The root systems of onion and *Allium fistulosum* in the context of organic farming: a breeding approach

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The root systems of onion and *Allium fistulosum* in the context of organic farming: a breeding approach

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 29 oktober 2003
des namiddags te vier uur in de Aula

Melo, P.E. de (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. With references and summaries in English, Dutch and Portuguese.

ISBN: 90-5808-915-0

Abstract

A massive amount of synthetic fertilizers is needed to grow onions (*Allium cepa* L.) due to their meager and inefficient root system. While the sustainability of such high-input systems is being questioned, low-input systems, such as organic agriculture, are gaining ground. For organic agriculture, plants have to be good nutrient scavengers. Therefore, productivity and stability of onion production in organic systems can be problematic. Plant breeding can improve the efficiency of onion roots, but breeding relies on available variation and there was no information about that in onions or in *Allium*. The aim of this thesis was to search for variation in root morphology in onion and in its allied species *A. fistulosum* L., to understand the role this variation could play in organic agriculture and to perform a genetic analysis of root traits. The variation found in root traits in onion was limited, although old onion cultivars had a higher root length density than modern ones. Huge variation was observed between onion and *A. fistulosum*. *A. fistulosum* developed substantially more stem-borne and lateral roots and, consequently, a much denser root system. Experiments carried out in an organic farm revealed that onion explored a smaller volume of soil and had a lower root density than *A. fistulosum*. In addition, onion, contrary to *A. fistulosum*, showed a reduction in total and fine root density when cultivated in a soil with low nitrogen content. It was also demonstrated that *A. fistulosum* was very responsive to indigenous and inoculated AMF (50 to 60% increase in both shoot biomass and root length). A Quantitative Trait Locus (QTL) analysis was done on the genetic linkage map of the progeny from the cross *A. cepa* x (*A. roylei* x *A. fistulosum*) to locate some of the genes responsible for the better performance of the *A. fistulosum* root system. All traits were evaluated in a replicated trial using *in vitro* cloned plants. QTLs were found for number of bulbs (1) and stem-borne roots (2) and, more interesting for breeding, for the number of lateral roots (1) and for the relative root length of fine and thick roots (1). The results showed the feasibility of breeding for onions with improved root systems using the interspecific hybrid between *A. roylei* and *A. fistulosum* as a genetic source. Some perspectives on the use of cultivars carrying such roots traits in onion organic production are highlighted.

Keywords: *Allium cepa*, *Allium roylei*, Japanese bunching onion, introgression breeding, organic agriculture, linkage map, AFLP, QTL analysis, Arbuscular Mycorrhizal Fungi, *in vitro* multiplication

Contents

Chapter 1

General introduction

A number of important topics for the general understanding of our research are presented in a 'birds eye view' to introduce the readers to this thesis. The work was completed in the context of breeding of onion for organic agriculture and, therefore, the general principles of this agricultural system are highlighted. Subsequently, the main implications that organic agriculture has for organic plant breeding are described, including more specifically how the research performed on root systems of onion and its allies fits into the general framework of organic plant breeding. Finally, the aim of the thesis is presented and brief descriptions of the various chapters are outlined.

Organic agriculture

In the last two decades organic agriculture gained momentum as there is a raising concern of the general public towards the so-called conventional agriculture, with its high inputs and substantial use of synthetic fertilizers and biocides. Doubts about the environmental and social sustainability and about the safety of the conventionally produced food are currently in the spotlight. An intense society questioning on these matters has triggered governmental and non-governmental initiatives to develop and to support alternatives in the direction of a more sustainable agriculture, of which organic agriculture is one example. Nowadays, approximately 23 million hectares are farmed organically worldwide (Yussefi, 2003). In Europe, USA and Japan, retail sales for organically produced food have doubled in value in the last five years, reaching US$ 19 billion in 2002 (Kortbech-Olesen, 2003). Although still small, the percentage of agricultural land devoted to organic farming is also increasing all over the world, with countries like Austria (11.3%), Switzerland (9.7%) and Italy (7.9%) taking the lead (Yussefi, 2003).

The question how to define organic farming is easier asked then answered. This is due to the fact that organic farming is a movement that consists of several streams, such as the bio-dynamical and the ecological (van Bruggen, 1995). The British Soil Association, in an attempt to define organic farming, presented the following description (MacKerron et al., 1999): '*organic farming encompasses the research, development and promotion of sustainable relationships between the soil, plants, animals and people and the biosphere, in order to produce healthy food and other products while protecting and enhancing the environment*'. In a Dutch study on organic agriculture, Lammerts van Bueren et al. (1998) defined it as '*a sustainable agricultural production method in which crop yield is being reached in an*

economically accountable manner, without exceeding the carrying capacity of the natural resources (i.e. water, air, soil fertility) present at the site of production'. Lyson (2002) states that '*a truly sustainable agriculture is the one that is economically profitable for the farmers, preserves and enhances environmental quality, contributes to the well-being of farm households and nurtures local community development*'. What comes out from these definitions is that organic agriculture is all about sustainability: environmental, economical and social sustainability.

In practice, organic agriculture can be currently characterized by the avoidance of biocides to control diseases, pests and weeds (van Bruggen, 1995) and also of synthetic fertilizers. Instead, manure, plant composts and legume cultivation are used to build and sustain soil fertility (Clark et al., 1998). In addition, organic agriculture seeks the accomplishment of closed nutrient cycles (Watson et al., 2002; Berry et al., 2003) and, through meticulous crop rotation, mixed cropping and use of field margins, for example, at the stimulation of agro-biodiversity (Altieri, 1999; van Elsen, 2000). The application of the aforementioned general practices, in combination with site-specific ones, ultimately leads to the stability of the system (van Bruggen and Semenov, 1999; Mäder et al., 2002).

Organic plant breeding

Organic plant breeding consists in the development of cultivars optimized for completing a successful ecological and economical (re) production cycle in an organic farming environment, making exclusive use during the cultivar development of breeding techniques that respect the principle of integrity of life. Lammerts van Bueren (2002) used the following criteria to characterize organic plant breeding: the safeguarding of plant self-reproducing ability in all stages of their development, the preservation of enough adaptive potential in the crop and the respect to plant integrity through the recognition of species barriers and species characteristics.

Organic plant breeding should target also at improving plant vitality. Plants with enhanced vitality are less damaged by biotic and abiotic stresses. Studies into the direction of rooting behavior, nutrient uptake and use efficiency, weed suppression and disease resistance are all examples of breeding research focussed on increasing plant vitality (Lammerts van Bueren et al., 1999). Through a comprehensive understanding of the plant in the context of its complex environment, research on plant vitality tries to find new opportunities for reducing outbreaks of diseases and pests (van Bruggen, 1995; van Bruggen and Semenov, 1999).

To incorporate these key concepts into the cultivar development process, a breeder could think in case of cross-pollination species into the direction of bulk population breeding (evolutionary plant breeding sensu Suneson, 1956). In case of

self-fertilizing species, breeding can be based on the modified pedigree selection (Allard, 1960), leading to the development of mixed cultivars with isogenic and isophenic lines (Lammerts van Bueren, 2002). The development of molecular markers increased even more the power of these 'old' breeding methods. Molecular markers allow that changes in allele frequencies are closely followed during breeding and, consequently, raises the efficiency of both early and direct selection for traits with high phenotypic plasticity (Kumar, 1999; Stuber et al., 1999; Asins, 2002). As important points to study in organic plant breeding, genotype x environment (management) and yield stability have been put forward. Furthermore, the organic agriculture community perceives that it is essential to involve farmers in the breeding process, exercising the participatory or collaborative plant breeding (Cleveland et al., 2000).

The development of cultivars specifically adapted to organic agriculture has been very slow. This is mostly due to two facts: (a) it can be foreseen that the seed prices will be higher for organic than they are for conventional cultivars, which already happens for organic seeds, due to inherent difficulties of producing high quality seeds in an organic environment and (b) the market is much smaller for organic than for conventional cultivars. Therefore (conventional) breeding companies are not willing to pay the risk of developing organic cultivars on large scale. Instead, most of them sell currently organic seed, *i.e.*, seeds from conventionally bred cultivars which are harvested in organically managed fields. According to the European Union Regulation 2092/91 for organic agriculture, which will be effective from January 2004 onwards, organic seeds are still within the limits of organic agriculture. But organic farmers are not completely pleased with organic seeds. Their demand is for cultivars adapted to organically managed environments, with high yield stability rather than high productivity, for example (Lammerts van Bueren et al., 2002). This will be achieved only when the whole breeding process was carried out in organic farming systems.

Onions and organic agriculture

Onion cultivation is not simple due to a number of reasons. The first one is that onions have a biennial life cycle, therefore plants grow slowly and are weak competitors with weeds (Hewson and Roberts, 1971). In organic agriculture, where biocides are abandoned, onion producers have to rely for a great part on hand weeding. However, considering the concept of agro-biodiversity, it is desirable that plants growing in organic farming have the ability to tolerate some levels of coexistence with weeds (Altieri, 1999). Another difficulty in cultivating onion comes from below ground. Onions have a very superficially growing root system (Portas, 1973). Only the top 20 cm of the soil are explored, which makes onions more than other crops vulnerable for drought (Strydom, 1964). To make the bad worse, onion roots seldom

branch and lack root hairs (Weaver and Brunner, 1927; Föhse et al., 1991). As result, onion has very low root densities (Greenwood et al., 1982) and is very inefficient in exploiting the soil. These root characteristics make onions very prone to nutrient deficiency and, back to the issue of weeds, enhance their inability to stand competition.

All in all, one wonders how is it possible that high yields are the rule and not the exception? To date, onion growers have circumvented this problem with a massive use of fertilizers, in detailed plans, spreading the application throughout the growing season (Fontes and Nogueira, 1984; Wiedenfeld, 1986; Greenwood et al., 1992; Patel and Vachhani, 1994). In addition, high-yielding fields are always irrigated (Galbiatti and Castellane, 1990; Chopade et al., 1997). Furthermore, it should be considered that arbuscular mycorrhiza fungi (AMF) play an important role in onion nutrition (Stribley, 1990). However, the raising costs of fertilizers, the strengthening of regulation restricting their use and a crescent environmental awareness are making it difficult to sustain such a production model (Sattelmacher et al., 1994; Matson et al., 1997). Simultaneously, low-input systems, such as organic agriculture, are gaining ground worldwide. However, the particularities of the soil fertility management in organic farming demand plants that develop abundant and active roots (Lammerts van Bueren et al., 2002), which definitely exclude the current onion cultivars. Therefore, it can be envisaged that problems related to onion nutrition and their effects on productivity and stability of production in organic farming systems might become dramatic.

