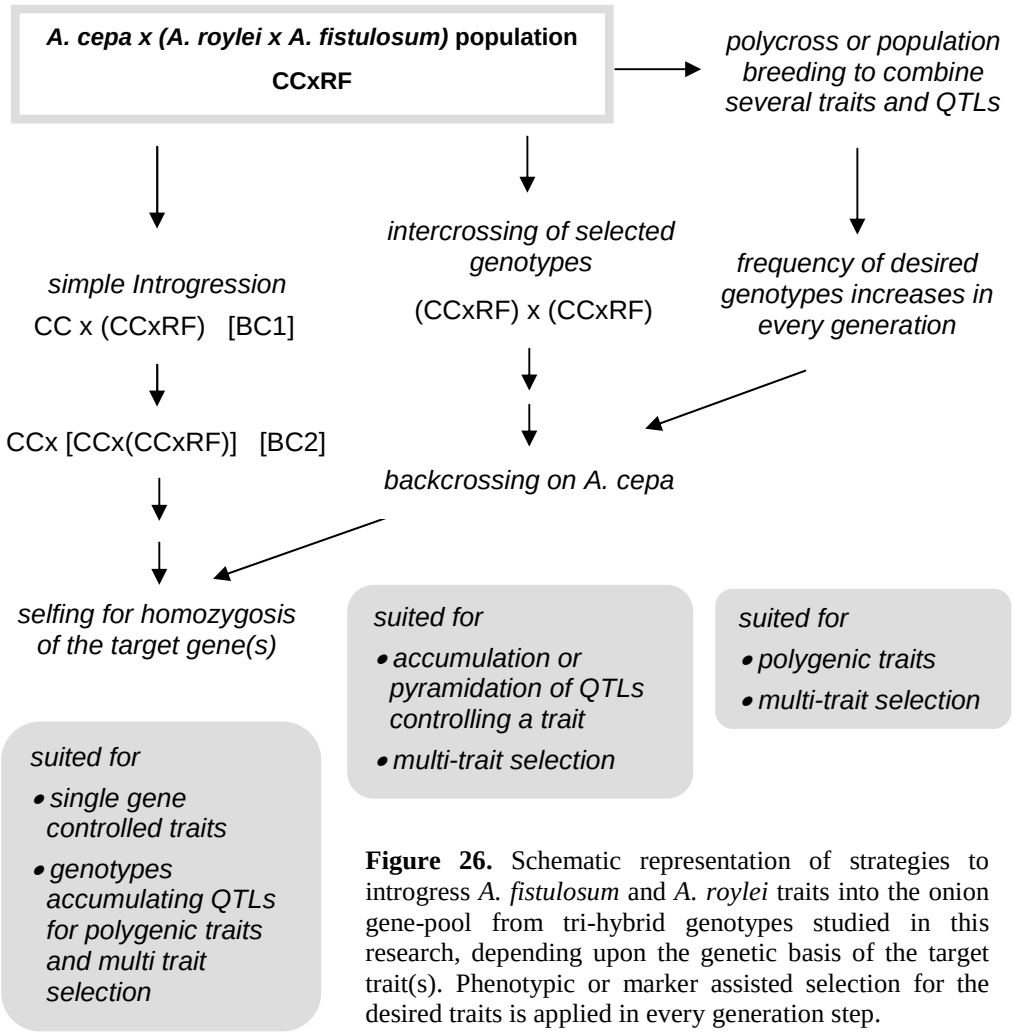


Figure 26. Schematic representation of strategies to introgress *A. fistulosum* and *A. roylei* traits into the onion gene-pool from tri-hybrid genotypes studied in this research, depending upon the genetic basis of the target trait(s). Phenotypic or marker assisted selection for the desired traits is applied in every generation step.

using marker-aided selection, and is followed by selfing aiming for homozygosity of the target gene, as described for the introgression of downy mildew resistance in onion (Scholten et al. 2007). Simple backcrossing can also be applied when a tri-hybrid genotype already harbours the target QTLs. Otherwise, intercrossing of selected tri-hybrid genotypes can be carried out when various QTLs should be combined, either for polygenic traits or for multiple trait selection. Even a polycross (population breeding or recurrent selection) may be suitable in order to

increase the frequency of genotypes combining several (not always detected) alleles for the target trait(s) in a favourable genetic background (Charcosset and Moreau 2004).

Availability of molecular markers facilitates selection in all these paths of pre-breeding activities. For example, marker-assisted selection for disease resistance genes can be more efficient if the set up of screening assays is complex and expensive. The efficiency of using molecular markers will be clearer for accumulating or pyramiding QTLs for quantitatively expressed traits, because in such a case there are usually different genotypic combinations that can be hardly distinguished phenotypically. Even for a trait controlled by a single gene, molecular markers can be very efficient when applied not only for the target gene, but also to speed up the recovery of the desired genetic background (Charcosset and Moreau 2004). The development of co-dominant markers would be of great help in the phase of distinguishing homozygous from heterozygous genotypes for the QTLs under selection.

Activities described in Figure 26 end up with plant material ready to be used as parental material in the development of open-pollinated onion cultivars or inbred lines to be used as parents of onion F1 hybrids.

Resistance to Fusarium basal rot

I identified several *Fusarium* species pathogenic to onion, indicating that Fusarium basal rot is caused by a complex of species, although *F. oxysporum* is the most important one in onion cultivation (Chapter 2). Several species causing FBR in onion were reported in the past (summarized by Entwistle 1990), and were also reported for Fusarium wilt in *A. fistulosum* in Japan, which was caused by *F. oxysporum*, *F. solani* and *F. verticillium* (Dissanayake et al. 2009a). Complex pathogen populations with diverse requirements in environmental conditions for disease development may lead to complex disease patterns in the field (Kistley 2001).

Genetic diversity studied by AFLPs, which scans the whole *Fusarium* genome, was in agreement with a phylogeny on the basis of sequencing the EF1-alfa gene (Geiser et al. 2004). This result supports the alternative use of these molecular tools for phylogenetic studies. Moreover, this finding supports the correspondence between evolutionary events at the genome and single gene levels (O'Donnell et al. 1998, Kistley 2001). I showed in Chapter 2 that *F. oxysporum* isolates causing onion basal rot were grouped into two main clades. This finding supports the polyphyletic origin of isolates pathogenic to onion. Polyphyly was also reported for *Fusarium* isolates causing wilting in *A. fistulosum* (Dissanayake

2009b) and other formae speciales (discussed in Chapter 2). Therefore, the forma specialis within *F. oxysporum* becomes a concept with practical applications although it is uninformative from a phylogenetic point of view.

Fusarium oxysporum is a ubiquitous inhabitant in soils worldwide (Kistler 2001). Evolution of *F. oxysporum* towards pathogenicity on a host plant can be explained by a few genes controlling key factors for infection and exploitation of plant tissues. These factors are the ability of the spores to produce germlings that attach to the root surface and penetrate the plant tissue by a primary infection peg (Recorbet et al. 2003). Then, branching and proliferation of hyphae come along with a front-line of exo-enzymes produced by the pathogen (pectinases and exo-polygalacturonases, among others) causing cell death, followed by collapse, necrosis and rotting of plant tissues (Holz and Knox-Davies 1986, Roncero et al. 2003). In different steps of this process, the pathogen overcomes defence reactions triggered by the plant.

Three hypotheses to explain why more distantly related isolates (and even different species) have pathogenic ability on the same host were discussed by Kistley (2001): (a) an ancient common origin of pathogenicity genes, (b) independent pathogenicity emerged in unrelated taxa, and (c) horizontal gene transfer. The analysis of the molecular basis of pathogenicity may contribute to elucidate whether the same genes are present in different *Fusarium* clusters or species, or have evolved independently. The outcome of this analysis may condition the extent of the effectiveness of a resistance source.

Breeding for resistance in *A. fistulosum* to Fusarium basal rot has been carried out since the sixties of the previous century, and highly resistant cultivars were developed since then (Shigyo and Kik 2008). The use of FBR resistance from *A. fistulosum* in the development of new onion cultivars was hampered by the poor crossability between *A. cepa* and *A. fistulosum*. I confirmed the high level of resistance found in *A. fistulosum* to isolates pathogenic to onion of *F. oxysporum* clade 2, clade 3, and to a *F. proliferatum* isolate (Chapter 2). Nevertheless, root and basal plate infections were observed in plants of *A. fistulosum*, indicating that this species is host for *Fusarium*, and that its resistance is not based on immunity.

Fusarium wilt in *A. fistulosum* was reported in countries where it is widely cultivated, like Japan (Shinmura et al. 1998) and Colombia (Navia and Gómez 1999). Dissanayake et al. (2009a) compared 32 *Fusarium* isolates obtained from *A. fistulosum* wilting plants in Japan. Using seedling tests, five *F. oxysporum* isolates were aggressive to *A. fistulosum*, and the rest of the isolates were intermediate to weakly pathogenic. Onion cultivars were highly susceptible to this

set of *Fusarium* isolates (Dissanayake et al. 2009a). These results, therefore, confirm positive prospects for the use of FBR resistance from *A. fistulosum* in onion breeding. However, they also suggest evolution of some *Fusarium* isolates to overcome mechanisms of defence in *A. fistulosum* (Dissanayake et al. 2009a).

Plant resistance involves diverse recognition events that trigger defence reactions. One strategy involves R genes of the plant (NBS-LRR proteins) that trigger a hypersensitive reaction after recognition of a specific elicitor produced by the pathogen (reviewed by McDowell and Woffenden 2003). Another strategy involves the recognition by pathogen associated molecular patterns (PAMPs), which triggers diverse defence cascades leading to basal (or partial) resistance, including MAP-kinases, pathogenesis related proteins (PR), and the ethylene, salicylic acid and jasmonic acid pathways (reviewed by Hammond-Kosak and Parker 2003, Koornneef and Pieterse 2008, Niks and Marcel 2009). Basal resistance comprises also active morphological barriers, like deposition of callose and cell-wall thickening. Defence mechanisms supporting basal resistance, which may delay or completely suppress infection and establishment of the pathogen, have shown to be durable, race non-specific, and usually controlled by various genes. Broad spectrum and durable resistance, in some specific pathosystems, is also mediated by a recessive host gene mutation encoding members of a protein family only found in plants. This is the case of *Mlo* gene in the barley – *Blumeria graminis* f. sp. *hordei* system, and *Ol-2* gene in tomato – *Oidium neolycopersici* (Bai et al. 2008).

With a few exceptions, *Fusarium* diseases cannot be distinguished by races of the pathogen, nor by race-specific resistance in the host. At the molecular level, some of the defence mechanisms described above have been identified for *Fusarium* diseases. Resistance gene analogues (RGA) can be identified on the basis of highly conserved motifs in the NBS region (Van der Linden et al. 2004). Then, the association between the presence of these RGA loci and the resistant phenotype in segregating populations can be analysed aiming to identify candidate genes. In this way, a gene coding for a non-TIR NBS-LRR protein was found to be associated with resistance to *F. oxysporum* f. sp. *cubense* race 4 in wild banana *Musa acuminata* (Peraza-Echeverria et al. 2008). Similar associations were found for resistance to *F. oxysporum* f. sp. *zingiberi* in *Zingiber officinale* (Swetha Priya and Subramanian 2008) and for the resistance to *F. oxysporum* f. sp. *lycopersici* race 2 in tomato (Simons et al. 1998). Certainly, for the QTLs involved in FBR resistance from *A. fistulosum* and *A. roylei* that were identified in this research, the quest for candidate genes by NBS profiling is an interesting future research line in order to elucidate the molecular basis of the resistance.

The molecular basis involved in other defence mechanisms against *Fusarium* diseases have been studied to a lesser extent. For resistance to *F. graminearum* in maize, one of the two QTLs determining resistance proved to be a NBS-LRR gene, but not the second one (Yuan et al. 2009). The role of MAP-kinases against *F. graminearum* was studied *in vitro* (Ramamoorthy et al. 2007), and the role of oxidative burst in basal resistance against *F. oxysporum* was demonstrated in *Arabidopsis thaliana* (Davies et al. 2006).

AMF diversity and agriculture

At the start of my PhD research, virtually nothing was known about the presence and frequency of naturally occurring arbuscular mycorrhizal fungi (AMF) in Dutch onion cultivation. Therefore, I studied AMF diversity as a first step to study *Allium*–AMF relationships. High levels of AMF colonization were found in onion roots obtained from Dutch agricultural soils under either organic or conventional cultivation farming systems (Chapter 4). It was striking that AMF were abundant even in high-tech agricultural systems, and that AMF were present in soils with high P levels. Moreover, the level of AMF colonization seemed to have a positive effect on yield.

The inability of onions to take up adequate amounts of P is likely the consequence of their poor rooting system, which explores a limited volume of soil. AMF allows for the exploration of larger volumes of soil.