It is possible to breed for improved root systems, as it is for other plant traits, but it is far from simple (Clarke and McCaig, 1993). Root evaluation is laborious and root traits display a large phenotypic plasticity in response to various soil condition, which might make direct selection rather inefficient (Lynch and Brown, 2001). In these circumstances, Marker Assisted Breeding can be of great help and it proved to be efficient in rice (Shen et al., 2001). But breeding relies on variation and there is no information in onion, in particular, or in *Allium*, in general, about genetic variation of root traits. Actually, literature on root systems of onions and its wild allies is scant. A brief review on this topic can be found in Brewster (1994). Therefore, it was considered at the onset of this work that there was justifiable space for a thorough investigation on identifying in onion and in allied germplasm root characteristics that could improve onion suitability to organic growing conditions. In addition, we were also very much keen on knowing which role plant breeding could play in this context.

Aim and outline of this thesis

The general aim of this thesis was to study the variation present in root morphology and its genetic basis in onion and in its allied species *Allium fistulosum* L.

and to understand the functionality of this variation for an organic agricultural environment.

Chapter 1 gives a brief overview of organic agriculture and organic plant breeding. It also highlights the main aspects of the onion root system that make the crop so inadequate to organic cultivation. **Chapter 2** describes root morphology and development in several onion cultivars. It compares the root systems of Eastern European and Dutch cultivars and, among the Dutch, it compares root traits of cultivars bred and released in the 40's and 50's to root traits of modern F_1 hybrids. In addition to onion, the allied species *Allium fistulosum*, the Japanese bunching onion, was analyzed. **Chapter 3** covers the morphology and functionality aspects of the root systems of onion and *A. fistulosum* when plants were grown in organically managed soils. It deals with the changes in the above and below ground development in both species as response to a three-fold variation in soil nitrogen availability. **Chapter 4** analyses the influence of Arbuscular Mycorrhizal Fungi (AMF) on plant and root growth of *A. fistulosum*. Plants were pre-inoculated with AMF and then grown in an organically managed soil in the presence of active indigenous AMF. Therefore, the contribution and interaction between indigenous and inoculated AMF could be compared and discussed. **Chapter 5** presents a reliable *in vitro* propagation multi-tissue approach to safeguard unique *Allium* plant material from losses. Explants derived from different sources and from three distinct *Allium* species, namely *A. cepa*, *A. fistulosum* and *A. roylei*, were tested. **Chapter 6** reports the development of a genetic map for the interspecific hybrid *A. roylei* x *A. fistulosum* based on species-specific AFLP markers segregating on the progeny of the cross *Allium cepa* x (*A. roylei* x *A. fistulosum*). It discusses interesting segregation ratios, the search of genomic regions putatively involved with important root traits and their applicability in onion breeding. **Chapter 7** summarizes the results and puts in perspective the contribution of breeding for root traits to the sustainability of the onion production.

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Chapter 2

Variation in root morphology in *Allium fistulosum* L. and in onion cultivars from different origins and release dates

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Abstract

Onion has a shallow, poor laterally spread and scarce root system, demanding high fertilization rates to achieve high yields. In the search for variation in root traits that could be ultimately used in breeding genotypes adapted to more sustainable fertilization practices, 16 onion populations were evaluated. The populations included cultivars that were originated from East Europe and from the Netherlands. Among the Dutch, cultivars bred in the 40's and 50's were used along with recently released or still experimental F_1 hybrids. Three accessions of *Allium fistulosum* were also evaluated. Two experiments were carried out, one in the greenhouse including all onion and *A. fistulosum* populations and another at an organic farm, using two onion cultivars and two *A. fistulosum* accessions. Results obtained in the greenhouse and in the field agreed to a large extent. *A. fistulosum* had significantly more stem-borne and lateral roots than onion and, as consequence, a root length density twice to three times larger, especially due to the increase in the length density of fine roots. There was also variation among onion cultivars, although rather limited. When grouped by origin, Dutch cultivars had significantly more lateral roots than Eastern-European, while within Dutch cultivars, old cultivars had denser root systems than modern ones. This indicates that onion breeding in the Netherlands, with genotype selection under high fertilization levels, led to the reduction in root development.

Keywords: *Allium cepa* L., Japanese bunching onion, Welsh onion, introgression breeding, stem-borne roots, root length density, lateral roots, soil exploitation, soil exploration, root branching

Introduction

Onion is one of the leading vegetable crops worldwide. In 2002, it was cultivated in more than 100 countries, on nearly 3 million ha, producing more than 50 million tons (FAO, 2003). The reason for such a global distribution is certainly the universal acceptance of onion as food and condiment, but not the simplicity of growing. Actually, onion is a difficult crop to grow and one of the main challenges resides in giving an onion plant a proper fertilization. Brewster (1994) summarized the main difficulties in fertilizing onions: (1) P and K in the soil should be high, although onions remove less P and K from the soil than many other vegetables that demand lower availability levels; (2) high yielding onion crops have a shoot N content similar to other species, but high yields are only achieved with heavy fertilization. The recovery rate of the applied N is in average 37% (Greenwood et al., 1992), which means that a considerable residual N is left in the soil, a risk for underground water contamination (Power and Schepers, 1989).

Most of these difficulties should be credited to the peculiarities of the onion root system. Onions have a shallow, poor laterally spread and scarce root system (Thompson, 1921; Weaver and Brunner, 1927; Drinkwater and Janes, 1955; Strydom, 1964; Portas, 1973; Greenwood et al., 1982). The bulk of the root system is made up of stem-borne roots that are continuously produced by the plant (Jones and Mann, 1963). Stem-borne roots seldom branch; hence onions have sparse and, in addition, short lateral roots, which rarely rebranch (Weaver and Brunner, 1927). Such a root system pattern leads to the characteristic low density of roots in onion (Weaver and Brunner, 1927; Greenwood et al., 1982), the main cause of onion fertilizer requirements being so much higher than what is actually used by the crop. In addition to the scarce branching, in onion root hairs are either missing or very rare and short (Jones and Mann, 1963; Föhse et al., 1991), which reduces even more the potential uptake surface of the root system.

Several factors emphasize the importance of developing plants more efficient in nutrition: costs of fertilization, increasing legislative regulation restricting the use of fertilizers, so as to minimize environmental hazards, and the integration to agriculture of marginal lands (Sattelmacher et al., 1994), where crop performance is specially dependent on root system development (Mia et al., 1996). To these factors, it must be added the mounting importance given to better-balanced and sustainable agricultural systems, as, for example, organic cultivation. Although still representing a low percentage of the total land dedicated to agriculture, the area managed organically is increasing steeply all over the world, reaching currently nearly 23 million hectares (Yussefi, 2003). Retail sales of organically produced food in the European Union, USA, Canada, Japan and Oceania are estimated to get to 23 to 25 US$ billion dollars

in 2003 (Kortbech-Olesen, 2003). With respect to fertilization, organic systems are characterized by an extensive use of resources and absence of mineral fertilizers (van Bruggen, 1995). Instead, nutrients are applied using more complex sources, resulting in less promptly availability (Atkinson, 2000; Mäder et al., 2002). Hence organic agricultural systems demand plants with high nutrition efficiency, which are defined as those able to realize a yield above average under conditions of suboptimal nutrient supply (Graham, 1984). High nutrition efficiency has two major components: nutrient use and nutrient uptake (Sattelmacher et al., 1994), the latter related to and dependent on several root traits. Many of these traits were already shown to have a genetic component with a relatively high heritability (for reviews see O'Toole and Bland, 1987; Clarke and McCaig, 1993; Hoad et al., 2001). Yet, plant breeding has been given little direct attention to plant components below ground. Clarke and McCaig (1993) and Atkinson (2000) attributed this apparent lack of interest to the inherent difficulties in screening root systems, as well as to the fact that links between root morphology and function are still not well understood.

The objective of this study was to identify variation in root traits in onion and in the allied species *A. fistulosum* that could be ultimately used in breeding to improve onion root development. To raise chances of finding variation, onion cultivars from diverse origins were studied, namely from the Netherlands and from Eastern European countries, and also cultivars bred in different periods of time in the Netherlands. *A. fistulosum* was included because while dealing with this species in our research program, it was noticed that it develops a more abundant root system than onion. *A. fistulosum* is known as the Japanese bunching onion or Welsh onion. It has been cultivated for more than 2000 years and remains as an important vegetable in China and Japan (Brewster, 1994). To our understanding, this is the first report on root morphology and development in this species.

Material and Methods

Greenhouse

Sixteen onion culti- and candivars, hereafter referred to as populations, and three *Allium fistulosum* accessions (Table 1) were assessed. Seeds were sown in 1.3-L pots, filled with fertilized sand (1.0 g of 12-10-18 NPK per pot) and irrigated by surface. The experiment was carried out in a greenhouse, in a completely randomized design, with each onion population or *A. fistulosum* accession replicated three times.

Plants were harvested 90 days after sowing, at full vegetative growth, and roots were washed while still attached to the stem, over a sieve. Once roots were cleaned, the stem-borne roots were counted. Following, a gentle water stream was used to flat

the root system over a surface. Roots were then cut off from the stem and the flattened root system was divided longitudinally in two parts. From one part, three stem-borne roots were randomly taken and the lateral roots in each of them were counted. Roots in the other part of the root system were cut in 5-cm segments, stirred in water and sampled five times to form the final sample used for measuring root length. Following, root samples were stained overnight (500 ppm Safranin), at 4°C. The next day, the stained roots were rinsed in water to remove the excess of dye, spread in a transparent tray and scanned with a table flatbed scanner. Total root length and the root length in 13 classes of root diameter, from 0 to 1.2 mm or above, with 0.1-mm increments, were obtained from root images using the software WinRhizo®. Subsequently, the two parts of the root system, including the three stem-borne roots used for counting root branching, were dried (72°C, forced ventilation, 96 hours) separately. The dry weight of the parts was summed up to obtain total dry weight. Total root length was estimated by multiplying the root length obtained in the scanned sample by the ratio between total root dry weight and root dry weight in the scanned sample. Root length was expressed as volumetric root length density (L_V), in cm of root per cm^3 of soil (Smit and Zuin, 1996; Atkinson, 2000).

Table 1. Origin and identification of *A. fistulosum* accessions and onion populations used to study the variation in root traits.

Species	Name	Origin and date of release	Seed Source[1]	Population Type
A. fistulosum	Bunc. Onion Straight-Leaf	Australia	CGN 16.369	-
A. fistulosum	Sapporo Nebuka Futonegi	Japan	CGN 16.442	-
A. fistulosum	Wild	Odessa Bot. Garden	CGN 14.763	-
A. cepa	Bessonovskii	Central Russia	CGN 16.360	OP Cultivar
A. cepa	Franzisko	East Europe	Nickerson-Zwaan	OP Cultivar
A. cepa	Strigunovskii	East Europe	CGN 14.755	OP Cultivar
A. cepa	Vsetatskà	Czech Republic	CGN 16.366	OP Cultivar
A. cepa	Wolska	Poland	CGN 14.726	OP Cultivar
A. cepa	Maelstede	Netherlands – 1953	CGN 14.739	OP Cultivar
A. cepa	Plastro	Netherlands – 1958	HRI 3480	OP Cultivar
A. cepa	Zeeuwese Bruine	Netherlands – 1943	CGN 14.756	OP Cultivar
A. cepa	Westerloo	Netherlands – 1944	CGN 14.721	OP Cultivar
A. cepa	Wijbo	Netherlands – 1951	HRI 4120	OP Cultivar
A. cepa	Jumbo	Netherlands – 1973[2]	Syngenta Seeds	OP Cultivar
A. cepa	ADV 98.454	Netherlands – Exper.[3]	Advanta	F_1 Hybrid
A. cepa	Drago	Netherlands – 2000	Nickerson-Zwaan	F_1 Hybrid
A. cepa	Profit	Netherlands – 1999	Advanta	F_1 Hybrid
A. cepa	SG 8282	Netherlands – Exper.[3]	Syngenta Seeds	F_1 Hybrid
A. cepa	SG 8286	Netherlands – Exper.[3]	Syngenta Seeds	F_1 Hybrid

[1]/ CGN: Center for Genetic Resources, Wageningen, the Netherlands; HRI: Horticulture Research International, Wellesbourne, United Kingdom; [2]/ Not included in the group of old, nor in the group of modern Dutch populations; [3]/ Included in the group of modern Dutch populations.

Analysis of variance was performed over the data for all populations and accessions. Significant differences between means were established using LSD, at 5%

probability level. Analysis of variance and contrasts between means were also performed over data broken by species, by origin and, for the Dutch onion populations, by release date. Cultivar Jumbo was excluded from this last comparison, since it did not fit in either category. Whenever two means were contrasted, the T test for unequal variances was used.

Field

A. fistulosum accessions CGN 16.369 and CGN 16.442 (Table 1) and two onion populations, the F_1 hybrid Drago and the open-pollinated cultivar Jumbo, were used. Seeds were sown in trays and 45-day old uniform seedlings were transplanted to the field. The experiment was carried out at an organic farm, in completely randomized blocks, with four replications and nine plants per plot. Plants were spaced by 0.50 x 0.50 m to avoid mixing roots of neighboring plants. No mineral fertilizing was used and the main soil chemical properties in the onset of the experiment were as follows: 1.39 mg.100g^{-1} of N-mineral, 31 mg P_2O_5.100g^{-1} of P-available (P_W), 173 mg.100g^{-1} K_2O (K-HCl) and pH (KCl) 7.4. No irrigation supplementary to precipitation was used.

Sixty-five days after transplanting, 35 x 50 cm pin-boards (Oliveira et al., 2000) were applied to the soil profile at a trench excavated adjacently to the plants. One plant per population was sampled to survey the root system spatial distribution. After overnight immersion under vacuum in a 5% pyrophosphate solution to dissolve the clay, pinboards were washed using a gentle water stream to avoid disturbing the roots. Pin-boards showed that roots, independent of the onion or *A. fistulosum* population, did not go further than 30 cm far from the plant and deeper than 30 cm in the soil profile. Thus, this was the zone used for sampling roots using the auger method (Oliveira et al., 2000). Auger samples were collected 70 days after transplanting from all populations, in all replications, from two plants per replication, using 7-cm diameter x 10-cm high cores (385 cm^3). Samples were collected in the soil profile in increments of 10 cm up to 30 cm deep; and in three lateral positions: adjacently to the plant up to 7 cm, from 10 to 17 cm and from 20 to 27 cm far from the plant. Samples were first frozen (-18^{o}C), then washed. Washing was carried out by immersion of the frozen samples for 15 minutes in bubbling running water. Roots were retained in sieves and then separated from debris by flotation and elutriation. Clean roots were stained and scanned as described for the greenhouse experiment to obtain total root length and root length in classes or root diameter. The volumetric root length density (L_V) was obtained dividing the root length measured in each sample by the volume of the core. Plants whose roots were sampled were harvested in order to count the number of stem-borne roots. Data were studied by analysis of variance. Contrasts

between species and between populations in a given species were considered significant at a 5% probability level.

Results

Greenhouse

Stem-borne roots

A. fistulosum and onion root systems were extremely distinct. The first prominent difference between the species was the number of stem-borne roots. *A. fistulosum* genotypes had much more stem-borne roots and contrasted significantly with onion (Table 2). Within onion, contrasts were not significant either to the origin of the populations, Eastern European or Dutch, or within Dutch populations, to the period when it was bred. When accessions and populations were compared one by one, disregarding their species, origin and age, *A. fistulosum* accessions CGN 14.763 and CGN 16.442 had significantly more stem-borne roots than all other genotypes (Table 3). A. *fistulosum* accession CGN 16.369 and the onion populations Wijbo and SG-8286 formed a second group, not differing from each other, although *A. fistulosum* CGN 16.369 differed significantly from all other onion populations. Among onion populations, cultivar Wijbo had statistically more stem-borne roots than cultivars Westerloo and Wolska, and than candivar ADV-98.454, with the other genotypes ranging in between.

Lateral roots

The occurrence of lateral roots, due to root branching, was another characteristic that remarkably differentiated the root system of the two species. *A. fistulosum* had significantly more lateral roots than onion (Table 2). Within onion, the number of lateral roots varied significantly only with origin: Dutch populations had more lateral roots than those coming from East Europe. When accessions and populations were compared to each other, the three *A. fistulosum* accessions had significantly more lateral roots than any onion populations and did not differ from each other (Table 3). When onion populations were considered, candivar ADV-98.454 had significantly more lateral roots than cultivars Franzisko and Vsetatskà. All other onion populations varied between those and did not differ significantly from one or from another.

Root length density (L_V)

Populations, but especially, species, differed significantly in the overall size of the root system. To assess this difference, the root length density (L_V) was used. *A. fistulosum* L_V was significantly higher than onion's (Table 2). Among onions, L_V did

not differ between Eastern European and Dutch populations (Table 2). However, when Dutch populations were contrasted by release date, it was observed that old cultivars had a significantly higher L_V than modern populations (Table 2).

Table 2. Means for root morphological traits and significance of contrasts between (a) *A. fistulosum* and onion, (b) Eastern European and Dutch onion populations and (c) Dutch onion populations differing in the period when they were bred, for plants cultivated in the greenhouse.

Groups[1]	Stem-Borne Roots	Lateral Roots[2]	Root Length Density (cm.cm^3)		
			L_V	L_{Vfine}[3]	L_{Vthick}[4]
A. fistulosum	38[5]	21	1.90	0.67	0.18
Onion	17	8	1.08	0.15	0.07
Contrast	***	***	***	***	***
Dutch	17	9	1.11	0.14	0.07
E. European	16	6	1.02	0.17	0.05
Contrast	**n.s.**	***	**n.s.**	**n.s.**	**n.s.**
Old	17	10	1.21	0.15	0.08
Modern	17	9	1.03	0.13	0.06
Contrast	**n.s.**	**n.s.**	**	**n.s.**	**n.s.**

[1]/ The identification of the populations belonging to a given group is given in table 1; [2]/ Number of lateral roots per stem-borne root; [3]/ L_{Vfine}: root length density of fine roots, with diameter greater than or equal to 0.2 and smaller than 0.4 mm; [4]/ L_{Vthick}: root length density of thick roots, with diameter greater than 1.2 mm; [5]/ Each mean represents the average for all populations belonging to a given group.

In the population-basis comparison, the three *A. fistulosum* accessions had similar L_V, which were significantly higher than L_V in any onion population (Table 3). Among onion populations, cultivar Maelstede had a higher L_V than candivars SG-8282 and ADV-98.454 and than cultivar Wolska. The other onion populations had intermediate values, not differing statistically from the extremes.

In *A. fistulosum* there was a massive presence of fine roots, not seen in onion, which was very likely a consequence of the much higher number of lateral roots in the former in relation to the latter. To quantify this difference, the specific root length could have been used. The specific root length is the ratio between root length and root dry weight and gives an indication of root average diameter (Atkinson, 2000). However, because *A. fistulosum* and onion differed not only in the amount of fine roots, but also in the amount and thickness of thick roots, specific root length was not applicable, since it would be confounding two opposite effects. Instead, the root length density (L_V) per root diameter was calculated. This was a successful approach to reveal classes of root diameter characteristic to one or another species (Figure 1).