In Chapter 4 AMF species diversity (phylotype diversity) was assessed based on rDNA RFLP patterns and subsequent sequencing. This method yields a conservative estimate of diversity, since more than one AMF species may belong to the same phylotype. Two phylotypes (belonging to *Glomus*-A) were most abundant and found in most or almost all fields, whereas the other phylotypes were infrequent. This result is in agreement with earlier studies on AMF diversity in arable lands (e.g. Oehl et al. 2003, Daniell et al. 2001). A few farms, either organic or conventional, had higher AMF phylotype diversity. I conclude that AMF diversity does not differ between organic and conventional farming systems, but also that both organic and conventional farming may not be homogeneous groups with regard to farming practices that influence AMF diversity and activity. Minimum requirements for labelling as ‘organic farming’ (as defined by SKAL, www.skal.com) imply, for example, the exclusion of synthetic fungicides and pesticides. However, farming practices with impact on AMF community composition are usually ‘recommended’ management practices: reduced ploughing, rotation with mycorrhizal-host crops, green cover crops instead of bare fallow periods, moderate level of phosphorus in the soil (Gosling et al. 2006, Hijri et al. 2006). Our finding that some organic and conventional

farms had larger AMF diversity compared to other farms irrespective of the farming system, revealed that agriculture effects are not uniform, as found earlier by Hijri et al. (2006).

The relevance of this diversity for agriculture deserves more attention. For natural grasslands a positive correlation between AMF diversity and biodiversity of above-ground plant communities and total biomass production has been noted (Van der Heijden et al. 1998). Biodiversity in natural ecosystems also supports stability and resilience of the whole system (Altieri 1999, Van der Heijden et al. 2006). Therefore, larger diversity of AMF in agri-ecosystems may be regarded as an indicator for a higher level of sustainability.

However, AMF diversity indices were not related with onion yields (Chapter 4). Currently, it is not known whether agricultural yield is maximized in the presence of the 'best' AMF, or whether a species mixture of AMF creates higher yields through complementarity, either directly through P-uptake patterns or indirectly, through a simultaneous better protection of the crops. The first view is in line with a schematic view of agriculture as an industrial process in which all resources are devoted to allocate maximum biomass to the harvestable organ. Under this hypothesis, crop yield may be favoured by a single efficient AMF strain acting at the right time, avoiding carbon allocation to inefficient AMF strains. Under the alternative hypothesis, larger AMF diversity may offer possibilities for better interaction with diverse host species in the rotation, access to diverse sources of nutrients, differential action along the season and soil depths. On the long term, yield stability will be guaranteed. As discussed in Chapter 4, studies comparing actions of single vs. mixed inocula yielded variable results (Van der Heijden et al. 2006, Jansa et al. 2008). More research is warranted to address these important questions in the framework of low-input and organic agricultural systems.

Measuring and improving the response to mycorrhiza in *Allium*

The establishment and functioning of the mycorrhizal symbiosis is the result of compatible genetic backgrounds between both partners: the fungus and the plant (Gollotte et al. 2002). Numerous genes are involved in signalling, re-programming colonized root cells, and triggering systemic reactions (reviewed by Balestrini and Lanfranco 2006, Massoumou et al. 2007). The presence and expression of such genes may differ between plant genotypes and, as a consequence, may cause differences in the functioning of the symbiosis.

It has been suggested in earlier research that plant genetic variation in response to mycorrhizal fungi allows plant breeding for this trait (Chapter 5). However,

research aiming at analysis of the genetic basis of variation in plant populations has been scarce. Furthermore, a theoretical framework describing the plant-AMF relationship has not been completely developed yet (Sawers et al. 2008). By analyzing variation among *Allium* species, the hybrid between *A. fistulosum* and *A. roylei*, and a tri-hybrid *Allium* population (Chapter 5), I contributed to the knowledge of the response of these *Allium* taxa to AMF. At the same time, I contributed with experimental data towards the development of new concepts to analyse plant-mycorrhiza interactions from a breeding point of view.

Allium species (including onion) benefitted to a large extent from mycorrhiza, which is in agreement with previous reports (Hayman and Mosse 1971, Tawaraya et al. 2001). This large benefit was a consequence of the inability of these species to grow in the absence of mycorrhiza in a P-limiting environment (Charron et al. 2001).

The ultimate expression of plants better exploiting the symbiosis would be an enhancement of plant growth and crop yields. In literature, the response to mycorrhizal fungi has usually been calculated by measuring plant weight of plants colonized with AMF compared with non-colonized control plants, on a proportional basis (Planchette et al. 1983, Smith 2000, Janos 2007, Sawers et al. 2008). However, responsiveness is not an appropriate index because it is always negatively correlated with NM plant weight (Chapter 5; Kuyper et al., in prep.). Therefore, while responsiveness may be a useful concept in many areas of mycorrhizal research, from a plant breeding point of view, selection for responsiveness is misleading, because it would result in selection for low-yielding plants in the absence of AMF.

Instead of responsiveness, breeders and growers are interested in high-yielding plants in the presence of AMF in the soil. While this may be the main selection criterion, such plants may or may not have similar yield in the absence of AMF. If environmental conditions for AMF colonization are not favourable, plants that still achieve high yields would also be preferred. Therefore, from a plant breeding perspective two indices were proposed to evaluate the response to AMF: mycorrhizal benefit (MB), the difference in plant weight with and without AMF inoculation; and mycorrhizal breeding value (MV), the average between these two plant weight measurements. Genetic variation for the response to AMF was studied in the tri-hybrid *Allium* population, and QTLs for MB and MV were located on the linkage map of the RF-hybrid.

MB and MV were positively correlated with plant weight of mycorrhizal and non-mycorrhizal plants (Chapter 5). QTLs for plant weight coincided with QTLs

for MB or MV. This correlation implies that the larger the plant in the mycorrhizal or non-mycorrhizal treatments, the larger will be the profit obtained from the symbiosis. Positive relationships between MB or MV and the number of roots per plant were also observed. These observations in *Allium* differ from other species (e.g. wheat, maize), where a larger ability to growth in the absence of AMF was correlated with a lower benefit from AMF (MB) (Kuyper et al., in prep.). These latter results may be explained by the fact that when a genotype is able to grow in the absence of AMF, it will receive little additional benefit when inoculated with AMF. However, for *Allium* species, even genotypes that grew best in the absence of AMF were still below the point in which the plant itself can substitute the action of AMF.

As the observed benefit from AMF depends upon the level of P, Janos (2007) proposed to assess mycorrhizal benefit along a range of P concentrations in the soil, in order to define the largest AMF effect on a response curve (see Figure 1). This proposal has serious practical constraints (high costs) when working with large number of plant genotypes, as well as theoretical problems, because for various genotypes maximum responsiveness may be found at different P concentrations. This source of variation makes comparisons very complicated. Another limitation is that P availability is not the only environmental factor that influences AMF-plant relationships, but rather the experimental conditions as a whole: temperature, pH of the soil, water availability, Ca content, organic matter, presence of other soil micro-organisms, etc. (Stribley 1990, Sawers et al. 2008).

As *Allium* species did perform (very) poorly in the non-mycorrhizal condition, the comparison between mycorrhizal and non-mycorrhizal treatments became rather meaningless (Chapter 5). It is therefore not surprising that the survey of AMF in onion cultivation revealed that AMF were abundant in all agricultural soils (Chapter 4). In fact, a non-mycorrhizal control for onion could only be experimentally created. Alternative control treatments may be used in future research.

Protective effect from AMF against diseases

As my thesis deals with interactions between *Allium* species and pathogenic soil-borne fungi, and between *Allium* species and AMF, the synthesis of the interaction between the pathogen and AMF may come up as an important line of future research.

AMF can have a protective effect against soil-borne diseases in many pathosystems (Pozo and Azcon-Aguilar 2007). The protective effect may be the

result of increased plant growth, or the result of direct plant defence reactions to other fungi elicited by AMF, turning the roots more prone to react against pathogens.

Numerous studies have shown the protective effect of AMF against soil-borne *Fusarium* diseases. Incidence of *Fusarium* root rot decreased in asparagus *(Asparagus officinalis)* from 90% to 20-50% when seedlings were previously colonized by *Glomus* species (Matsubara et al. 2001), in chickpea *(Cicer arietinum)* from 100% to 54% (Rakesh et al. 2004), and in strawberry *(Fragaria x annanasa)* from 100% in non-mycorrhizal plots to 22% in plots previously inoculated with *Glomus mosseae* (Schnitzler 2004). Microscopic studies showed that cortical cells having AMF arbuscules were never infected by *Fusarium* (Matsubara et al. 2001). The growth of *F. oxysporum* f. sp. *chrysanthemi* was restricted to the epidermis and outer cortical cells in AMF colonized axenic carrot roots, whereas the pathogen invaded the root and the vascular stele in non-mycorrhizal roots (Benhamou et al. 1994).

The protective effect can be caused by direct interaction between AMF and the pathogen through antagonism and competition. In co-cultures of carnation (*Dianthus caryophyllus*, a non-mycorrhizal species) and *Tagetes patula* colonized by *G. intraradices*, the survival of carnation exposed to *F. oxysporum* f. sp. *dianthi* more than doubled (St-Arnaud et al. 1997). This phenomenon was tentatively explained by a direct interaction between micro-organisms in the soil, or the induction of resistance mechanisms in carnation.

Differential gene expression was observed between AMF inoculated plants and plants affected by pathogenic attacks. The expression of chitinase class III in *Medicago truncatula* was enhanced by the symbiosis with *Glomus intraradices*, but not by *F. solani* f. sp. *phaseoli* and other pathogens (Salzer et al. 2000). In another study, common bean (*Phaseolus vulgaris*) showed marked increase in the expression of defense-related genes like chitinase, β-1-3-glucanases and phenyl-alanine ammonia-lyase when inoculated with *F. solani* f. sp. *phaseoli*, whereas the expression of these genes did not change when inoculated with *G. mosseae* (Mohr et al. 1998).

These studies indicate that plants respond differently to AMF than to pathogens. Furthermore, general defence mechanisms are triggered by AMF colonization. These defences include systemic effects leading to protective effects to aboveground diseases and pests (Pozo et al. 2002). In AMF-induced resistance, the jasmonate acid (JA) pathway is up-regulated, whereas the salicylic acid (SA) pathway is down-regulated. This differential response explains why AMF-

induced systemic resistance has been effective against necrotrophic pathogens and generalist chewing insects (mediated by JA), but not against fungal biotrophics, viruses and sap-feeding insects (mediated by SA). An explanation for these directional changes in the host plant is that AMF establish a relationship similar to biotrophic pathogens, and requires the suppression of SA defences (Pozo and Azcón-Aguilar 2007). For example, mycorrhiza-defective *myc*$^{-}$ mutants fail in the suppression of SA defence pathway (García Garrido and Ocampo 2002).

Interactions between AMF and pathogenic *Fusarium* can occur in onion cultivation and may result in a protective effect to the host plant, since both AMF and *Fusarium* are present in the field. Because onion plants are colonized by AMF early in the season (Chapter 4), when the environmental conditions for FBR infection are normally not yet achieved, defence reactions triggered by AMF could occur before FBR infections, and therefore a protective effect given by AMF against FBR in onion crops is possible. It would be worthwhile to measure the importance of this interaction by appropriate experimental setups (simultaneous versus sequential inoculation, variation in inoculum density of AMF and *Fusarium*).

Synthesis

Onion cultivations are hampered by numerous biotic and abiotic stresses in conventional, as well as in low-input and organic farming systems. Among these threats, Fusarium basal rot (FBR) and the limited ability of onion to take up nutrients can be tackled by means of breeding for resistance and improved symbiosis with AMF.

I therefore studied genetic diversity in the causal agent of Fusarium basal rot, and the genetic basis of FBR resistance in onion cultivars by exploiting the genetic diversity present in *A. fistulosum* and *A. roylei*. Furthermore, I demonstrated the relevance of AMF for *Allium* species, and showed the presence of a low AMF diversity in commercial onion cultivation in The Netherlands, with most samples containing one or two *Glomus*-A species only. No differences in the presence and frequency of AMF phylotypes between organic and conventional cultivation systems were observed. By introgressing genes from *A. fistulosum* and *A. roylei* into onion, I showed that a high response to AMF in onion can be maintained or enhanced while simultaneously improving the rooting system. New indices to analyse *Allium*-AMF interactions were proposed from a breeding point of view. For *Allium*, it seems that genetic variation concerning the response to AMF highly depends on plant growth rate. This indicates that selection for plant

weight in mycorrhizal environments can be successfully used for indirect selection of genotypes with enhanced response to AMF.

In conclusion, *A. fistulosum* and *A. roylei* are rich sources of genetic variation for valuable traits in onion breeding, both for resistance to FBR and for exploitation of the benefit from AMF. The development of advanced backcross populations and the use of molecular markers will precise these findings and facilitate even more their exploitation. Breeding onion cultivars with FBR resistance and enhanced benefit from AMF, capable of growing in a wide range of environments, will therefore become a realistic goal in the near future.

References

Abawi GS, Lorbeer JW (1971). Pathological histology of four onion cultivars infected by *Fusarium oxysporum* f. sp. *cepae.* Phytopathology 61:1164-1169.

Alkan N, Gadkar V, Coburn J, Yarden O, Kapulnik Y (2003). Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. New Phytologist 161:877–885.

Altieri MA (1999). The ecological role of biodiversity in agroecosystems. Agriculture, Ecosystems and Environment 74:19-31.

Altschul SF, Thomas LM, Alejandro AS, Jinghui Z, Webb M, David JL (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Appoloni S, Lekberg Y, Tercek MT, Zabinski CA, Redecker D (2008). Molecular community analysis of arbuscular mycorrhiza fungi in roots of geothermal soils in Yellowstone National Park (USA). Microbial Ecology 56:649-659.