In *A. fistulosum*, roots with diameter equal to or greater than 0.2 and smaller than 0.4 mm, yielded a L_V at least three times higher than in onion. L_V for roots with diameter equal to or greater than 0.4 and smaller than 0.5 mm was also higher in *A.*

fistulosum than in onion. However, the difference between the two species was not as marked as in the previous classes. As the root caliber increased, the difference between species was totally vanished, to appear back only for thick roots, with diameter greater than 1.2 mm (Figure 1). Assuming that roots with diameter equal to or greater than 0.2 and smaller than 0.4 mm were those that represented best the lateral roots, a root length density for roots exclusively within this diameter range (L_{Vfine}) was calculated. As expected, there was a huge and significant difference in L_{Vfine} between *A. fistulosum* and onion (Table 2). Within onion there was no major variation and contrasts for origin and for cultivar release date were not significant (Table 2). When the analysis was based on the population individual behavior, L_{Vfine} discretely separated populations according to their species. Nor *A. fistulosum* accessions differed from each other, neither onion populations, and *A. fistulosum* accessions were significantly higher than onion populations (Table 3).

Table 3. Means for root morphological traits of *Allium fistulosum* and onion populations from Eastern Europe and from the Netherlands, cultivated in the greenhouse.

Populations[1]	**Stem-Borne Roots**	**Lateral Roots**[2]	**Root Length Density (cm.cm^3)**		
			L_V	**L_{Vfine}**[3]	**L_{Vthick}**[4]
A. fistulosum					
CGN 14.763	49	23	2.00	0.68	0.20
CGN 16.369	28	23	1.86	0.62	0.17
CGN 16.442	38	20	1.82	0.72	0.16
Onion					
Bessonovskii	17	7	1.11	0.17	0.06
Franzisko	16	6	1.03	0.13	0.09
Strigunovskii	19	7	1.12	0.21	0.06
Vsetatskà	14	6	0.98	0.18	0.04
Wolska	11	7	0.84	0.17	0.04
Maelstede	18	11	1.30	0.16	0.09
Plastro	16	8	1.13	0.16	0.05
Z. Bruine	18	10	1.26	0.14	0.08
Westerloo	13	9	1.09	0.13	0.07
Wijbo	22	8	1.23	0.17	0.09
Jumbo	17	8	1.07	0.09	0.09
ADV-98.454	13	12	0.92	0.09	0.05
Drago	19	10	0.95	0.10	0.06
Profit	15	10	1.13	0.21	0.05
SG-8282	19	8	0.93	0.15	0.04
SG-8286	21	8	1.20	0.13	0.08
LSD (p<0.05)	**9**	**5**	**0.35**	**0.13**	**0.06**

[1]/ The identification of a population origin and release date is given in table 1; [2]/ Number of lateral roots per stem-borne root; [3]/ L_{Vfine}: root length density of fine roots, with diameter greater than or equal to 0.2 and smaller than 0.4 mm; [4]/ L_{Vthick}: root length density of thick roots, with diameter greater than 1.2 mm.

A clear-cut difference between species for the thickest roots, with diameter greater than 1.2 mm, was also observed (Figure 1). This difference is very likely due to

the higher abundance of strong stem-borne roots in *A. fistulosum* in comparison to onion. When the L_V for thick roots was analyzed, contrasts were significant only for species, with the L_V for thick roots in *A. fistulosum* being significantly higher than in onion (Table 2). There were no significant effects of origin of the onion population or of its release date, in case of Dutch populations, over L_V for thick roots (Table 2). Individual differences among populations were exactly as described to L_V for fine roots: populations from the same species did not differ significantly from each other and *A. fistulosum* accessions scored values significantly higher than onion populations (Table 3).

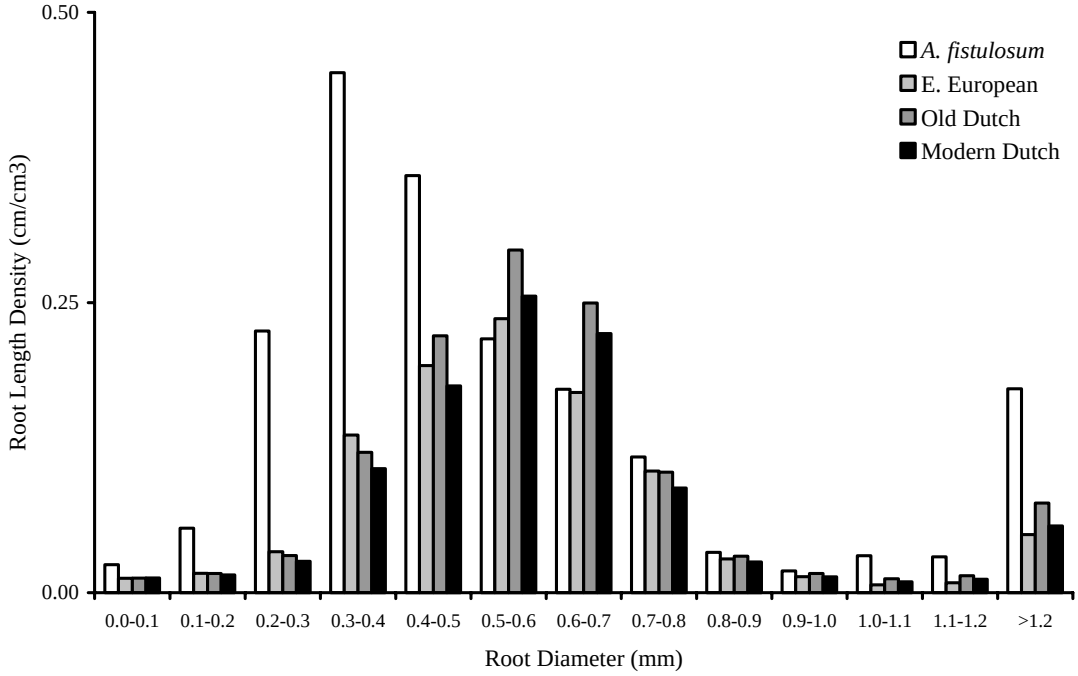

Figure 1. Distribution of root length density in classes of root diameter in *A. fistulosum* and in Eastern European and old and modern Dutch onion cultivars, grown in the greenhouse. The identification of the populations belonging to a given group is given in table 1. Bars represent the mean of all populations belonging to a given group.

Field

Stem-borne and lateral roots

As expected, roots developed to a much larger extent in the field than in the greenhouse, independent of species or population. Even then, the differences related to root morphology found between species in the greenhouse could be retrieved in the field. Stem-borne roots were three times more abundant in *A. fistulosum* than in onion (Table 4). There were no significant differences in the number of stem-borne roots between populations in a given species. The number of lateral roots was not evaluated

in the field because the Auger method does not allow lateral roots to be counted accurately.

Root length density (L_V)

To evaluate root length density (L_V), root samples were collected at nine points in the soil, in a combination of three rooting depths and three lateral positions. At the deepest sampling layer, from 20 to 30 cm deep in relation to soil surface, roots were present only at the first lateral position, but were too scarce, below 0.01 cm.cm^{-3}, and therefore were not considered in the analysis. In the two upper layers, *A. fistulosum* and onion differed significantly in L_V in all sampled positions. At the farthest position, from 20 to 27 cm from the plant, at the second soil layer, from 10 to 20 cm deep in relation to soil surface, L_V between species could not be compared because onion roots were absent. Nevertheless, wherever the two species could be compared, *A. fistulosum* had a significantly higher L_V than onion, often two to three times larger (Table 4). Onion cultivars did not differ from each other in any sampling point (data not shown). *A. fistulosum* accession CGN 16.369 had a significantly higher L_V (3.75 cm.cm^{-3}) than CGN 16.442 (2.64 cm.cm^{-3}) at the distance from 0 to 7 cm far from the plant in the top soil layer and, again, at the distance from 10 to 17 cm far from the plant, at the second soil layer, from 10 to 20 cm deep in relation to soil surface (CGN 16.369 = 0.67 cm.cm^{-3}; CGN 16.442 = 0.32 cm.cm^{-3}). At the other sampling points, *A. fistulosum* accessions did not differ from each other.

Table 4. Means and significance of contrasts for number of stem-borne roots and root length density (L_V) in *Allium fistulosum* and in onion grown in an organically managed field, with the root systems sampled in three depths and in three distances from the plant.

	Stem-Borne Roots[1]	**Lateral Position (cm from the plant)**					
		0-7	**10-17**	**20-27**	**0-7**	**10-17**	**20-27**
		L_V (cm.cm^{-3})			**L_{Vfine}[2] (cm.cm^{-3})**		
				Depth: 0 - 10 cm			
A. fistulosum[3]	99	3.20	2.30	1.92	1.26	0.92	0.82
Onion[4]	32	1.42	1.05	0.42	0.28	0.24	0.14
Contrast	***	***	***	***	***	***	***
				Depth: 10 - 20 cm			
A. fistulosum		0.93	0.49	0.16	0.35	0.20	0.08
Onion		0.37	0.15	-	0.05	-	-
Contrast		**	**	-	**	-	-

[1]/ Counted directly in the stem; [2]/ LVfine: root length density of fine roots, with diameter greater than or equal to 0.2 and smaller than 0.4 mm; [2]/ Mean for the two *A. fistulosum* accessions grown in the field; [3]/ Mean for the two onion cultivars grown in the field.

As observed in the greenhouse, *A. fistulosum* had the bulk of its root system made up of roots much finer than onion also in the field. Roots with diameter greater

than or equal to 0.2 and smaller than 0.5 mm stood out as the most prominent classes in *A. fistulosum* (Figure 2). In onion, the majority of the root system was made up of roots with a diameter greater than or equal to 0.4 and smaller than 0.7 mm (Figure 2). Thus, roots of *A. fistulosum* were mostly finer than roots of onion, with roots with diameter from 0.4 to 0.5 mm representing an intersection class simultaneously important for both species. L_V per root diameter for the second soil layer, from 10 to 20 cm deep in relation to soil surface had smaller figures, but the same distribution as observed in the top 10 cm of soil (data not shown).

Based on the species-specific behavior, a main class of root diameter ($0.2 \leq x < 0.4$) was formed to represent the fine roots, as it was done for the greenhouse experiment. L_V for this class was calculated (L_{Vfine}) and analyzed. *A. fistulosum* and onion differed significantly in L_{Vfine} in all sampling points where the two species could be contrasted and, in all cases, L_{Vfine} in *A. fistulosum* was overwhelmingly higher than in onion (Table 4). *A. fistulosum* accessions differed significantly from each other for L_{Vfine} only at the top soil layer, in a distance from 0 to 7 cm far from the plant, where CGN 16.369 had L_{Vfine} of 1.44 $cm.cm^{-3}$, while CGN 16.442 scored 1.07 $cm.cm^{-3}$. Onion cultivars did not differ from each other in any sampling point.