Atkinson D, Baddeley JA, Goicoechea N, Green J (2002). Arbuscular mycorrhizal fungi in low input agriculture. In: Ginaninazzi S, Schuepp H, Barea JM, Haselwandter K (eds.), Mycorrhizal Technology in agriculture: from genes to bioproducts. Birkhausen. Basel-Boston-Berlin. pp. 211-222.

Baayen RP, O'Donnell K, Bonants PJM, Cigelnik E, Kroon LP, Roebroeck EJ, Waalwijk C (2000a). Gene genealogies and AFLP analysis in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic *formae speciales* causing wilt and rot disease. Phytopathology 90:891-900.

Baayen RP, Van den Boogert PHJF, Bonants PJM, Poll JTK, Blok WJ, Waalwijk C (2000b). *Fusarium redolens* f.sp. *asparagi*, causal agent of asparagus root rot, crown rot and spear rot. European Journal of Plant Pathology 106:907-912.

Bai Y, Pavan S, Zheng Z, Zappel NF, Reinstädler A, Lotti C, De Giovanni C, Ricciardi L, Lindhout P, Visser R, Theres C, Panstruga R (2008). Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of Mlo function. Molecular plant microbe interaction 21:30–39.

Balestrini R, Lanfranco L (2006). Fungal and plant gene expression in arbuscular mycorrhizal simbiosis. Mycorrhiza 16:509-524.

Bandyopadhyay R, Mwangi M, Aigbe SO, Leslie JF (2006). *Fusarium* species from the cassava root rot complex in West Africa. Phytopathology 96:673-676.

Baon JB, Smith SE, Alston AM (1993). Mycorrhizal responses of barley cultivars differing in P efficiency. Plant and soil 157:97–105.

Barker SJ, Duplessis S, Tagu D (2002). The application of genetic approaches for investigations of mycorrhizal symbiosis. Plant and soil 244:85-95.

Benhamou N, Fortin JA, Hamel C, St-Arnoud M, Shatilla A (1994). Resistance responses of mycorrhizal Ri T-DNA-transformed carrot roots to infection by *Fusarium oxysporum* f.sp. *chrysanthemi*. Phytopathology 84:958-968.

Bergquist RR, Lorbeer JW (1971). Reaction of *Allium* spp. and *Allium cepa* to *Botryiotinia (Botrytis) squamosa.* Plant Disease reporter 55(5):394-398.

Blancard D, Villeneuve F, Chamont S (2003). Le deperissement du peireau: bilan phytosanitaire en 2001. Infos CTIFL 188:46-49.

Boddington CL, Dodd JC (2000). The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. II. Studies in experimental microcosms. Plant and Soil 218:145-157.

Bosch-Serra AD, Currah L (2002). Agronomy of onions. In: Rabinowitch HD, Currah L (eds.), , *Allium* Crop Science: Recent Advances. CAB International, Wallingford, Oxon. UK, pp. 187-232.

Buso JA, Aragao FAS, Reinfschneider FJB, Boiteux LS, Peloquin SJ (2002). Assesment under short-day conditions of genetic materials derived from three potato breeding strategies: 4x-4x (intra-Tuberosum), 4x-2x (FDR 2n pollen), and 4x-4x (diplandrous tetraploid). Euphytica 126:437-446.

Chani E, Ashkenazi V, Hillel J, Veilleux RE (2002). Microsatellite marker analysis of an anther-derived potato family: skewed segregation and gene-centromere mapping. Genome 45:236-242.

Chahal GS, Gosal SS (2006). Principles and procedures of plant breeding, biotechnological and conventional approaches. Harrow, Alpha Science International, 604 p.

Charcosset A, Moreau L (2004). Use of molecular markers for the development of new cultivars and the evaluation of genetic diversity. Euphytica 137:81-94.

Charron G, Furlan V, Bernier-Carou M, Doyon G (2001). Response of onion plants to arbuscular mycorrhizae. 1. Effects of inoculation method and phosphorus fertilization on biomass and bulb firmness. Mycorrhiza 11:187-197.

Cheng X, Baumgartner K (2004). Survey of arbuscular mycorrhizal fungal communities in northern California vineyards and mycorrhizal colonization potential of grapevine nursery stock. Hortscience 39:1702-1706.

Cox DR, Hinkley DV (1979). Theoretical Statistics. Chapman and Hall, London. 511 pp.

Cramer CS (2000). Breeding and genetics of Fusarium basal rot resistance in onion. Euphytica 115: 159-166.

Cramer CS (2006). Onion trait heritability and response from selection. Journal of American Society Horticultural Science 131:646-650.

Currah L, Maude RB (1984). Laboratory tests for leaf resistance to *Botrytis squamosa* in onions. Annals of Applied Biology 105:277-283.

Daniell TJ, Husband R, Fitter AJ, Young JP (2001). Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiology and Ecology 36:203-209.

Dann PR, Derrick JW, Dumaresq DC, Ryan MH (1996). The response of organic and conventionally grown wheat to superphosphate and reactive phosphate rock. Australian Journal of Experimental Agriculture 36:71-78.

Davies DR, Bindschedler LV, Strickland TS, Bolwell GP (2006). Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*: implications for basal resistance. Journal of experimental botany 57:1817-1827.

De Melo PE (2003). The root systems of onion and *Allium fistulosum* in the context of organic farming: a breeding approach. Ph.D. Thesis, Wageningen University and Research Centre, the Netherlands. 136 pp.

De Visser CLM (1999) Fusarium in uien en rasverschillen in aantasting, evaluatie van een biotoets. PAV Bulletin Vollegrondsgroenteteelt, December 1999, p.4-7.

De Vries JN, Wietsma WA, De Vries T (1992a). Introgression of leaf blight resistance from *Allium roylei* Stearn into onion (*A. cepa*. L). Euphytica 62:127-133.

De Vries JN, Wietsma WA, Jongerius MC (1992b). Introgression of characters from *Allium roylei* Stearn into *A. cepa* L. In: Hanelt P, Hammer K, Knüpfer K (eds.) The genus *Allium*: taxonomic problems and genetic resources. Buch- und Offsetdruck Lüders, Halberstadt, pp.321-325.

De Vries JN, Wietsma WA (1992). *Allium roylei* Stearn restores cytoplasmic male sterility of Rijnsburger onion (*A. cepa* L.). Journal of Genetics and Breeding 46:379-381.

Dhingra OD, Coelho Netto RA (2001). Reservoir and non-reservoir hosts of bean-wilt pathogen, *Fusarium oxysporum* f.sp. *phaseoli*. Journal of Phytopathology 149:463-467.

Dissanayake MLMC, Kashima R, Tanaka S, Ito S (2009a) Pathogenic variation and molecular characterization of *Fusarium* species isolated from wilted Welsh onion in Japan. Journal of general plant pathology 75:37-45.

Dissanayake MLMC, Kashima R, Tanaka S, Ito S (2009b) Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from wilted Welsh onion in Japan. Journal of General Plant Pathology 75:125-130.

Douds DD, Janke RR, Peters SE (1993). VAM fungus spore populations and colonization of roots of maize and soybean under conventional and low-input sustainable agriculture. Agriculture, Ecosystems and Environment 43:325–335.

Dugan FM, Hellier BC, Lupien SL (2003). First report of *Fusarium proliferatum* causing rot of garlic bulbs in North America. Plant Pathology 52:426.

Du Toit LJ, Inglis DA, Pelter GQ (2003). *Fusarium proliferatum* pathogenic on onion bulbs in Washington. Plant Disease 87:750.

Eason WR, Scullion J, Scott EP (1999). Soil parameters and plant responses associated with arbuscular mycorrhizas from contrasting grassland management regimes. Agriculture, Ecosystems and Environment 73:245–255.

Entwistle AR (1990). Root diseases. In: Rabinowitch HD, Brewster JL (eds.). Onions and allied crops. CRC Press. Boca Raton, Florida. pp. 103-154.

Fageria NK, Baligar VC, Li YC (2008). The role of nutrient efficient plants in improving crop yields in the Twenty First century. Journal of Plant Nutrition 31:1121-1157.

FAOSTAT (February 25, 2008), http://faostat.fao.org/ (periodically updated).

Felix EL (1933). Disease resistance in *Allium fistulosum* L. Phytopathology 23:109-110.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Finkers R, Van den Berg P, Van Berloo R, Ten Have A, Van Heusden AW, Van Kan JAL, Lindhout P (2007). Three QTLs for *Botrytis cinerea* resistance in tomato. Theoretical and Applied Genetics 114:585–593.

Finlay RD (2004). Mycorrhizal fungi and their multifunctional roles. Mycologist 18:91-96.

Friesen M, Fritsch RM, Blattner FR (2006). Phylogeny and new intrageneric classification of *Allium* (*Alliaceae*) based on nuclear ribosomal DNA ITS sequences. Aliso (Rancho Santa Ana Botanic Garden) 22:372–395.

Fritsch RM, Friesen N (2002). Evolution, domestication and taxonomy. In: Rabinowitch HD, Currah L (eds.). *Allium* crop science: recent advances. CABI Publishing, pp.5-30.

Galmarini CR, Goldman IL, Havey MJ (2001). Genetic analysis of correlated solids, flavour, and health-enhancing traits in onion (*Allium cepa* L.). Molecular genetics and genomics 265:543-551.

Galván GA, Koning-Boucoiran CFS, Koopman WJM, Burger K, González PH, Waalkwijk C, Kik C, Scholten OE (2008). Genetic variation among *Fusarium* isolates from onion and resistance to Fusarium basal rot in related *Allium* species. European Journal of Plant Pathology 121:499-512.

Galván G, Wietsma WA, Putrasemedja S, Permadi AH, Kik C (1997). Breeding for resistance against *Colletotrichum gloeosporioides* in *Allium* section *cepa*. Euphytica 95:173-178.

Galván GA, Parádi I, Burger K, Baar J, Kuyper TW, Scholten OE, Kik C (2009). Molecular diversity of arbuscular mycorrhizal fungi in onion roots from organic and conventional farming systems in the Netherlands. Mycorrhiza 19:317-328.

Gao X, Kuyper TW, Zou C, Zhang F, Hoffland E (2007). Mycorrhizal responsiveness of aerobic rice genotypes is negatively correlated with their zinc uptake when nonmycorrhizal. Plant and Soil 290:283-291.

García-Garrido JM, Ocampo JA (2002). Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. Journal of Experimental Botany 53:1377-1386.

Geiser DM, Jiménez-Guasco MM, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell K (2004). FUSARIUM-ID v. 1.0: A DNA sequence database for identifying Fusarium. European Journal of Plant Pathology 110:473-479.

George E, Marschner H, Jakobsen I (1995). Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Critical Reviews in Biotechnology 15:257–270.

Gollotte A, Brechenmacher L, Weidmann S, Frankel P, Gianinazzi-Pearson V (2002). Plant genes involved in arbuscular mycorrhiza formation and functioning. In: Ginaninazzi et al. (eds.), Mycorrhizal Technology in agriculture. Birkhäuser Verlag, Basel-Boston-Berlin. pp. 87-102.

Gosling P, Hodge A, Goodlass G, Bending GD (2006). Arbuscular mycorrhizal fungi and organic farming. Agriculture, Ecosystems and environment 113:17-35.

Greenwood DJ, Gerwitz A, Stone DA, Barnes A (1982). Root development of vegetable crops. Plant and Soil 68:75-96.

Gurushidze M, Mashayekhi S, Blattner FR, Friesen N, Fritsch RM. (2007). Phylogenetic relationships of wild and cultivated species of *Allium* section *Cepa* inferred by nuclear rDNA ITS sequence analysis. Plant Systematic and Evolution 269:259–269.

Gutierrez JA, Cramer CS (2005). Screening short-day onion cultivars for resistance to Fusarium basal rot. HortScience 40:157-160.

Gutierrez JA, R Molina-Bravo and CS Cramer (2006) Screening winter-sown, intermediate-day onion cultivars for resistance to Fusarium basal rot. Hort-Technology 16(1):177-181.

Hajjar R, Hodgkin T (2007). The use of wild relatives in crop improvement: a survey of developments over the last 20 years. Euphytica 156:1-13.

Hammond-Kosack KE, Parker JE (2003). Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. Current Opinion in Biotechnology 14:177–93.

Hanelt P (1990). Taxonomy, evolution and history. In: Brewster JL and Rabinovitch HD (eds.), Onions and allied crops, Vol. 1. Botany, physiology, and genetics. CRC Press, Boca Raton, Florida, pp. 1-26.

Havey M (1991). Molecular characterization of the interspecific origin of viviparous onion. Journal of Heredity 82:501-503.

Havey MJ, Wehner TC (1999). Vegetable cultivar descriptions for North America, List 24, 1999. Hortscience 34:961-968.

Hayman DS, Mosse B (1971). Plant growth responses to vesicular-arbuscular mycorrhiza. I. Growth of Endogone-inoculated plants in phosphate-deficient soils. New Phytologist 70:19–27.

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JP (1998). Ploughing up the wood-wide web? Nature 394:431.

Hermsen JGT (1992). Introductory considerations on distant hybridization. In: Kallo G, Chowdhury JB (eds). Distant hybridization in crop plants. Springer-Verlag, Berlin – New York. p. 1-14.