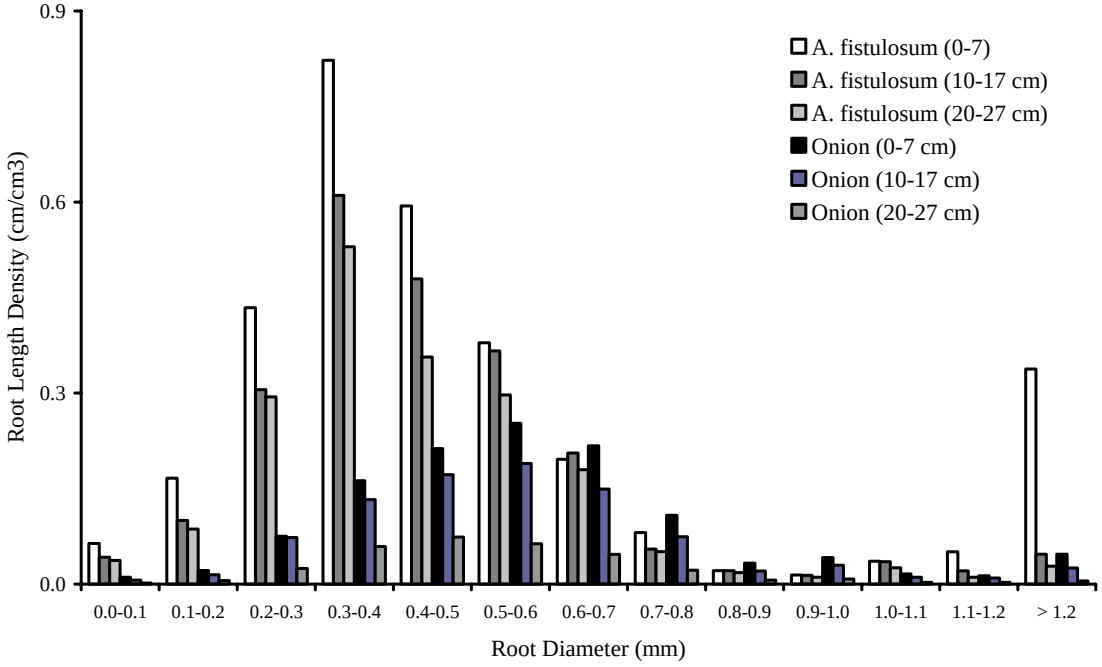

Figure 2. Distribution of root length density in classes of root diameter in the top 10 cm of soil, in three distances from the plant, in *A. fistulosum* and onion grown in an organically managed field. Bars represent the mean for the two *A. fistulosum* accessions or for the two onion cultivars grown in the field.

Thick roots, with diameter above 1.2 mm, also significantly differentiated *A. fistulosum* and onion root systems, but only at the topsoil layer and close to the plant

(Figure 2). In this sampling point, L_V for thick roots in *A. fistulosum* and onion were respectively 0.34 and 0.05 $cm.cm^{-3}$ and populations, in a given species, did not differed from each other. At one lateral distance further, still in the top 10 cm of soil, *A. fistulosum* and onion L_V for thick roots were respectively 0.05 and 0.03 $cm.cm^{-3}$ and did not differ significantly. Only *A. fistulosum* showed thick roots at the farthest sampling position, at the top 10 cm of soil (L_V = 0.03 $cm.cm^{-3}$). In the second soil depth, in the first lateral position, both species presented very few thick roots with L_V values of 0.02 and 0.01 $cm.cm^{-3}$ respectively for *A. fistulosum* and onion. In all other sampling points, thick roots were absent in both species.

Discussion

Root system variation in onion

Onion populations varied within a short range for all root traits evaluated, although populations from fairly different geographic origins were tested (Table 1). Actually, geographic origin was a significant source of variation only for number of lateral roots, but differences were not large enough to produce significant effects on either total or fine root length density (Table 2). Although there are a few reports on root research in onion involving simultaneously more than one population (Weaver and Brunner, 1927; Drinkwater and Janes, 1955; Portas, 1973; Greenwood et al., 1982), no studies were found that compared root traits variation among populations as it was performed here.

Root length density in Dutch onion cultivars was significantly different when recently released cultivars were compared to cultivars released some decades ago (Table 2), although the pattern of length distribution along root diameter (Figure 1) and the number of stem-borne and lateral roots were not altered (Table 2). Modern Dutch onion cultivars had lower root length density or, in other words, shorter root systems than older ones. Brown et al. (1987) compared a landrace to a high-yielding modern cultivar of barley and found that the root system in the former was longer than in the latter, especially in the soil layer from 20 cm and below. In wheat, modern cultivars also showed a lower root fraction than older ones and, consequently, were more responsive improved environments, where nutrient availability was higher (reviewed by Hoad et al., 2001). High levels of fertilization and ideal supplementation of water through irrigation, which assure a wealthy nutrient availability, are characteristics of modern agricultural systems. In such an environment, plants that privilege carbohydrate allocation to above- in detriment of below-ground development are more likely to be selected, which creates an opportunity to favor changes in root morphology that occur in association with such a partitioning behavior (O'Toole and Bland, 1987). This might have been the case after years of onion breeding and selection in favorable

conditions of soil fertility in the Netherlands. A neat example of such effect is the maize mutant called "rootless", described by Sattelmacher et al. (1993). The genotype "rootless", with confers a phenotype with root development drastically reduced, out-yielded its normal rooting near-isogenic line in optimal P supply. However, in sub-optimal P conditions, where roots should be apt to efficiently forage for nutrients, the advantage of "rootless" was not only vanished, but the yield in "rootless" was significantly lower than in the normal line.

Root system variation between onion and A. fistulosum

Breeders always tried to extend onion genetic base to avoid the lack of variation (reviewed by Kik, 2002). Our results, in both greenhouse and field showed that, when breeding for root traits in onion, *A. fistulosum* is definitely a species to consider. The degree of variation between the two species regarding number of both stem-borne and lateral roots, with huge quantitative and qualitative consequences on root length density, completely overshadowed the variation found within *A. cepa*. In addition, Khrustaleva and Kik (2000) elegantly demonstrated that the inter-specific sterility barriers that have posed difficulties to a more routinely use of *A. fistulosum* in onion breeding can be circumvented to a large extent if *A. roylei* was used to bridge the introgression of *A. fistulosum* into onion.

Stem-borne roots

Number of stem-borne roots was one of the traits that markedly and consistently differentiated onion and *A. fistulosum* root systems. In the greenhouse experiment, onion produced in average 17 stem-borne roots per plant (Table 2), and nearly twice as much in the field (Table 4). In literature, the number of stem-borne roots in onion grown in the field, at the half of its vegetative cycle, has varied around 30 to 35 (Weaver and Brunner, 1927; Jones and Mann, 1963; Nandekar and Sawarkar, 1992), although Leskovar and Vavrina (1999) reported up to 49 roots per plant. In our experiments, *A. fistulosum* produced twice and three times more stem-borne roots than onion respectively in the greenhouse (Table 2) and in the field experiments (Table 4), where the *A. fistulosum* vigorous root system had an impressive average of 99 stem-borne roots per plant. Reports of number of roots in *A. fistulosum* are rare in literature and when present, they refer to the densely sowed and early-harvested *A. fistulosum* plants cultivated for their tender and good-flavor leaves. Under such conditions, in greenhouse experiments, 12 to 14 week-old *A. fistulosum* plants had from 15 to 25 roots per plant (Matsubara et al., 1994; 1995).

Stem-borne roots constitute the bulk of the onion root system and since the cotyledon stage they take over from the primary root the leading role in nutrient and water uptake (Weaver and Brunner, 1927; Jones and Mann, 1963; Brewster, 1994).

Actually, since this stage, the stem is nearly the solely source of new roots (Jones and Mann, 1963), due to the early death of the primary root and to the poor branching characteristic of onion roots (Weaver and Brunner, 1927; Brewster, 1994). In adventitious root systems, as such of onion and *A. fistulosum*, stem-borne roots represent also the front line in the chase of water and mobile nutrients and, if strong enough, they are crucial for overcoming soil mechanical resistance for root elongation (Sattelmacher et al., 1994).

Lateral roots

Another major difference between the root systems of onion and *A. fistulosum* was the much higher number of lateral roots the latter had in relation to the former (Table 2). In *Allium*, lateral roots originated from the stem-borne roots through branching. There were also significant differences in number of lateral roots related to the origin of the onion cultivar, but the variation was restricted if compared to the differences found between species and did not have significant consequences on root length (Table 2). Onion roots are well known for the lack or rarity of branching, with short lateral roots being found at low rates, hardly ever rebranching (Weaver and Brunner, 1927; Brewster, 1994). Pulgarin et al. (1988) studied the branching pattern of onion stem-borne roots and determined that branching was found only far from the root tip and concentrated in one side of root, with longitudinal inhibition between branches. Birdsall and MacLeod (1990) noticed that lateral-root primordia, which later develop into root branches, were absent in the first-formed stem-borne roots, at least for the 4-week period after seed germination their observation lasted. No report related to the development of lateral roots in *A. fistulosum* was found.

A higher number of branches contributes to a better architectural arrangement of the root system and increases root length (Bray, 1954; Mia et al., 1996; Rao and Ito, 1998). In addition, young lateral roots are usually the most efficient in nutrient uptake (Burns, 1980; Robinson et al., 1991; Rao and Ito, 1998). Consequently, root systems with prolific lateral root development are well adapted to intense soil exploitation and high nutrient uptake even in conditions of non-luxurious availability (Atkinson, 1990; Rao and Ito, 1998; Casimiro et al., 2003). In case of organic cultivation, where it is not possible or desired to supply nutrients as abundantly as in conventional cultivation (van Bruggen, 1995; Lammerts van Bueren et al., 2002) the higher number of lateral roots in *A. fistulosum* provides an obvious advantage over onion.

Root length density (L_V)

As consequence of the higher number of both stem-borne and lateral roots in *A. fistulosum* in relation to onion, root length in *A. fistulosum* was much larger than in onion, both at the greenhouse (Table 2) and in almost all sampling points in the field

(Table 4). Length is the most obvious root attribute to be related to the rate of nutrient uptake, with a clear functional significance, especially when expressed as density of roots in a given volume of soil or root length density (L_V) (Hoad et al., 2001). L_V gives an indication of possible limitations regarding soil exploitation for nutrients and water (Atkinson, 2000). There is little doubt that L_V is important in determining the uptake efficiency of low-mobility nutrients, such as P. There is mounting evidence that this holds true also for mobile ones, such as nitrogen, especially in cases of very low L_V or shortage of the nutrient or water (Sattelmacher et al., 1994). *Allium* crops have low L_V when compared to other crops (Brewster, 1994; Smit and Zuin, 1996) and *A. fistulosum*, although with a much larger L_V than onion (Tables 2 and 4), is not an exception. Brussels sprouts, cauliflower and pea have L_V of respectively 10.0, 9.4 and 3.8 $cm.cm^{-3}$, while lettuce and leek, the latter also an Alliaceae, have L_V of respectively 2.1 and 2.0 $cm.cm^{-3}$ (Greenwood et al., 1982; Smit et al., 1996), which is comparable to the values found for *A. fistulosum*. The maximum onion L_V reported by Greenwood et al. (1982) was 1.1 $cm.cm^{-3}$, similar to that found in this study (Tables 2 and 4). Interesting enough, onion maximum L_V is around the threshold below which L_V starts to be limiting for N uptake, considered to be around 1 to 1.5 $cm.cm^{-3}$, although variable with plant demand and nitrate and/or ammonium concentration in the soil (Sattelmacher et al., 1994; Wiesler and Horst, 1994).

Root length density (L_V) was not only quantitative, but also qualitatively different between onion and *A. fistulosum*. L_V distribution along root diameter showed that the bulk of *A. fistulosum* root system was made up of roots much finer than in onion, both in greenhouse and field conditions (Figures 1 and 2). L_V, discussed above, already indicated that *A. fistulosum* had higher soil exploitation potential than onion (Hoad et al., 2001). However, when it is considered that (1) L_V exclusively for fine roots (L_{Vfine}) constituted an even more striking difference between the two species and (2) fine roots are more efficient in uptake (see Discussion, *Lateral roots*); one should consider that the difference between the two species regarding soil exploitation is even larger. Reduction in root diameter is indeed an efficient approach to increase the absorbing root surface with reduced carbohydrate allocation and reaches its optimum with the formation of root hairs (Sattelmacher et al., 1994). The smaller the root diameter, the larger the volume of soil delivering nutrients to the roots and the higher the uptake rates (Föhse et al., 1991; Rao and Ito, 1998). It makes a larger L_V for fine roots especially valuable under conditions of limited nutrient supply, when plant nutrition is much more dependent on uptake than in utilization efficiency (Sattelmacher et al., 1993). In this context, Krannitz et al. (1991) observed in *Arabdopsis thaliana* that in genotypes with abundant fine root development due to

formation of lateral roots phosphate was depleted to much lower concentrations than in genotypes with less lateral roots.

Soil Exploration

In addition to a higher potential for soil exploitation, the volume of soil explored by *A. fistulosum* was larger than that of onion. *A. fistulosum* roots were present at a distance from 20 to 27 cm far from the plant, at the second soil layer studied, where onion roots were absent (Table 4). Even then, rooting in both species was shallow, as it was restricted to a depth of 20 cm with a high concentration of roots in the top 10 cm, where the horizontal root spread of both species was similar. Rooting depth and horizontal distribution indicate soil exploration and the potential availability of nutrients and water (Atkinson, 2000). The existing studies agree that onion is a plant of superficial rooting, with roots concentrated in a radius of 15 to 20 cm around the plant, with few roots seldom going further (Thompson, 1921; Weaver and Brunner, 1927; Drinkwater and Janes, 1955; Strydom, 1964; Portas, 1973; Greenwood et al., 1982). No reports were found on soil exploration patterns of *A. fistulosum*. The thick *A. fistulosum* stem-borne were expected to have gone deeper in soil than what was actually seen. However, the field experiment was carried out in a very wet season. So, there was enough of water in the soil upper layers. Moreover, nutrient concentration in the topsoil was not a limiting factor to divert roots to a different foraging behavior.

Conclusions

Our results showed that there was some variation for root traits among onion populations and that onion breeding for aboveground traits in the Netherlands resulted in cultivars with less dense root systems. However, the variation observed among onion populations in relation to root traits was restricted if compared to the variation found between *A. fistulosum* and onion. *A. fistulosum* had much more stem-borne roots than onion and, since they branched far more often, they produced much more lateral roots. These morphological differences conferred to *A. fistulosum* a root length density twice to three times larger than that of onion, especially due to the increase in length density of fine roots. The behavior of different populations in a given species was highly consistent even when greenhouse and field results were compared. The meager root system of all onion cultivars indicates that the development of an onion genotype with an *A. fistulosum*-like root system will very likely have a considerable impact in onion nutrient requirement and hence on fertilization practices. We consider that this approach is a valid and sensible way to effectively develop onion genotypes better adapted to balanced and sustainable agricultural systems, such as organic cultivation.

Acknowledgments

We would like to thank Jaap van der Berg for looking after the plants at the greenhouse; Andries Siepel and Henk van Rein, from the Lovinkhoeve Experimental Farm, for their help with the field experiment; and Bruno Ligthart, from the HAS – Den Bosch, for his help in collecting and cleaning the roots. We would like to thank also Prof. Evert Jacobsen and Dr. Sjaak van Heusden, from Wageningen UR, for the valuable comments on the manuscript.

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Chapter 3

Differential soil exploration and exploitation by onion and *A. fistulosum* L. cultivated in an organic farming system with two contrasting nitrogen levels

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Abstract

A. fistulosum produces more stem-borne roots than onion and, because they branch far more often, its root system has also a higher density. However, it is disputable if such differences would give any advantage to *A. fistulosum* in conditions of nitrogen shortage, an important issue in the organic production of onions. The objective of this work was to study plant and root development in onion and *A. fistulosum* grown in two organically managed soils with a three-fold contrast in N availability. Root samples were taken from three depths, up to 30 cm, and at three lateral positions, up to 27 cm far from the plant. Onions rooted up to 20-cm deep, disregard of the N availability, while *A. fistulosum* rooted up to 30 cm where less N was available. Reduction in N availability reduced severely onion root length density (L_V), while *A. fistulosum* experienced only a mild and marginal drop. In none of the species, reduction in L_V was associated with changes in number of stem-borne roots, which were always more abundant in *A. fistulosum*. Only onion had aboveground fresh and dry matter depressed by the reduction in N availability. The ratios between below- (root dry weight and length) and above-ground (shoot dry weight) growth were not altered by soil N availability in any of the species and were significantly higher in *A. fistulosum*. The results indicated that the development of an onion with an *A. fistulosum*-like root system might be a rewarding approach towards onion cultivation in more sustainable agricultural systems.

Keywords: *Allium cepa* L., Japanese bunching onion, stem-borne roots, lateral roots, root length density, nitrate, carbohydrate allocation, Auger sampling, organic agriculture, plant breeding

Introduction

Onions are notably known for its meager root system, which is made up of solely stem-borne roots with a few and short laterals that rarely rebranch (Weaver and Brunner, 1927; Pulgarin et al., 1988; Brewster, 1994; Chapter 2). As a consequence, onions have a very low root density and, hence, a low nutrient uptake surface (Baligar and Barber, 1978; Burns, 1980; Greenwood et al., 1982; Chapter 2). Concurring to reduce even more the potential for nutrient uptake, root hairs are either missing or, when present, are rare and short (Föhse et al., 1991). Such poor soil exploitation ability is not compensated by a good exploration pattern: onion root system is shallow and poor laterally spread (Weaver and Brunner, 1927; Drinkwater and Janes, 1955; Portas, 1973; Chapter 2).

The co-occurrence of such adverse root characteristics turns the proper supply of nutrients to onion into a real challenge. For nutrients delivered to the roots mostly by diffusion, such as P and K (Drew and Nye, 1969), onion needs a fertilization rate high enough to raise the ion concentration in the soil solution to levels in which the diffusion rate will not be a limiting factor to uptake (Baligar and Barber, 1978; Föhse et al., 1988). Nitrogen fertilization is by no means easier, even in cases when most N is available as nitrate and therefore mass-flow and not diffusion is the main N supply process (Tischner, 2000). The association of nitrate leaching in the soil with the shallow and limited rooting zone of onion makes a combination that restricts onion N uptake to not more than one third of the applied N, even under suboptimal N supply (Greenwood et al., 1992; Neeraja et al., 2001). Consequently, as the other nutrients, N has to be supplied in very high rates if reasonable yields are to be achieved (Greenwood et al., 1992; Brewster, 1994). Such inefficiency in N uptake places onion cultivation under pressure if one thinks about the increasing economic costs of fertilization (Moll et al., 1982; Greenwood et al., 1992) and the environmental contamination associated to the production and use of synthetic fertilizers (for reviews see (Power and Schepers, 1989).

To circumvent the excessive supply of N through synthetic fertilizers, the implementation of more sustainable agriculture systems, such as low-input systems, where the use of synthetic fertilizers is reduced, and organic agriculture, where it is totally avoided (Lammerts van Bueren et al., 2002), might be an alternative. In organic farming, N, as well as the other nutrients, are supplied through organic sources, as plant composts and animal manure (Power and Schepers, 1989; Poudel et al., 2002). These sources, when properly used, reduce risks of N leaching, since N-organic has still to be mineralized, while synthetic fertilizers supply N already as N-mineral (Poudel et al., 2002). However, the need for mineralization, which many times occur in a low pace, also means that N is not as promptly and abundantly available as when

synthetic fertilizers are used (Lammerts van Bueren et al., 2002; Mäder et al., 2002). For a crop as inefficient in N uptake as onion such restrictions in N availability can turn out to be too detrimental to plant development and yield.