Hetrick BAD, Wilson GWT, Cox TS (1993). Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. Canadian Journal of Botany 71:512–518.

Hetrick BAD, Wilson GWT, Cox TS (1995). Chromosome location of mycorrhizal responsive genes in wheat. Canadian Journal of Botany 73:891-897.

Hetrick BAD, Wilson GWT, Cox TS (1996). Mycorrhizal response in wheat cultivars: relationship to phosphorus. Canadian Journal of Botany 74, 19-25.

Hijri I, Sýkorová Z, Oehl F, Ineichen K, Mäder P, Wiemken A, Redecker D (2006). Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Molecular Ecology 15: 2277-2289.

Hogenboom NG (1984). Incongruity: non-funcioning of intercellular and intracellular partner relationships through non-matching information. In: Linskens HE, Heslop-Harrison J (eds). Cellular interactions. Springer-Verlag, Berlin – New York. p. 640-654.

Holz G, Knox-Davies PS (1974). Resistance of onion selections to *Fusarium oxysporum* f. sp. *cepae*. Phytophylactica 6:153-156.

Holz G, Knox-Davies PS (1986). Possible involvement of apoplast sugar in endo-pectin-trans-eliminase synthesis and onion bulb rot caused by *Fusarium exosyporum* f. sp. c*epae*. Physiological and molecular plant pathology 28:403-410.

IFOAM. 2007. The IFOAM basic standards for organic production and processing (available from www.ifoam.org, periodically updated).

Inden H, Asahira T (1990). Japanese bunching onion (*Allium fistulosum* L.). In: Brewster JL, Rabinovitch HD (eds.), Onions and allied crops, Vol. 3. Biochemistry, Food Science, and minor crops. CRC Press, Boca Raton, Florida, pp. 159-178.

Janos DP (2007). Plant responsiveness to mycorrhizas differs from dependence upon mycorrhizas. Mycorrhiza 17:75-91.

Jansa J, Mozafar A, Anken T, Ruh R, Sanders R, Frossard E (2002). Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225-234.

Jansa J, Mozafar A, Banke S, McDonald BA, Frossard E (2002). Intra- and intersporal diversity of ITS rDNA sequences in *Glomus intraradices* assessed by cloning and sequencing, and by SSCP analysis. Mycological Research 106:670–681.

Jansa J, Smith FA, Smith SE (2008). Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? New Phytologist 177:779-789.

Jastrow JD, Miller RM, Lussenhop J (1998). Contributions of interacting biological mechanisms to soil aggregate stabilization in restored prairie. Soil Biology and Biochemistry 30:905-916.

Jansen RC (1993). Interval mapping of multiple quantitative trait loci. Genetics 135:205-211.

Jansen RC, Stam P (1994). High resolution of quantitative traits into multiple loci via interval mapping. Genetics 136:1447–1455.

Jeuken M, Van Wijk R, Peleman J, Lindhout P (2002). An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L. sativa* x *L. saligna* F-2 populations. Theoretical and Applied Genetics 103:638-647.

Johnson NC (1993). Can fertilization of soil select less mutualistic mycorrhizae? Ecological Applications 3:749-757.

Jongman RHG, Ter Braak CJF, Van Tongeren OFR (1995). Data Analysis in Community and Landscape Ecology. Cambridge University Press. Cambridge, UK. 299 pp.

Kaeppler SM, Parke JL, Mueller SM, Senior L, Stuber C, Tracy WF (2000). Variation among maize inbred lines and detection of quantitative trait loci for growth at low phosphorus and responsiveness to arbuscular mycorrhiza fungi. Crop Science 40:358-364.

Keller ERJ, Schubert I, Fuchs J, Meister A (1996). Interspecific crosses of onion with distant *Allium* species and characterization of the presumed hybrids by means of flow cytometry, karyotype analysis and genome *in situ* hybridization. Theoretical and Applied Genetics 92:417-424.

Khrustaleva LI, Kik C (1998). Cytogenetical studies in the bridge cross *Allium cepa x (A. fistulosum x A. roylei*). Theoretical and Applied Genetics 96:8-14.

Khrustaleva LI, Kik C (2000). Introgression of *Allium fistulosum* into *A. cepa* mediated by *A. roylei*. Theoretical and Applied Genetics 100:17-26.

Khrustaleva LI, De Melo PE, Van Heusden AW, Kik C (2005). The integration of recombination and physical maps in a large-genome monocot using haploid genome analysis in a trihybrid *Allium* population. Genetics 169:1673-1685.

Kiers ET, West SA, Denison RF (2002). Mediating mutualisms: farm management practices and evolutionary changes in symbiont co-operation. Journal of Applied Ecology 39:745-754.

Kik C (2002). Exploitation of wild relatives for the breeding of cultivated *Allium* species. In: Rabinowitch HD, Currah L (eds.), *Allium* crop science: recent advances. CABI Publishing, Wallingford - New York. p. 81-100.

Kik C (2008). *Allium* genetic resources with particular reference to onion. Acta Horticulturae 770:135-137.

Kistler HC (1997). Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum.* Phytopathology 87:474-453.

Kistler HC (2001). Evolution of host specificity in *Fusarium oxysporum*. In: *Fusarium*, Paul E Nelson memorial symposium. APS Press. St. Paul. p.70-82.

Klaas M, Friesen N (2002). Molecular markers in *Allium*. In: Rabinowitch HD, Currah L (eds.), *Allium* crop science: recent advances. CABI Publishing, Wallingford-New York. p. 159-186.

Koike ST, GordonTR, Aegerter BJ (2003). Root and basal rot of leek caused by *Fusarium culmorum* in California. Plant Disease 87:601.

Koide RT, Mosse B (2004). A history of research on arbuscular mycorrhiza. Mycorrhiza 14:145-163.

Koopman WJM, Zevenbergen MJ, Van den Berg RG (2001). Species relationships in *Lactuca* sp. (*Lactuceae, Asteraceae*) inferred from AFLP fingerprints. American Journal of Botany 88:1881-1887.

Koornneef A, Pieterse, CMJ (2008). Cross talk in defense signalling. Plant physiology 146:839-844.

Kofoet A, Kik C, Wietsma WA, De Vries JN (1990). Inheritance of resistance to Downy Mildew (*Peronospora destructor* [Berk.] Casp.) from *Allium roylei* Stearn in the backcross *Allium cepa* L. x (*A. roylei x A. cepa).* Plant Breeding 105:144-149.

Kovacs GM, Balazs T, Penzes Z (2007). Molecular study of arbuscular mycorrhizal fungi colonizing the sporophyte of the eusporangiate rattlesnake fern (*Botrychium virginianum, Ophioglossaceae*). Mycorrhiza 17:597-605.

Krueger SK, Weinman AA, Gabelman WH (1989). Combining ability among inbred onions for resistance to Fusarium Basal Rot. Hortscience 24:1021-1023.

Lammerts van Bueren ET (2003). Organic plant breeding and propagation: concepts and strategies. Ph.D. Thesis, Wageningen University, 207p.

Lekberg Y, Koide RT (2005). Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. New Phytologist 168:189-204.

Li H, Smith SE, Holloway RE, Zhu Y, Smith FA (2006). Arbuscular mycorrhizal fungi contribute to phosphorus uptake by wheat grown in a phosphorus-fixing soil even in the absence of positive growth responses. New Phytologist 172:536–543.

Linderman RG, Davis EA (2004). Evaluation of commercial inorganic and organic fertilizer effects on arbuscular mycorrhizae formed by *Glomus intraradices.* Hort-technology 14:196-202.

Lopez J, Cramer CS (2002). Screening NPGS short-day onion accessions for resistance to Fusarium basal rot. *Allium* Improvement Newsletter 10:29-31.

Lorbeer JW, Kuhar TP, Hoffmann MP (2002). Monitoring and forecasting for disease and insect attack in onions and Allium crops within IPM strategies. In: Rabinowitch HD, Currah L (eds.), *Allium* crop science: recent advances. CABI Publishing, p.293-310.

Ludwin AC, Hubstenberger JF, Phillips GC, Southward GM (1992). Screening of *Allium* tester lines in vitro with *Pyrenochaeta terrestris* filtrates. Hortscience 27:166-168.

Lynch JP (2007). Roots of the second green revolution. Australian Journal of Botany 55:493–512.

Mace ES, Lester RN, Gebhardt CG (1999). AFLP analysis of genetic relationships among the cultivated eggplant, *Solanum melongena* L., and wild relatives (*Solanaceae*). Theoretical and Applied Genetics 99:626-633.

Mäder P, Edenhofer S, Boller T, Wiemken A, Niggli U (2000). Arbuscular mycorrhizae in a long-term field trial comparing low-input (organic, biological) and high-input (conventional) farming systems in a crop rotation. Biology and Fertility of Soils 31:150-156.

Mäder P, Fließbach A, Dubois D, Gunst L, Fried P, Niggli U (2002). Soil fertility and biodiversity in organic farming. Science 296:1694-1697.

Mangum PD, Peffley EB (2005). Central cell nuclear-cytoplasmic incongruity:a mechanism for segregation distortion in advanced backcross and selfed generations of (*Allium cepa* L. × *Allium fistulosum* L.) × *A. cepa* interspecific hybrid derivatives. Cytogenetics and Genome Research 109:400-407.

Martin WJ, McCallum J, Shigyo M., Jakse J., Kuhl JC, Yamane N., Pither-Joyce M., Gokce, AF, Sink K.C., Town C.D., Havey MJ (2005). Genetic mapping of expressed sequences in onion and

in silico comparisons with rice show scant colinearity. Molecular Genetics and Genomics 274:197-204.

Massoumou M, Van Tuinen D, Chatagnier O, Arnould C, Brechenmacher L, Sanchez L, Selim S, Gianinazzi S, Gianinazzi-Pearson V (2007). *Medicago truncatula* gene responses specific to arbuscular mycorrhiza interactions with different species and genera of *Glomeromycota*. Mycorrhiza 17:223-234.

Masuzaki S, Yaguchi S, Yamauchi N, Shigyo M (2007). Morphological characterisation of multiple alien addition lines of *Allium* reveals the chromosomal location of gene(s) related to bulb formation in *Allium cepa* L. Journal of Horticultural Science and Biotechnology 82:393-396.

Mathimaran N, Ruh R, Vullioud P, Frossard E, Jansa J (2005). *Glomus intraradices* dominates arbuscular mycorrhizal communities in a heavy textured agricultural soil. Mycorrhiza 16:61-66.

Matsubara Y, Ohba N, Fukui H (2001). Effect of arbuscular mycorrhizal fungus infection on the incidence of fusarium root rot in asparagus seedlings. Journal of the Japanese Society for Horticultural Science 70:202-206.

Maude RB (1990). Leaf diseases of onions. In: Rabinovitch HD, Brewster LJ (eds.). Onions and allied crops. Agronomy, Biotic Interactions, Pathology and Crop Protection, Vol. II. CRC Press. Boca Raton, Florida, pp.173–190.

McCallum J, Clarke A, Pither-Joyce M, Shaw M, Butler R, Brash D, Scheffer J, Sims I, Van Heusden S, Shigyo M, Havey MJ (2006). Genetic mapping of a major gene affecting onion bulb fructan content. Theoretical and Applied Genetics 112:958-967.

McCallum J, Pither-Joyce M, Shaw M, Kenel F, Davis S, Butler R, Scheffer J, Jakse J, Havey MJ (2007). Genetic mapping of sulfur assimilation genes reveals a QTL for onion bulb pungency. Theoretical and Applied Genetics 114:815-822.

McCollum GD (1982). Experimental hybrids between *A. fistulosum* and *A. roylei*. Botanical Gazette 143:238–242.

McCullagh R, Nelder JA (1989). Generalized Linear Models (2nd ed.). Monographs on Statistics and Applied Probability 37. Chapman and Hall, London. 511p.

McDowell JM, Woffenden BJ (2003). Plant disease resistance genes: resent insight and potential applications. Trends in Biotechnology 21(4):178-183.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. (1990). A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist 115:495-501.

Mengel K, Kirkby EA (2001). Principles of plant nutrition. Kluver Academic Publishers, 5th Ed. Dordrecht. 849p.

Merryweather J (2001). Comment: Meet the Glomales; the ecology of mycorrhiza. British Wildlife, Dec. 2001, pp.86-93.

Merryweather JW, Fitter HF (1998). Seasonal patterns of colonisation of the roots of Hyacinthoides non-scripta by arbuscular mycorrhizal fungi. Mycorrhiza 8:87-91.

Miller JC, Rajapakse S, Garber RK (1986). Vesicular-arbuscular mycorrhizae in vegetable crops. Hortscience 21:974-984.

Mohr U, Lange J, Boller T, Wiemken A, Vögeli-lange R (1998). Plant defence genes are induced in the pathogenic interaction between bean roots and *Fusarium solani*, but not in the symbiotic interaction with the arbuscular mycorrhizal fungus *Glomus mosseae.* New Phytologist 138:589-598.

Montes R, Nava RA, Flores HE, Mundo M (2003). Fungi and nematodes in roots and bulbs of onion (*Allium cepa* L) in the state of Morelos, Mexico. Revista Mexicana de Fitopatologia 21:300-304.