Therefore, the development of onion genotypes with enhanced efficiency in N uptake would be a valuable input to organic farming, applicable also to conventional growing. N uptake is dependent mainly on plant demand (Tischner, 2000). Nevertheless, under suboptimal conditions of supply, uptake is highly influenced by physiological and, even for a N-form as mobile as nitrate, also by morphological root characteristics (Clarkson, 1985; Sattelmacher et al., 1993; Robinson et al., 1994; Schröder et al., 1996). Hence, improvement in one or in both groups of characteristics should improve also N uptake. Nevertheless, links between root morphology and function are still poorly understood, due also to the difficulties in screening root systems (Atkinson, 1990; Clarke and McCaig, 1993).

We found variation in root morphology in onion, but in a very restricted magnitude, especially if compared to the pronounced differences found between onion and *A. fistulosum* (Chapter 2). *A. fistulosum*, commonly known as the Japanese bunching onion or Welsh onion, is closely related to onion. It is an important vegetable in the Far East (Jones and Mann, 1963; Brewster, 1994) and, in addition, it is an important gene reservoir for the breeding of new onion cultivars (reviewed by Kik, 2002). We observed that *A. fistulosum* had overwhelmingly more stem-borne and lateral roots than onion, which yields a root length density twice to three times higher than that of onion (Chapter 2). In plants with adventitious root systems like the Alliaceae, stem-borne roots are responsible for soil exploration, understood here as the expansion of the rooting zone in the chase for farther resources, including the ability to overcome soil mechanical resistance, if necessary (Materechera et al., 1992; Sattelmacher et al., 1994). Lateral roots are essential to raise the root length density and therefore to improve the soil exploitation, *i.e.*, the use of the soil to capture resources, which is an ability especially valuable in conditions of sub-optimal nutrient supply (Atkinson, 1990; Rao and Ito, 1998). In the present work, we intended to study soil exploration and exploitation in species with such a marked morphological differences in the roots, like onion and *A. fistulosum*, when plants were grown in two locations, with contrasting levels of soil N availability, in an organic farm.

Material and Methods

Two field experiments were set out in 2001 at the Lovinkhoeve Experimental Farm, located in the Flevoland Province, the Netherlands. This experimental farm is managed organically since 1995. Two field sites (locations), where contrasting amounts of organic amendment have being applied for several years (Table 1), were

used. The two locations were 50 m apart. Soil samples were analyzed at the onset of the experiment for N, P and K contents, as well as pH, and additionally in two other dates during the experiment to N (Table 2). Considering only the three main macronutrients, the most important difference between the two locations was the availability of mineral N, which varied in a order of 2.5- to 3-fold between them along the experiment (Table 2). Phosphate and potassium availability (P_w and K-HCl) was almost similar in the two locations (Table 2). The aforementioned soil contents of N-mineral, P_W and K-HCl are regular for the Dutch mineral soil conditions, although N-mineral and K-HCl are respectively in the low and in the high ends.

Table 1. Crop rotation and organic amendment applied at the Lovinkhoeve Experimental Farm, with nutrient contents and amounts used in plots OM_1 and OM_3.

Date	**Crop**	**Amendment**[1]	**Nutrient Content % of fresh weigh**			**Amount t.ha^{-1}**	
			N-total	**P_2O_5**	**K_2O**	**OM_1**	**OM_3**
April, 1997	Lucerne/Rye-grass	Pig slurry	0.60	0.34	0.58	0	20
May, 1998	Sugar beet/carrot	Cattle slurry	4.13	1.85	6.12	0	40
Oct., 1998	Wheat	Goat manure + straw	2.20	1.30	4.39	0	75
May, 1999	Potato	Cattle slurry	0.46	0.18	0.47	10	10
May, 2000	Corn	Pig slurry	0.30	0.07	0.37	0	30
April, 2001	Onion	Cattle slurry	0.25	0.12	0.28	10	40

[1]/ Solid manure was distributed in autumn and then incorporate by plowing up to 25 cm deep. Liquid manure was applied by injection, in spring, at 10-15 cm deep.

Table 2. Nutrient content and pH in the soil of plots OM_1 and OM_3 at the Lovinkhoeve Experimental Farm, in 2001.

Nutrient	**May**		**June**[1]		**August**[1]	
	OM_1	**OM_3**	**OM_1**	**OM_3**	**OM_1**	**OM_3**
NH_4^+ (mg.kg^{-1})	1.2	2.2	1.3	1.7	1.3	1.7
NO_3^- (mg.kg^{-1})	4.4	11.7	5.7	16.9	4.3	16.0
N-mineral (mg.kg^{-1})	5.6	13.9	7.0	18.6	5.6	17.7
P - total (mg P_2O_5.100g^{-1})	165	165	-	-	-	-
Pw (mg P_2O_5.100g^{-1})	27	31	-	-	-	-
K – HCl (mg.100g^{-1})	147	173	-	-	-	-
pH - KCl	7.3	7.4	-	-	-	-

[1]/ Only nitrogen was assessed in June and August

The experiments were carried out in a completely randomized block design, with four blocks per location. We transplanted 45-day old uniform seedlings from two accessions of *A. fistulosum*, namely CGN 16.369 and CGN 16.442 (CGN: Center for Genetic Resources, Wageningen, the Netherlands) and two onion accessions, the F_1 hybrid Drago and the open-pollinated cultivar Jumbo. Each accession was placed in a plot, making four plots per block. Fourteen days after transplant, experiments were thinned, leaving nine plants per plot, spaced by 50 x 50 cm. The large spacing between plants was used to allow free root development without mixing roots from neighboring

plants. No irrigation supplementary to rainfall was used and weeding was done mechanically.

Three evaluations were performed, 35, 65 and 95 days after transplanting. In the first and second evaluation, 0.35 x 0.60 cm (width x depth) pinboards (Oliveira et al., 2000), with 10-cm high pins, spaced by 5 x 5 cm, were applied to soil trenches excavated adjacently to the plants. Pin-boards were used to guide the Auger sampling and not as a source of data for analysis. One plant per accession was sampled in both locations at the first and second evaluations. The soil monoliths obtained with the pinboards were transferred to the laboratory and immersed overnight in a 5% pyrophosphate solution under vacuum to aid in dissolving the clay. In the following day, the pinboards were manually washed using gentle water streams. In the first and second evaluation dates, pinboards revealed roots dispersed respectively in a 20- and 30-cm radius around the plants. Those distances were then sampled, both vertical and horizontally, using the Auger method (Oliveira et al., 2000). No pin-boards were taken at the third evaluation date because at this time onions were at the harvest point, with roots already dead.

Auger samples were taken from two plants per accession in each block, from the four blocks per location, in the first and second evaluation dates. Samples of the same accession in the same block were combined in one. Cores with 7-cm diameter x 10-cm height (385 cm^3) were used. On the first evaluation date, samples were taken in two lateral positions in relation to the plant, from 0 to 7 and from 10 to 17 cm far from the plant, and in two depths, from soil surface to 10 cm deep and from 10 to 20 cm deep. On the second evaluation date, the same points as before were sampled, with one additional lateral position, from 20 to 27 cm far from the plant, and one additional depth, from 20 to 30 cm deep. Auger samples were frozen at -18^oC and then washed by immersion in running bubbling water for 15 minutes. Roots were retained in sieves and separated from debris by flotation and hand cleaning. Clean roots were stained overnight in a 500 ppm solution of safranin, at 4^oC. In the following day, the excess of dye was removed under running water and the roots were scanned using a flatbed scanner. Images were analyzed using the software WinRhizo®. Root length, as well as the root length per root diameter, were divided by the volume of the core to obtain the volumetric root length density (L_V) as well as length density for fine roots ($0.2 \leq x < 0.4$ mm) (Chapter 2), in each sampling point. Root length density at each lateral position was multiplied by the volume of soil represented by a given lateral position, considering the lateral positions as concentric rings around the plant. A total root length was then estimated for each plant by summing the root lengths found in each concentric ring. The total root length was used to calculate the root length : shoot dry weight ratio. Scanned roots were dried (72 hours, 72^oC) and weighted. A total root dry

weight was estimated as described for the total root length and used to calculate the root : shoot dry weight ratio. Plants whose roots were sampled were collected along for counting the number of stem-borne roots.

The fresh and dry weights of leaves and, in the case of onions, also bulbs, referred to as shoot weight, were measured in the plants whose root systems were sampled. Fresh weight was obtained in the same day plants were harvested, soon after plants arrived at the laboratory to avoid dehydration. Dry weight was obtained after placing chopped leaves and bulbs at 72^{o}C in an oven with forced ventilation, for 96 hours. On the third evaluation date, when onion bulbs were already formed, three plants per accession, including *A. fistulosum*, were harvested and their fresh and dry weights were assessed. We calculate the ratios between root length and shoot dry weight and between root and shoot dry weight using root figures from the second evaluation, when root development was at maximum, and shoot figures from the third evaluation, when bulbs were heavier in onion and leaves were larger in *A. fistulosum*.

Statistical analysis was performed using GenStat® 6.1. The analysis was done according to the model of a completely randomized block design on two locations, with locations corresponding to N availability levels. For analysis, N levels were tested against N x block level. Data for L_V of fine roots, root length : shoot dry weight and root : shoot dry weight were transformed to the square root before analysis.

Results

In the first evaluation, 35 days after transplant, plants were still small and had few roots (data not presented) while in the last evaluation, 95 days after transplanting, the root systems were already aged, showing mostly dead roots. Thus, root traits were evaluated and compared among accessions using the data collected in the second evaluation date, 65 days after transplanting. At this date, roots in all genotypes were healthy and no biotic or abiotic damages were apparent.

Soil Exploration

Onion roots explored the three lateral positions in the top 10 cm of soil and the two lateral positions closest to the plant in the second soil depth, from 10 to 20 cm deep, as indicated by values of root length density different from 0, at both soil plots (Table 3). There were no effects of N availability over the soil exploration pattern of onion. *A. fistulosum* roots spread laterally to all sampled positions, independent of N availability, in the two top soil layers, from 0 to 10 cm and from 10 to 20 cm deep (Table 3). Contrary to onion, changes in N availability affected the rooting depth in *A. fistulosum* (Table 3). At the high N availability level, no *A. fistulosum* roots were found below 20 cm, while when N availability was reduced, *A. fistulosum* developed a

significant root length density in the soil layer between 20 and 30 cm, in the vicinity of the plant (Table 3). As consequence, the rooting zone of *A. fistulosum* in the low N availability level had a V-like shape, contrary to the U-like format developed when plants grew where more N was available.