Mosse B (1973). Plant growth responses to vesicular-arbuscular mycorrhiza. IV. In soil given additional phosphate. New Phytologist 72:127–136.

Mosse B, Hayman DS (1971). Plant growth responses to vesicular-arbuscular mycorrhiza. II. In unsterilized field soils. New Phytologist 70:29–34.

Mueller GM, Bills GF, Foster MS (2004). Biodiversity of Fungi: Inventory and Monitoring Methods. Academic Press. 777p.

Mulè G, Susca A, Stea G, Moretti A (2004). Specific detection of the toxigenic species *Fusarium proliferatum* and *F. oxysporum* from asparagus plants using primers based on calmodulin gene sequences. FEMS Microbiology letters 110:495-502.

Navia E, Gómez J (1999). Determination of the causal agent of the pink root rot of Welsh onion (*Allium fistulosum*) in Guambia, municipality of Silvia, department of Cauca. ASCOLFI-Informa 25:56.

Nei M, Li WH (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences USA 76:5269-5273.

Netzer D, Rabinowitch HD, Weintal C (1985). Greenhouse technique to evaluate onion resistance to pink root. Euphytica 34:385-391.

Nicholson P, Simpson DR, Weston G, Rezanoor HR, Lees AK, Parry DW, Joyce D (1998). Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53:17-37.

Niks RE, Marcel TC (2009). Nonhost and basal resistance: how to explain specificity? New Phytologist 182: 817–828.

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998). Multiple evolucionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences USA 95:2044-2049.

Oehl F, Sieverding E, Ineichen K, Mäder P, Boller T, Wiemken A (2003). Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. Applied and Environmental Microbiology 69:2816-2824.

Oehl F, Sieverding E, Mäder P, Dubois D, Ineichen K, Boller T, Wiemken A (2004). Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhiza fungi. Oecologia 138:574-583.

Oehl F, Sieverding E, Ineichen K, Ris E-A, Boller T, Wiemken A (2005). Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytologist 165:273–283.

Özer N, Koycu D, Chilosi D, Magro P (2004). Resistance to Fusarium basal rot of onion in greenhouse and field and associated expression of antifungal compounds. *Phytoparasitica* 32:388-394.

Owusu-Bennoah E, Mosse B (1979). Plant growth responses to vesicular-arbuscular mycorrhiza. XI. Field inoculation responses in barley, lucerne and onion. New Phytologist 83:671-679.

Pandey A, Pandey R, Negi KS, Radhamani (2008). Realizing value of genetic resources of Allium in India. Genetic Resources and Crop Evolution 55:985-994.

Parke JL, Kaeppler SW (2000). Effects of genetic differences among crop species and cultivars upon the arbuscular mycorrhizal symbiosis. In: Kapulnik Y, Douds, DD (eds). Arbuscular

mycorrhizas: physiology and function. Kluver Academic Publ, Dordrecht-Boston-London. p.131-146.

Payne RW, DA Murray, SA Harding, DB Baird, Soutar DM (2006). GenStat for Windows (9th Edition) Introduction. VSN International, Hemel Hempstead.

Peraza-Echeverria S, Dale JL, Harding RM, Smith MK, Collet C. (2008). Characterization of disease resistance gene candidates of the nucleotide binding site (NBS) type from banana and correlation of a transcriptional polymorphism with resistance to *Fusarium oxysporum* f.sp *cubense* race 4. Molecular breeding 22(4):565-579.

Plenchette C, Fortin JA Furlan V (1983). Growth responses of several plant species to mycorrhizae in a soil of moderate P-fertility I. Mycorrhizal dependency under field conditions. Plant and Soil 70:199-209.

Plenchette C, Clermont-Dauphin C, Meynard JM, Fortin JA (2005). Managing arbuscular mycorrhizal fungi in cropping systems. Canadian Journal of Plant Science 85:31-40.

Portas, CAM (1973). Development of the root systems during the growth of some vegetable crops. Plant and Soil 39:507-518.

Porter DR, Jones HA (1933). Resistance of some of the cultivated species of *Allium* to pink root (*Phoma terrestris*). Phytopathology 23:290-298.

Powell CL, Clark GE, Verberne NJ (1982). Growth response of four onion cultivars to several isolates of VA mycorrhizal fungi. New Zealand Journal of Agricultural Research 25:465-470.

Pozo MJ, Azcón-Aguilar C (2007). Unraveling mycorrhiza-induced resistance. Current opinion in plant biology 10:393-398.

Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM, Azcon-Aguilar C (2002). Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. Journal of Experimental Botany 53:525-534.

Ramamoorthy V, Zhao XH, Snyder AK, Xu, JR, Shah, DM (2007). Two mitogen-activated protein kinase signalling cascades mediate basal resistance to antifungal plant defensins in *Fusarium graminearum*. Cellular microbiology 9:1461-1506.

Rakesh K, Jalali BL, Hari C (2004). Interaction between VA-mycorrhizal fungi and soil - borne plant pathogens of chickpea. Legume-Research 27:19-26.

Recorbet G, Steinberg C, Olivain C, Edel V, Trouvelot S, Dumas-Gaudot E, Gianinazzi S, Alabouvette C. (2003). Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum.* New Phytologist 159:73-92.

Redecker D (2000). Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73-80.

Redecker D, Hijri I, Wiemken A (2003). Molecular identification of arbuscular mycorrhizal fungi in roots: perspectives and problems. Folia geobotanica 38:113-124.

Ricroch A, Yockteng R, Brown SC, Nadot S (2005). Evolution of genome size across some cultivated *Allium* species. Genome 48:511-520.

Rieseberg LH and JH Willis (2007). Plant speciation. Science 317:910-914.

Righetti TL, Sandrock DR, Strik B, Vasconcelos C, Moreno Y, Ortega-Farias S, Bañados P (2007). Analysis of ratio-based responses. Journal of American Society Horticultural Science 132:3-13.

Roncero MIG, Hera, C, Ruiz-Rubio M, García Maceira FI, Madrid MP, Caracuel Z, Calero F, Delgado Jarana J, Roldán-Rodríguez R, Martínez-Rocha AL, Velasco C, Roa J, Martín-Urdiroz M, Córdoba D, Di Pietro A. (2003). *Fusarium* as a model for studying virulence in soilborne

plant pathogens. Physiological and molecular plant pathology 62:87-98.

Rossing WAH, Zander P, Josien E, Groot JCJ, Meyer BC, Knierim A (2007). Integrative modeling approaches for analysis of impact of multifunctional agriculture: a review for France, Germany and the Netherlands. Agriculture, Ecosystems and Environment 120:41-57.

Ryan MH, Chilvers GA, Dumaresq DC (1994). Colonisation of wheat by VA-mycorrhizal fungi was found to be higher on a farm managed in an organic manner than on a conventional neighbour. Plant and Soil 160:33–40.

Ryan MH, Graham JH (2002). Is there a role for arbuscular mycorrizal fungi in production agriculture? Plant and Soil 244:263-271.

Ryan MH, Small DR, Ash JE (2000). Phosphorus controls the level of colonisation by arbuscular mycorrhizal fungi in conventional and biodynamic irrigated dairy pastures. Australian Journal of Experimental Agriculture 40:663–670.

Salzer P, Bonanomi A, Beyer K, Vögeli-Lange R, Aeschbacher RA, Lange J, Wiemken A, Dongjin Kim D, Cook DR, Boller T (2000). Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. Molecular plant-microbe interaction 13: 763–777.

Sasa M, Zahka G, Jakobsen I (1987). The effect of pretransplant inoculation with VA mycorrhizal fungi on the subsequent growth of leeks in the field. Plant and Soil 97:279-283.

Sawers RJH, Gutjahr C, Paszkowski U (2008). Cereal mycorrhiza: an ancient symbiosis in modern agriculture. Trends in Plant Science 13:93-97.

Schnitzler WH (2004). Pest and disease management of soilless culture. Acta Horticulturae 648: 191-203.

Schultz PA, Miller RM, Jastrow JD, Rivetta CV, Bever JD (2001). Evidence of a mycorrhizal mechanism for the adaptation of *Andropogon gerardii* (*Poaceae*) to high- and low-nutrient prairies. American Journal of Botany 88:1650–1656.

Schwarzott D, Walker C, Schüßler A (2001). *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales) is nonmonophyletic. Molecular Phylogenetics and Evolution 21:190-197.

Scholten OE, Van Heusden AW, Khrustaleva LI, Burger-Meijer K, Mank RA, Antonise RGC, Harrewijn LJ, Van Haecke W, Oost EH, Peters RJ, Kik C (2007). The long and winding road leading to the successful introgression of downy mildew resistance into onion. Euphytica 156:345-353.

Scullion J, Eason WR, Scott EP (1998). The effectivity of arbuscular mycorrhizal fungi from high input conventional and organic grassland and grass-arable rotations. Plant and Soil 204:243-254.

Shigyo M, Kik C (2008). Onion. In: J. Prohens and F. Nuez (eds). Vegetables, Vol. 2. Handbook of Plant Breeding. Springer Verlag. Berlin. p.121-162.

Shinmura A (2002). Studies on the ecology and control of welsh onion root rot caused by *Fusarium redolens*. Journal of General Plant Pathology 68:265.

Shinmura A, Sakamoto N, Hayashi T, Hoshi H, Tanii A (1998). Occurrence of *Fusarium* root rot of Welsh onion caused by *F. oxysporum*. Bulletin of Hokkaido Prefectural Agricultural Exp. Stat. 74:35-41.

Simay EI (1990). Garlic rot caused by *Fusarium proliferatum* (Matsushima) Nirenferg var. *minus* Nirenferg in Hungary. Novenyvedelem 26:397-399.

Simons G, Groenendijk J, Wijbrandi J, Reijans M, Groenen J, Diergaarde P, Van der Lee T, Bleeker M, Onstenk J, de Both M, Haring M, Mes J, Cornelissen B, Zabeau M, Vos P (1998). Dissection of the fusarium I2 gene cluster in tomato reveals six homologs and one active gene copy. Plant Cell 10:1055–1068.

Sjöberg J, Persson P, Martensson A, Mattsson L, Adholeya A, Alström S. (2004). Ocurrence of *Glomrycota* spores and some arbuscular mycorrhiza fungal species in arable fields in Sweden. Acta Agriculturae Scandinavica B 54:202-212.

Smith FA (2000). Measuring the influence of mycorrhizas. New Phytologist 148:4-6.

Stadnik MJ, Dhingra OD (1995). Reaction of onion seeds and seedlings to *Fusarium oxysporum* f. sp. *cepae* and its relation to bulb basal rot. Fitopatologia Brasilera 20:429-433.

Stadnik MJ, Dhingra OD (1996) Response of onion genotypes to Fusarium oxysporum f. sp. cepae during the growth phase and in storage. Fitopatologia Brasileira 21:431-435.

Stankovic S, Levic J, Petrovic T, Logrieco A, Moretti A (2007). Pathogenicity and mycotoxin production by *Fusarium proliferatum* isolated from onion and garlic in Serbia. European Journal of Plant Pathology 118:165-172.

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995). Altered growth of *Fusarium oxysporum* f.sp. *chrysanthemi* in an *in vitro* dual culture system with the vesicular arbuscular mycorrhizal fungus *Glomus intraradices* growing on *D. carota* transformed roots. Mycorrhiza 5:431-438.

Stevenson M, Armstrong SJ, Ford-Lloyd BV, Jones GH (1998). Comparative analysis of crossover exchanges and chiasmata in *Allium cepa* × *fistulosum* after genomic *in situ* hybridization (GISH). Journal Chromosome Research 6:567-574.

Strack D, Fester T, Hause B, Schliemann W, Walter MH (2003). Arbuscular mycorrhiza: biological, chemical, and molecular aspects. Journal of Chemical Ecology 29:1955-1979.

Stribley DP (1990). Mycorrhizal associations and their significance. In: Rabinowitch HD, Brewster JL (eds.), Onions and Allied Crops. CRC Press, Boca Raton. Vol. II, pp. 85-101.

Stuthman DD (2002). Contribution of durable disease resistance to sustainable agriculture. Euphytica 124:253-258.

Swift CE, Wickliffe ER, Schwartz HF (2002). Vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cepae* from onion in Colorado. Plant Disease 86:606-610.

Swetha Priya R, Subramanian RB (2008). Isolation and molecular analysis of R-gene in resistant *Zingiber officinale* (ginger) varieties against *Fusarium oxysporum* f. sp. *zingiberi*. Bioresource technology 99(11):4540-4543.

Swofford DL (2003). PAUP*: Phylogenetic analysis using parsimony (* and other methods), version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts.

Sýkorová Z, Wiemken A, Redecker A (2007). Cooccurring *Gentiana verna* and *Gentiana acaulis* and their neighboring plants in two swiss upper montane meadows harbor distinct arbuscular mycorrhizal fungal communities. Applied and Environmental Microbiology 73:5426-5434.

Tanksley SD, McCouch SR (1997). Seed banks and molecular maps: unlocking genetic potential from the wild. Science 277: 1063-1066.

Tawaraya K, Tokairin K, Wagatsuma T (2001). Dependence of *Allium fistulosum* cultivars on the arbuscular mycorrhizal fungus, *Glomus fasciculatum*. Applied Soil Ecology 17:119-124.

Ter Braak CJF, Smilauer P (2004). Program CANOCO Version 4.53. Plant Research International, Wageningen University and Research Centre. Box 100, 6700 AC Wageningen, the Netherlands.

Valdez J, Makuch M A, Marini GV (2004). Patogenicidad de aislamientos de *Fusarium* spp. en plántulas de cebolla (*Allium cepa* L). 27th Congreso Argentino de Horticultura. Villa de Merlo, Argentina. p.60.

Van der Heijden MGA, Bardgett RD, Van Straalen NM (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11:296-310.

Van der Heijden MGA, Streitwolf-Engel R, Riedl R, Siegrist S, Neudecker A, Ineichen K, Boller T, Wiemken A, Sanders IR (2006). The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytologist 172:739-752.

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998). Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69-72.

Van der Linden CG, Wouters DCAE, Mihalka V, Kochieva EZ, Smulders MJM, Vosman B (2004). Efficient targeting of plant disease resistance loci using NBS profiling. Theoretical and Applied Genetics 109:384–393.

Van der Meer QP, De Vries JN (1990). An interspeciific cross between *Allium roylei* Stearn and *Allium cepa* L and its backcross to *Allium cepa*. Euphytica 47:29-31.

Van der Peer Y, De Watcher R (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Computer Applications in the Biosciences 10:569-570.

Van Heusden AW, Van Ooijen JW, Vrielink-van Ginkel R, Verbeek WHJ, Wiestma WA, Kik C (2000a). A genetic map of an interspecific cross in *Allium* based on amplified fragment length polymorphism (AFLP) markers. Theoretical and Applied Genetics 100: 118-126.

Van Heusden AW, Shigyo M, Tashiro Y, Vrielink-van Ginkel R, Kik C (2000b). AFLP linkage group assignment to the chromosomes of *Allium cepa* L. via monosomic addition lines. Theoretical and Applied Genetics 100:480–486.

Van Ooijen JW, Boer MP, Jansen RC, Maliepaard C (2002). MapQTL 4.0: software for the calculation of QTL positions on genetic maps. Plant Research International, Wageningen.

Van Ooijen JW, Voorrips RE (2001). JoinMap 3.0: software for the calculation of genetic linkage maps. Plant Research International, Wageningen.

Van Raamsdonk LWD, Ensink W, Van Heusden AW, Vrielink van Ginkel M, Kik C (2003). Biodiversity assessment based on cpDNA and crossability analysis in selected species of *Allium* subgenus *Rhizirideum*. Theoretical & Applied Genetics 107:1048-1058.

Van Raamsdonk LWD, Vrielink Van Ginkel M, Kik C (2000). Phylogeny reconstruction and hybrid analysis in *Allium* subgenus *Rhizirideum*. Theoretical and Applied Genetics 100:1000–1009.

Villanueva-Mosqueda E, Jiang J, Havey M (2000). GISH analysis reveals chromosome regions of *Allium fistulosum* among progenies from advances backcrosses to *Allium cepa*. *Allium* Improvement Newsletter 10:6-9.

Villeveille M (1996). Improvement of a screening test for *Fusarium oxysporum* Schlect. emend. Snyder and Hansen f.sp. *cepae*. Acta Botanica Gallica 143:109-115.

Voorrips RE (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. Journal of Heredity 93:77–78.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

Waalwijk C, Kastelein P, De Vries I, Kerényi Z, Van der Lee T, Hesselink T, Köhl J, Kema G (2003). Major changes in *Fusarium* spp. in wheat in The Netherlands. European Journal of Plant Pathology 109:743-754.

Walters TW, Ellerbrock LA, Van der Heide JJ, Lorbeer JW, LoParco DP (1996). Field and greenhouse procedures to evaluate onions for *Botrytis* leaf blight resistance. HortScience 31:436-438.

Wang YY, Vestberg M, Walker C, Hurme T, Zhang X, Lindström K (2008). Diversity and infectivity of arbuscular mycorrhizal fungi in agricultural soils of the Sichuan Province of mainland China. Mycorrhiza 18:59-68.

Wirsel SGR. 2004. Homogeneous stands of a wetland grass harbour diverse consortia of arbuscular mycorrhizal fungi. FEMS Microbiology Ecology 48:129-138.

Wright DP, Scholes JD, Read DJ, Rolfe SA (2005). European and African maize cultivars differ in their physiological and molecular responses to mycorrhizal infection. New Phytologist 167:881-896.

Xiao J, Grandillo S, Ahn SN, McCouch SR, Tanksley SD, Yuan L (1996). Genes from wild rice improve yield. Nature 384:223–224.

Yuan JZ, Tedman J, Ali L, Liu J, Taylor J, Lightfoot D, Iwata M, Pauls KP (2009). Different responses of two genes associated with disease resistance loci in maize (*Zea mays* L.) to 3-allyloxy-1,2-benzothiazole 1,1-dioxide. Current issues in molecular biology 11(suppl.1):I85-I94.

Yaguchi S., McCallum J, Shaw M., Pither-Joyce M., Onodera S., Shiomi N., Yamauchi N, Shigyo M (2008). Biochemical and genetic analysis of carbohydrate accumulation in *Allium cepa* L. Plant Cell Physiology 49:730-739.

Zhu YG, Smith SE, Barrit AR, Smith FA (2001). Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. Plant and soil 237:249-255.

Summary

Onion (*Allium cepa* L.) is one of the main vegetable crops worldwide. Onion cropping systems usually make use of large amounts of inputs. High-yielding crops rely on chemical control of diseases and large use of fertilizers. Agricultural systems involving more sustainable ways of production, like organic and low-input agricultural systems, gained interest in the last decade. In these systems, crop yield is in balance with other considerations like sustainability of the agro-ecosystem, management of biodiversity, and minimum impact on environment.

The search for a broader genetic background in cultivated species by combining or introducing new sources of resistance enhances the possibilities for sustainable agricultural systems. Below ground, arbuscular mycorrhizal fungi (AMF) have received increasing attention in the context of sustainable agriculture. Symbiosis with AMF usually improves the performance of cultivated host species under sub-optimal growing conditions, with benefits such as improved uptake of phosphorus and protection against diseases. Plant genetic variation for the benefit from AMF has been described for a number of cultivated species, including onion, and opens opportunities for plant breeding.

Fusarium basal rot (FBR) and the limited ability of onion rooting system to take up nutrients such as phosphorus are important threats for onion cultivation. Onion related species may be a source of genetic variation for resistance to FBR and for the benefit obtained from the symbiosis with AMF. This PhD research aimed to study the potential contribution from *A. fistulosum* and *A. roylei* to these traits, as well as the genetic basis of these traits in an *A. cepa* x (*A. roylei* x *A. fistulosum*) population.

The introductory **Chapter 1** deals with general aspects comprising the background of this thesis research: use of *Allium* species related to onion in breeding, breeding for resistance to onion basal rot caused by *Fusarium* species, AMF in agriculture, and the relevance of plant genetic variation in the benefit from AMF.

In **Chapter 2**, resistance to Fusarium basal rot in *Allium* species was investigated using different *Fusarium* isolates. A collection of isolates from Uruguay, The Netherlands and other countries was analysed using AFLP markers. The most abundant species was *F. oxysporum* followed by *F. proliferatum.* Isolates of *F. oxysporum* clustered in two clades, which suggested different

origins of *F. oxysporum* isolates pathogenic to onion. Onion and six related *Allium* species (*A. schoenoprasum, A. fistulosum, A. galanthum, A. pskemense, A. roylei, A. vavilovii*) were screened for FBR resistance using one *F. oxysporum* isolate from each clade, and one *F. proliferatum* isolate. *A. fistulosum* showed high level of resistance to these three isolates, and *A. roylei* intermediate levels of resistance. *A. fistulosum, A. roylei* and *A. galanthum* were identified as potential sources of resistance to *Fusarium* in onion.

Chapter 3 presents the genetic analysis of resistance to FBR in a tri-hybrid *A. cepa x (A. roylei x A. fistulosum)* population. Screening was carried out on adult plants, in a greenhouse, using an aggressive *F. oxysporum* isolate as inoculum. Symptoms were scored as wilting (before harvest), as basal rot observed at harvest, and rotting after four weeks storage at 27°C. Resistance to FBR from *A. fistulosum* was dominantly expressed in the *A. roylei* x *A. fistulosum* parental hybrid and the tri-hybrid population. FBR reduced weight of *A. cepa* and susceptible genotypes in comparison to non-inoculated controls. A molecular linkage map based on AFLP markers was developed for the *A. roylei x A. fistulosum* parent, with 111 AFLP markers on the eight linkage groups assigned to chromosomes. One quantitative trait loci (QTL) for FBR resistance from *A. roylei* was mapped on a distal region of chromosome 2, and one QTL from *A. fistulosum* on the long arm of chromosome 8. These two QTLs showed additive effects, and accounted for 30 to 40% of the total variation for FBR incidence and FBR severity, at harvest and after storage. Each QTL separately had significant effect on FBR, but did not confer complete resistance. Therefore, more QTLs from *A. fistulosum* remain to be discovered. The AUDPC summed up for differences in timing of the disease regarding wilting during the season, harvest and post-harvest scores. Four QTLs for AUDPC were located: the two QTL described and two additional ones on chromosomes 4 and 8 from *A. fistulosum*. The identification of QTLs for FBR resistance from *A. fistulosum* and *A. roylei* open positive prospects for the introgression of these resistances into onion.

In **Chapter 4**, as a first step in studying the importance of AMF for onion cultivation, genetic diversity and colonization levels of naturally occurring AMF in onion roots were studied to compare organic and conventional farming systems in the Netherlands. In 2004, twenty onion fields were sampled in a balanced survey between farming systems and between two regions, namely Zeeland and Flevoland. In 2005, nine conventional and ten organic fields were additionally surveyed in Flevoland. Ten plants per field were randomly sampled. All plants were colonized by AMF, with 60% for arbuscular and 84% hyphal colonization as grand means. In Zeeland, onion roots from organic fields had higher colonization levels than those from conventional fields. Onion yields in conventional farming

were positively correlated with colonization level. AMF phylotypes were identified by rDNA sequencing. Overall, fourteen AMF phylotypes were identified. The number of phylotypes per field ranged from one to six. Two phylotypes associated with *Glomus mosseae – coronatum* and *G. caledonium – geosporum* species complexes were the most abundant, whereas other phylotypes were infrequently found. Organic and conventional farming systems had similar number of phylotypes per field and Shannon diversity indices. A few organic and conventional fields had larger number of phylotypes, comprising phylotypes associated with *Glomus*-B, *Archaeospora* and *Paraglomus*. This research suggests that farming systems as such did not influence AMF diversity, but rather specific environmental conditions or agricultural practices.

Allium fistulosum is a source to improve rooting system in onion, and also has a large response to AMF. Therefore, *A. fistulosum* may be a potential source to improve these traits in onion. In **Chapter 5**, the response to inoculation with *Glomus intraradices* of *Allium* species and progeny plants of the cross between *A. cepa* x (*A. roylei* x *A. fistulosum*) was evaluated in two independent greenhouse experiments (2006 and 2007). Two indices were employed: mycorrhizal benefit (MB), as the difference in plant weight between mycorrhizal (AM) and non-mycorrhizal (NM) treatments, and mycorrhizal breeding value (MV) as the average plant weight between these treatments. AM plant weights of *Allium* species were significantly larger than the corresponding NM. Tri-hybrid genotypes showed transgressive segregation for plant weight, MB, and MV. QTLs contributing in both experiments to the response to mycorrhiza were located on the linkage map of the *A. roylei x A. fistulosum* parental genotype. Two QTLs from *A. roylei* for MB, MV, and plant weight of AM plants were detected on chromosomes 2 and 3. A QTL associated with *A. fistulosum* alleles for MV (but not MB), plant weight of AM and NM plants, and the number of stem-borne roots was detected on linkage group 9. Positive interactions between plant weight, larger rooting system and enhanced response to mycorrhiza were observed, which open prospects to combine these traits in the development of more robust onion cultivars. Mycorrhizal responsiveness (MR), as defined in the literature, was negatively correlated with plant weight in the NM treatment, and was rejected as an index for breeding purposes.

A general discussion on the findings of this PhD research is presented in **Chapter 6**. The value of the tri-hybrid population for introgressions of traits from *A. roylei* and *A. fistulosum* is discussed and specifically the potential contribution to improve the levels of resistance to FBR and the benefit from AMF in onion. Other topics addressed are the genetic variation among *Fusarium* isolates pathogenic to onion and the extent of resistance from related species, the

significance of AMF diversity for agriculture, and the response to mycorrhiza as a goal in plant breeding. Arising future research lines are indicated, like the exploitation of AMF to obtain protection against Fusarium basal rot and other onion diseases.

Samenvatting

Ui (*Allium cepa* L.) is een van de belangrijkste groentegewassen wereldwijd. Om uien te kunnen telen worden gewoonlijk grote hoeveelheden inputs gebruikt. Hoog-productieve gewassen zijn afhankelijk van chemische gewasbescherming en grote hoeveelheden meststoffen. Teeltsystemen gericht op een meer duurzame productiewijze, zoals biologische en low-input landbouw, hebben de laatste jaren meer aandacht gekregen. In dergelijke landbouwsystemen wordt niet alleen gestreefd naar een hoge gewasopbrengst maar wordt ook rekening gehouden met duurzaamheid van het agro-eco systeem, management van de biodiversiteit en een minimale schade aan de omgeving.

De zoektocht naar een bredere genetische achtergrond in cultuurgewassen door het combineren of introduceren van nieuwe resistentiebronnen vergroot de mogelijkheden voor duurzame landbouwsystemen. Ondergronds is er meer aandacht voor arbusculaire mycorrhizaschimmels (AMF) in de context van duurzame landbouw. Symbiose met AMF verbetert over het algemeen de groei van gecultiveerde gewassen onder sub-optimale teeltomstandigheden, doordat planten profijt hebben van bijvoorbeeld een verbeterde opname van fosfor en weerbaarheid tegen ziekten. Genetische variatie binnen één soort voor profijt van AMF is beschreven voor een aantal cultuurgewassen waaronder ui en biedt kansen voor veredeling.

Fusarium bolrot (FBR) en de geringe mogelijkheid van uienwortels om voedingsstoffen op te nemen zoals fosfor zijn belangrijke bedreigingen voor de uienteelt. Soorten die verwant zijn aan ui kunnen een bron zijn van genetische variatie voor resistentie tegen FBR en voor het profijt van de symbiose met AMF. Dit promotie-onderzoek had als doel om de potentiële bijdrage aan eerder genoemde eigenschappen uit *A. fistulosum* en *A. roylei* te onderzoeken, alsmede de genetische basis ervan in een *A.cepa* x (*A. roylei* x *A. fistulosum*) populatie.

Het inleidende **Hoofdstuk 1** beschrijft algemene aspecten over de achtergrond van dit promotie-onderzoek: het gebruik van aan ui verwante *Allium*-soorten voor veredeling, resistentieveredeling tegen *Fusarium* bolrot, AMF in

landbouwkundige systemen en de waarde van genetische variatie voor het profijt van AMF.

In **Hoofdstuk 2** wordt een beschrijving gegeven van resistentie die gevonden is tegen *Fusarium* bolrot in *Allium*-soorten door gebruik te maken van verschillende *Fusarium* isolaten. Een collectie van stammen uit Uruguay, Nederland en andere landen is onderzocht met behulp van AFLP merkers. De meest voorkomende soort bleek *F. oxysporum* te zijn, gevolgd door *F. proliferatum*. Isolaten van *F. oxysporum* waren geclusterd in twee evolutionaire groepen, een aanwijzing voor verschillende oorsprong van *F. oxysporum*isolaten pathogeen op ui. Ui en zes verwante *Allium* soorten (*A. schoenoprasum, A. fistulosum, A. galanthum, A. pskemense, A. roylei, A. vavilovii*) zijn getoetst op FBR resistentie door gebruik te maken van één *F. oxsporum* isolaat van elke groep en één *F. proliferatum* isolaat. *A. fistulosum* had het hoogste resistentieniveau tegen deze drie isolaten en *A. roylei* een intermediair niveau. *A. fistulosum, A. roylei* en *A. galanthum* zijn geïdentificeerd als potentiële resistentiebronnen tegen *Fusarium* in ui.

Hoofdstuk 3 beschrijft de genetische analyse van resistentie tegen FBR in een tri-hybride populatie van *A.cepa* x (*A. roylei* x *A. fistulosum*). De screening is uitgevoerd op volwassen planten in een kas, waarbij gebruik gemaakt werd van een agressief *F. oxysporum* isolaat als inoculum. De volgende symptomen zijn waargenomen: verwelking (voor de oogst), bolrot op het moment van oogst en na vier weken bewaring bij 27 °C, en bolgewicht. Resistentie tegen FBR uit *A. fistulosum* bleek dominant tot expressie te komen in de *A. roylei* x *A. fistulosum* hybride en in de tri-hybride populatie. Planten met FBR hadden een lager gewicht, zoals *A. cepa* en de vatbare genotypes, in vergelijking tot de niet-geïnoculeerde controleplanten. Een moleculaire merkerkaart gebaseerd op AFLP merkers is ontwikkeld voor de *A. roylei* x *A. fistulosum* ouder bestaande uit 111 AFLP merkers verdeeld over de acht chromosomen. Er is een QTL (Quantitative Trait Locus) gevonden voor FBR-resistentie afkomstig uit *A. roylei* op het uiteinde van chromosoom 2 en voor *A. fistulosum* op de lange arm van chromosoom 8. Deze twee QTLs vertonen additieve effecten en dragen 30-40% bij aan de totale variatie voor FBR incidentie en aantasting, zowel bij oogst als na bewaring. Elk QTL afzonderlijk had een significant effect op FBR, maar resulteerde niet in een complete resistentie. Dat betekent dat er waarschijnlijk nog meer QTLs gevonden kunnen worden uit *A. fistulosum*. De AUDPC (Area Under Disease Progress Curve) werd berekend om het verloop van de ziekteverschijnselen zoals verwelking gedurende het seizoen, aantasting bij oogst en na bewaring in kaart te brengen. Vier QTLs werden gevonden voor AUDPC:

de twee QTLs die al eerder beschreven werden en twee extra QTLs op chromosoom 4 en 8 uit *A. fistulosum*. De identificatie van QTLs voor *A. fistulosum* en *A. roylei* bieden nieuwe mogelijkheden voor de introgressie van deze resistenties in ui.

In **Hoofdstuk 4** is een eerste stap gemaakt in het bestuderen van het belang van AMF voor de uienteelt. Dit is gedaan door uienwortels te verzamelen in biologische en gangbare teeltsystemen in Nederland en de diversiteit van AMF en het percentage kolonisatie door AMF te bestuderen. In 2004 zijn 20 uienvelden gekozen voor een vergelijking tussen teeltsystemen en regio's in Nederland, namelijk Zeeland en Flevoland. In 2005 zijn 9 gangbare en 10 biologische bedrijven in Flevoland opnieuw onderzocht. Per veld zijn 10 planten verzameld. Alle planten bleken gekoloniseerd door AMF, over het gehele experiment gemiddeld was 60% van de wortellengte bezet met arbuscules en 84% met hyphen. In Zeeland werden hogere niveaus van kolonisatie gevonden in biologisch geteelde uien dan in gangbaar geteelde uien. De uienopbrengst bleek positief gecorreleerd met het niveau van kolonisatie in de gangbare velden. AMF -"soorten" zijn geïdentificeerd met moleculaire methoden. Over het gehele experiment zijn 14 AMF-soorten gevonden. Het aantal soorten per veld varieerde van één tot zes. De twee soortendie het meest voorkwamen, behoorden tot het *Glomus mosseae – G. coronatum* complex en het *G. caledonium – G. geosporum* complex. De andere soorten kwamen minder vaak voor. Het aantal soorten per veld en de diversiteit was gelijk voor biologische en gangbare velden. Enkele biologische en gangbare velden hadden een groter aantal soorten per veld. Het onderzoek liet zien dat teeltsystemen als zodanig de AMF diversiteit niet beïnvloeden, maar dat diversiteit eerder afhangt van specifieke omgevingsfactoren of landbouwkundige handelingen.

Allium fistulosum kan gebruikt worden om het wortelstelsel van ui te verbeteren en heeft ook een grote respons op AMF. Daarom wordt *Allium fistulosum* gezien als een potentiële bron om deze eigenschappen in ui te verbeteren. In **Hoofdstuk 5** is de groei, in aanwezigheid van de AMF-schimmel *G. intraradices*, van *Allium* soorten en de nakomelingen van de kruising tussen *A. cepa* x (*A. roylei* x *A. fistulosum*) onderzocht in twee kasproeven. Twee indices zijn berekend: MB (mycorrhiza benefit), het verschil in plantgewicht tussen planten met (AM) en zonder (NM) mycorrhiza; en MV (mycorrhiza breeding value), het gemiddelde van deze twee behandelingen. Het gewicht van *Allium* planten met mycorrhiza was significant hoger dan dat van de bijbehorende NM-planten. De tri-hybride genotypen vertoonden transgressieve uitsplitsing voor plantgewicht, MB en MV. QTLs voor mycorrhizarespons die in beide

experimenten gevonden werden zijn geplaatst op de koppelingskaart van de *A. roylei x A. fistulosum* ouder. Er zijn twee QTLs gevonden uit *A. roylei* voor MB, MV, en plantgewicht van AM-planten op chromosomen 2 en 3 en één QTL geassocieerd met *A. fistulosum* voor MV (maar niet MB), plantgewicht van AM- en NM-planten, en het aantal primaire wortels op koppelingsgroep 9. Positieve interacties zijn gevonden tussen plantgewicht, groter wortelstelsel en grootte van de respons op mycorrhizaschimmels. Dit biedt perspectief voor het ontwikkelen van robuuste uienrassen. Mycorrhizaresponsiviteit (MR), zoals gedefinieerd in de literatuur, is als index voor veredeling verworpen, omdat deze negatief gecorreleerd is met plantgewicht van de NM-planten.

In **Hoofdstuk 6** wordt de waarde van de tri-hybride populatie voor introgressie van eigenschappen uit *A. roylei* and *A. fistulosum* bediscussieerd, naast specifiek de potentiële bijdrage aan het verbeteren van de resistentie tegen FBR en het profijt van AMF in ui. Andere onderwerpen, die aan bod komen zijn genetische variatie tussen *Fusarium*-isolaten, die pathogeen zijn voor ui, het gebruik van aan ui verwante soorten, de betekenis van AMF diversiteit voor de landbouw en de mycorrhizarespons als doel voor plantenveredelaasrs. Toekomstige onderzoekslijnen worden aangegeven, zoals het gebruik van AMF om de weerbaarheid tegen FBR en andere uienziekten te vergroten.

Resumen

La cebolla (*Allium cepa* L.) es una de las principales hortalizas en el mundo. Los sistemas de producción de cebolla generalmente hacen uso de grandes cantidades de insumos. La obtención de altos rendimientos se basa en el control químico de enfermedades y en altas cantidades de fertilizantes. Los sistemas agrícolas que involucran formas más sostenibles de producción como la producción orgánica y la producción de bajos insumos ganaron interés en la última década. En estos sistemas, el rendimiento del cutlivo está balanceado con otras consideraciones como la sostenibilidad del agro-ecosistema, el manejo de la biodiversidad, y el impacto reducido sobre el ambiente.

La búsqueda de una base genética amplia en las especies cultivadas a través de la combinación o introducción de nuevos fuentes de resistencia, amplía las posibilidades en los sistemas agrícolas sostenibles. En el suelo, los hongos micorríticos arbusculares (AMF) han recibido creciente atención en el contexto de la agricultura sostenible. La simbiosis con AMF generalmente mejora el crecimiento de las especies huéspedes cultivadas bajo condiciones sub-óptimas,

como resultado de beneficios como la mejor absorción de fósforo y la protección contra enfermedades. La variación genética en la especie huésped en el beneficio obtenido de la asociación con AMF ha sido descripta en viarias especies cultivadas, incluida la cebolla, y abre oportunidades para el mejoramiento genético.

La podredumbre basal causada por Fusarium (FBR) y la limitada habilidad del sistema radicular de la cebolla para absorver nutirentes como el fósforo, son importantes limitantes para el cultivo. Especies emparentadas a la cebolla pueden ser una fuente de variación genética para la resistencia a FBR y para el beneficio obtenido a partir de la simbiosis con AMF. Esta investigación de doctorado buscó estudiar la contribución potencial de *A. fistulosum* y de *A. roylei* en estos caracteres, así como estudiar la base genética de estos caracteres en una población *A. cepa* x (*A. roylei* x *A. fistulosum*).

El **capítulo 1**, introductorio, trata aspectos generales que comprenden los antecedentes de esta investigación de doctorado: el uso en el mejoramiento genético de especies del género *Allium* relacionadas a la cebolla, el mejoramiento por resistencia a la podredumbre basal causada por especies de *Fusarium*, las micorrizas en la agricultura, y la relevancia de la variación genética vegetal en el beneficio obtenido de las micorrizas.

En el **capítulo 2**, la resistencia a la podredumbre basal en especies del género *Allium* fue investigada utilizando diferentes aislamientos de *Fusarium.* Una colección de aislamientos de Uruguay, Holanda y otros países fue analizada utilizando marcadores AFLP. La especie más abundante fue *F. oxysporum* seguida de *F. proliferatum.* Los aislamientos de *F. oxysporum* fueron agrupados en dos clades, lo que sugiere diferentes orígenes de los aislamientos de *F. oxysporum* patogénicos de cebolla. Accesiones de cebolla y de seis especies relacionadas (*A. schoenoprasum, A. fistulosum, A. galanthum, A. pskemense, A. roylei, A. vavilovii*) fueron evaluadas en su resistencia a FBR utilizando un aislamiento de *F. oxysporum* de cada clade, y un aislamiento de *F. proliferatum. A. fistulosum* mostró alta resistencia a estos tres aislamientos y *A. roylei* tuvo niveles intermedios de resistencia. *A. fistulosum, A. roylei* y *A. galanthum* fueron identificadas como potenciales fuentes de resistencia al *Fusarium* de la cebolla.

El **capítulo 3** presenta el análisis genético de la resistencia a FBR en una población tri-híbrida *A. cepa x (A. roylei x A. fistulosum)*. La evaluación se llevó a cabo con plantas adultas en un invernadero, utilizando como inóculo un aislamiento agresivo de *F. oxysporum.* Los síntomas fueron evaluados como marchitamiento (antes de la cosecha), como podredumbre basal observada a la

cosecha, y podriciones observadas después de cuatro semanas de almacenamiento a 27°C. La resistencia a FBR en *A. fistulosum* se expresó en forma dominante en el híbrido *A. roylei* x *A. fistulosum* y en la población tri-híbrida. FBR redujo el peso por planta de *A. cepa* y de genotipos tri-híbridos susceptibles en comparación con los controles no inoculados. Se construyó un mapa de ligamiento para el padre *A. cepa* x *A. fistulosum* basado en marcadores AFLP, con 111 marcadores AFLP en los ocho grupos de ligamientos asignados a cromosomas. Un locus de característica cuantitativa (QTL) para la resistencia a FBR fue situado en una región distal del cromosoma 2 de *A. roylei*, y un QTL de *A. fistulosum* fue situado sobre el brazo largo del cromosoma 8. Estos dos QTLs mostraron efecto aditivo y explicaron 30 a 40% de la variación total en incidencia y severidad de FBR, evaluadas a la cosecha y luego del almacenamiento. Cada QTL separadamente tuvo efecto significativo sobre FBR, pero no confirió resistencia completa. Por tanto, más QTLs de *A. fistulosum* podrían ser descubiertos. El área bajo la curva de progreso de la enfermedad (AUDPC) evidenció diferencias en el momento de aparición de la enfermedad, considerando marchitamiento previo a la cosecha, y la evaluación de podredumbres basales en la cosecha y la poscosecha. Cuatro QTLs para el AUDPC fueron localizados: los dos QTLs descriptos en los cromosomas 2 y 8, y dos QTLs adicionales sobre los cromosomas 4 y 8 de *A. fistulosum.* La identificación de QTLs para la resistencia a FBR provenientes de *A. fistulosum* y *A. roylei* abre posibilidades para la introgresión de estas resistencias en el cultivo de cebolla.

En el **capítulo 4**, como primer paso para el estudio de la importancia de los hongos micorríticos arbusculares (AMF) para el cultivo de cebolla, se estudió la diversidad genética y los niveles de colonización que naturalmente ocurren en las raíces de cebolla, comparando los sistemas de producción orgánicos y convencionales en Holanda. En 2004, veinte cultivos de cebolla fueron muestreados en un relevamiento balanceado entre los dos sistemas de producción y dos regiones: Zeeland y Flevoland. En 2005, nueve cultivos convencionales y diez orgánicos adicionales fueron relevados en Flevoland. Se muestrearon diez plantas por cultivo al azar. Todas las plantas estaban colonizadas por AMF, con 60% de colonización arbuscular y 84% de colonización con hifas como grandes medias. En Zeeland, las raíces de cebolla de cultivos orgánicos tuvieron mayores niveles de colonización que aquellas de cultivos convencionales. Los rendimientos de cebolla en cultivos convencionales estuvieron correlacionados positivamente con los niveles de colonización. Filotipos de AMF se identificaron por sequenciado del rDNA. En total, se distinguieron 14 filotipos de AMF. El número de filotipos por cultivo varió desde uno a seis. Dos filotipos asociados con

especies de los complejos *Glomus mosseae – coronatum* y con *G. caledonium – geosporum* fueron los más abundantes, mientras que otros filotipos fueron infrecuentes. Los sistemas de cultivos orgánicos y convencionales tuvieron similar número de filotipos por cultivo y similar índice de diversidad de Shannon. Unos pocos cultivos orgánicos y convencionales tuvieron mayor número de filotipos, y comprendieron filotipos asociados con *Glomus*-B, *Archaeospora* and *Paraglomus*. Esta investigación sugirió que el sistema de cultivo como tal no influencia la diversidad de AMF, sino que condiciones ambientales específicas o prácticas agrícolas específicas determinarían una mayor diversidad.

Allium fistulosum es una fuente para mejorar el sistema radicular de la cebolla, y también muestra una gran respuesta a AMF. Por lo tanto, *A. fistulosum* puede ser una fuente potencial para mejorar estos caracteres en la cebolla. En el **capítulo 5**, la respuesta a la inoculación con *Glomus intraradices* en especies de *Allium* y en plantas de la progenie del cruzamiento entre *A. cepa* x (*A. roylei* x *A. fistulosum*) fueron evaluadas en dos experimentos independientes (2006 y 2007). Dos índices fueron empleados: el beneficio micorrítico (MB), como la diferencia en el peso de la planta entre los tratamientos con micorrizas (AM) y sin micorrizas (NM), y el valor de mejoramiento micorrítico (MV), como el peso promedio de las plantas en estos dos tratamientos. Los pesos por planta de las especies de *Allium* parentales fueron significativamente mayores en el tratamiento AM que en los correspondientes NM. Los genotipos tri-híbridos mostraron segregación transgresiva para el peso por planta, MB y MV. QTLs que contribuyeron en ambos experimentos a la respuesta a las micorrizas fueron situados sobre el mapa de ligamiento del parental *A. roylei x A. fistulosum.* Dos QTLs de *A. roylei* para MB, MV y para el peso de las plantas en el tratamiento AM fueron detectados sobre los cromosomas 2 y 3. Un QTL asociado con alelos de *A. fistulosum* para MV (pero no MB), para el peso por planta en los tratamientos AM y NM, y para el número de raíces del disco basal fue detectado sobre el grupo de ligamiento 9. Fue observada interacción positiva entre el peso por planta, el mayor sistema radicular, y la mejor respuesta a micorrizas, lo cual abre perspectivas para combinar estos caracteres en el desarrollo de cultivares de cebolla más rústicos. La responsividad (MR), tal como es definida en la literatura, estuvo negativamente correlacionada con el peso por planta en el tratamiento NM, y fue descartada como índice de selección para fines de mejoramiento.

Una discusión general de los resultados de esta investigación de doctorado es presentada en el **capítulo 6.** El valor de la población tri-híbrida para la introgresión de características de *A. roylei* y de *A. fistulosum* es discutido, y en particular, las contribuciones potenciales para mejorar los niveles de resistencia a

FBR y el beneficio obtenido de AMF por la cebolla. Otros puntos abordados son la variación genética entre aislamientos de *Fusarium* patogénicos de la cebolla, la relevancia de la resistencia en especies relacionadas a la cebolla, el significado de la diversidad de AMF para la agricultura, y la respuesta a las micorrizas como objetivo en el mejoramiento genético vegetal. Futuras líneas de investigación son sugeridas, como la explotación de AMF para la obtención de protección contra la podredumbre basal causada por *Fusarium* y contra otras enfermedades del cultivo.

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"… a toda esa gente que quiso un camino nuevo pa' su pago
pero que no precisa un camino nuevo pa' llegar a mi memoria."

'… to all people who dreamed of a new way for their land
though they do not need a new way to come into my mind…'

Ruben Lena (1925-1995), Uruguay

Following a PhD programme is a rather long and important project in life. At least, that was the case for me, and the PhD experience changed me in several ways beyond the target and expected formation. Of course, such a programme required the contribution from many people who I would like to acknowledge here. So many colleagues and friends contributed direct or indirectly to come to this end that make impossible to mention everybody here. But as Ruben Lena wrote, I will have them in my mind forever.

Firstly, I thank the permanent support from Chris Kik and Olga Scholten, my daily supervisors. Olga took over the responsibility of guiding me from the second year of my research. I learnt very much from Olga on the topics covered in this research, as well as on scientific communication, and organization of research. I appreciate she managed to visit my University in Uruguay in the frame of this project. During these years Olga and I spent uncountable long conversations, and that was a good opportunity to build a friendly relationship. We had even frequent meetings in her home place, with Theo cooking for us and the *meisjes* running around, and therefore I must extend my gratitude to her whole family.

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One of the main gifts in Wageningen was the opportunity to meet Karin. Always active and lovely, Karin is the best assistant that a researcher can dream of. We shared many hours installing and evaluating experiments and working in the labs, and I learnt many experimental details from her skills. I must acknowledge the support she gave me also on all kind of issues to live in Wageningen, and the friendship we made with her and her husband Jan. Thus, I am grateful having Karin as paranymph in my defence.

Working at Plant Breeding department was a great experience, as I learnt from comments and suggestions from many people, and I found always the best attitude from everybody to help me. Firstly I would like to thanks my co-authors: *Madame* Carole Boucoiran, who taught me on molecular techniques and lab skills, Wim Koopman on phylogenetic analysis, Cees and Ineke on the Fusarium, Paul Keizer on statistical methods and philosophy, Istvan Páradi from *Magyar* and Patrick *uit Limburg* on sequencing and horses...

Sandwich programme, moving back and on, and I shifted my office more that the whole Department did... I express my gratitude to my office-mates along the years for the support and friendship I always found. My first year began with Karin, her mandarins at noon, and a really good man as Remmelt. I had the honour of taking the desk from Dirk, a marvellous gay. Then I shared office with Reza, Mishia, and Anoma, to finally move in my third year with Stefano. We made a deep friendship mixed up with *pasta* and guitars after working time in Dijkgraaf 5C with Stefano and Sonia, Pavel and Daniella. And I appreciate very much having Stefano as a paranymph in my defence.

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Curriculum Vitae

Guillermo A. Galván was born in Montevideo, Uruguay, on 14 February 1963. He obtained the degree of Agronomist Engineer in 1991 at the Facultad de Agronomía, Universidad de la República, Uruguay. He early joined the Vegetable Production team of the same University, from 1988. His research activities were centred on local genetic resources and breeding of vegetable crops. In 1991, an onion breeding programme was initiated. In 1996, he obtained a M.Sc. degree in Crop Science (Plant Breeding specialization) in Wageningen University. After that, he participated in the release of onion cultivars and the organization of onion seed certification programme. He maintained also the interest on local genetic resources of vegetables, as well as on native genetic resources of potato in a multidisciplinary team. In 2002 he was appointed assistant professor in Plant Breeding (vegetables). Several granted research projects were formulated and conducted since then. In the period 2004-2009, he began a sandwich PhD programme in Wageningen University that ended with the production of this thesis. This project opened windows for future cooperation and for increasing use of molecular tools in his breeding and genetic resources activities in Uruguay.

Address:

Centro Regional Sur (CRS)
Dept Producción Vegetal de la Facultad de Agronomía
Universidad de la República
Camino Follé km 36
90100 Progreso, Uruguay

Email:

ggalvanv@gmail.com
horticrs@fagro.edu.uy

www.fagro.edu.uy

Education Certificate of the Graduate School Production Ecology and Resource Conservation

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of Literature (5.6 ECTS)

- Literature reviews presented at PRI: breeding for resistance to FBR in onion and plant genetic control in Mycorrhiza formation and functioning (2005 and 2006)

Writing of Project Proposal (7.0 ECTS)

- Genetic analysis of resistance to soil-borne diseases in onions (Allium cepa L) and its wild relatives; proposal granted by fellowship Alban Program, European Union (2004)

Laboratory Training and Working Visits (1.4 ECTS)

- Methods in screening for resistance to Fusarium basal rot; INTA Mendoza Argentina (2005)

Post-Graduate Courses (2.8 ECTS)

- Sampling and evaluation strategies in Mycorrhiza diversity and characterization; Cost Action, European Union; Lisboa , Portugal (2005)
- Screening techniques for resistance in late blight potato; EUCABLIGHT European Conc. Action; Wageningen (2004)

Competence Strengthening / Skills Courses (2.8 ECTS)

- Writing and presenting scientific papers; SENSE Education Desk (2007)
- Academic writing; CENTA, Wageningen Univ. (2005)

Discussion Groups / Local Seminars and Other Scientific Meetings (8.4 ECTS)

- Agricultural production systems in temperate systems; Prof. A. van Bruggen (2005)
- Seminars at the PRI Biodiversity and Breeding Business Unit ; one hour weekly (2004 -07)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.2 ECTS)

- Who pull the strings? (over the policy of scientific journals); PE&RC (2006)
- Collapse of civilizations; PE&RC (2007)
- Mycorrhiza workshop; oral presentation; Wageningen (2005)

International Symposia, Workshops and Conferences (11 ECTS)

- Regional symposium on Genetic resources; oral presentation; Montevideo, Uruguay (2005)
- 5th International conference on Mycorrhiza; poster; Granada, Spain (2006)
- 5th International symposium on Edible Alliaceae; oral presentation; Dronten, the Netherlands (2007)
- 9th International congress of Plant Pathology; oral presentation; Torino, Italy (2008)

Courses in Which the PhD Candidate Has Worked as a Teacher

- Preparation of teaching material in Spanish for a refresher course on ”Breeding for durable resistance”; Dr. Rients Niks, Plant Breeding; Quito, Ecuador, 5 days (2004)
- Breeding for resistance to diseases; MSc course; Plant Production Dept.; 12 lectures; Uruguay (2005 and 2008)

Supervision of MSc Students (3 students; 50 days)

- Breeding for benefit from Mycorrhiza; Wageningen
- Breeding for resistance to Fusarium; Wageningen
- Plant defense enzymes involved in FBR infection on onion; Uruguay

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