Table 3. Root length density ($cm.cm^{-3}$) of onion and *A. fistulosum* grown at an organic farming system, with two levels of N available in the soil, in three different depths and three lateral positions in relation to the plant.

Soil Depth (cm)	**Lateral Positions (cm from the plant)**					
	0 – 7		**10 – 17**		**20 – 27**	
0 – 10	**Onion[2]**	***A. fist*[2]**	**Onion[2]**	***A. fist*[2]**	**Onion[2]**	***A. fist*[2]**
High N[1]	1.44 aB[3]	3.20 aA	1.06 aB	2.30 aA	0.43 aB	1.92 aA
Low N	0.96 aB	2.97 aA	0.54 bB	2.31 aA	0.13 bB	1.62 bA
10 – 20						
High N	0.37 aB	0.93 aA	0.14 aB	0.49 aA	0.0	0.16 a
Low N	0.35 aB	0.95 aA	0.10 aB	0.43 aA	0.0	0.14 a
20 – 30						
High N	0.00	0.00 b	0.0	0.0	0.0	0.0
Low N	0.00 B	0.30 aA	0.0	0.0	0.0	0.0

[1]/ N levels are indicated in table 2; [2]/ Each mean represent the average of the two populations belonging to a given species; [3]/ Numbers followed by the same small letter in the column did not differ significantly ($p < 0.05$) from each other, indicating that there is no difference between N levels within a given species. Numbers followed by the same capital letter in the row did not differ significantly ($p < 0.05$) from each other, indicating that there is no difference between species in the same N level. Comparisons are restricted to the same soil depth, at the same lateral position.

Soil Exploitation

A decrease in root length density (L_V) as response to the reduction in N availability was observed in both species, although onion was significantly more affected than *A. fistulosum* (Table 3). Changes in L_V were most prominent in the upper soil layer (0 to 10 cm), where in onions it was depressed by 33.3 to 69.8% when N availability was reduced (Table 4). In *A. fistulosum*, the decrease in L_V as response to the lowering of N availability was of only 0 to 15.6% (Table 4). No significant interactions were found between accessions and locations for a given species.

Table 4. Percentage of reduction in root length density (L_V) and in root length density of fine roots (L_{Vfine}) in onion and *A. fistulosum* grown in an organic farming system, as response to a three-fold reduction in the soil N availability.

Soil Depth (cm)	**Lateral Positions (cm from the plant)**					
	0 – 7		**10 – 17**		**20 – 27**	
	Onion[2]	***A. fist*[2]**	**Onion[2]**	***A. fist*[2]**	**Onion[2]**	***A. fist*[2]**
L_V						
0 – 10	33.3	7.2	49.1	0.0	69.8	15.6
10 – 20	5.4	0.0	28.6	12.2	-	12.5
L_{Vfine}[1]						
0 – 10	44.8	- 3.2	60.0	- 8.2	75.0	12.2
10 – 20	20.0	5.7	50.0	40.0	-	12.5

[1]/ Fine roots = roots with diameter greater than or equal to 0.2 and smaller than 0.4 mm (Chapter 2); [2]/ Each mean represent the average of the two populations belonging to a given species.

As a portion of the root system of onions (± 20%) and the major part of it in *A. fistulosum* (± 60%) consisted of fine (lateral) roots and these roots play a key role in soil exploitation, changes in the L_V of fine roots (L_{Vfine}) in response to changes in N availability were also studied. In all sampling points where L_{Vfine} could be compared between species it was significantly higher in *A. fistulosum* than in onion, without any significant interaction between accessions and locations (N availability level) for a given species (Table 5). Considering the response to the change in N availability in soil, similar trends were observed in L_{Vfine} when compared to what was found in the overall root system in both species. In onion, there was a severe reduction L_{Vfine} as response to changes in the N availability in all three lateral positions at the top 10 cm of soil, while in *A. fistulosum* the reduction in L_{Vfine} was much slighter and restricted to the farthest lateral position (Table 5).

Table 5. Root length density ($cm.cm^{-3}$) of fine roots (diameter greater or equal to 0.2 mm and smaller than 0.4 mm) of onion and *A. fistulosum* grown at an organic farming system, with two levels of N available in the soil, in three different depths and three lateral positions in relation to the plant.

Soil Depth	**Lateral Positions (cm from the plant)**					
(cm)	**0 – 7**		**10 – 17**		**20 – 27**	
0 – 10	**Onion[2]**	***A. fist*[2]**	**Onion[2]**	***A. fist*[2]**	**Onion[2]**	***A. fist*[2]**
High N[1]	0.29 aB[3]	1.26 aA	0.25 aB	0.97 aA	0.12 aB	0.82 aA
Low N	0.16 bB	1.30 aA	0.10 bB	1.05 aA	0.03 bB	0.72 bA
10 – 20						
High N	0.05 aB	0.35 aA	0.02 aB	0.20 aA	0.0	0.08 a
Low N	0.04 aB	0.33 aA	0.01 aB	0.28 aA	0.0	0.07 a
20 – 30						
High N	0.00	0.00 a	0.0	0.0	0.0	0.0
Low N	0.00 A	0.04 aA	0.0	0.0	0.0	0.0

[1]/ N levels are indicated in table 2; [2]/ Each mean represent the average of the two populations belonging to a given species; [3]/ Numbers followed by the same small letter in the column did not differ significantly ($p < 0.05$) from each other, indicating that there is no difference between N levels within a given species. Numbers followed by the same capital letter in the row did not differ significantly ($p < 0.05$) from each other, indicating that there is no difference between species in the same N level. Comparisons are restricted to the same soil depth, at the same lateral position.

N availability did not affect significantly the number of stem-borne roots in neither species (Table 6). Species, however, differed markedly in this trait. *A. fistulosum* produced three times more stem-borne roots than onion in both fertility levels. There were no significant interactions between accessions and locations in a given species.

Aboveground fresh and dry matter

The objective of this work was to study how onion and *Allium fistulosum* responded to changes in nutrient availability and not to compare yield or productivity between species, also because they are morphologically very different, onion

developing a bulb and *A. fistulosum* not. Hence, comparisons for aboveground matter were restricted to figures obtained from the same species in the two different fertility levels. Onion cultivars produced significantly less fresh and dry weight in the low than in the high N availability level, while for *A. fistulosum* the reduction in N availability did not had any significant effect on either fresh or dry weight (Table 6). No significant interactions between accessions and locations for a given species were detected.

Table 6. Number of stem-borne roots and aboveground weight in onion and *A. fistulosum* cultivated in an organic field, with two levels of N available in the soil.

N Level[1]	Stem-Borne Roots		Fresh Weight (g)		Dry Weight (g)	
	Onion	*A.fistulosum*	Onion	*A.fistulosum*	Onion	*A.fistulosum*
High	32.2 aB[2]	99.0 aA	325.8 a	405.2 a	33.6 a	43.4 a
Low	32.9 aB	92.5 aA	200.4 b	372.3 a	24.1 b	46.1 a

[1]/ N levels are indicated in table 2; [2]/ Numbers followed by the same small letter in the column did not differ significantly ($p < 0.05$) from each other. Numbers followed by the same capital letter in the row did not differ significantly ($p < 0.05$) from each other.

Root : shoot dry weight and root length : shoot dry weight ratios

Species differed significantly from each other in relation to both the root : shoot dry weight and the root length : shoot dry weight and ratios, but N availability did not significantly affect the ratio in a given species (Table 7). Onion had ratios significantly smaller than *A. fistulosum* independent on the N availability. There were no significant differences between populations of a given species.

Table 7. Ratios between above and below ground development in onion and *A. fistulosum* cultivated in an organic field, with two levels of N available in the soil.

N Level[1]	Root : Shoot Dry Weight ($mg.g^{-1}$)		Root Length : Shoot Dry Weight ($cm.mg^{-1}$)	
	Onion[2]	*A. fistulosum*[2]	Onion[2]	*A. fistulosum*[2]
High	48.9 aB[3]	106.6 aA	0.52 aB	1.29 aA
Low	38.5 aB	93.8 aA	0.37 aB	1.11 aA

[1]/ N levels are indicated in table 2; [2]/ Figures for onion and *A. fistulosum* represent the average for the populations in a given species; [2]/ Numbers followed by the same small letter in the column did not differ significantly ($p < 0.05$) from each other. Numbers followed by the same capital letter in the row did not differ significantly ($p < 0.05$) from each other.

Discussion

Soil Exploration

Onion rooting zone was not altered by changes in N availability and at both levels it was smaller than *A. fistulosum*'s (Table 3). In opposition, A. *fistulosum* responded to the change in N availability by increasing rooting depth in the lower N availability level (Table 3). Increase in root development at deeper layers in association with sub-optimal supply of nutrients was observed before in other crop

species when grown under shortage of nitrogen (Sattelmacher et al., 1994). Other factors reported to induce roots to forage in deeper regions of the soil profile are competition between plants or even between roots of the same plant in case of high density (Burns, 1980) and drought (reviewed by Hoad et al., 2001). Competition between roots could have occurred in *A. fistulosum*, but drought was not the case in this study, due to very wet season.

Concerning access to soil resources, the relevance of the ability to explore larger volumes of soil that *A. fistulosum* showed in relation to onion is unquestionable, especially if nutrients are dispersed in the soil in patches. Actually, localization of nutrients is rather common in organic agriculture, due to the distinct mineralization rates of organic matter in both time and space (van Vuuren et al., 1996). If a radial symmetric root distribution around the plant is assumed (van Noordwijk et al., 1985), *A. fistulosum* exploration of the farthest lateral position in the second soil layer increased the volume of its rooting zone by 30% in comparison to onion. The rooting zone gained by *A. fistulosum* in depth, in the low N availability level, is important especially in cases of drought. However, the increase in volume in the rooting zone was small, only 2%, and hence its value for plant nutrition should not be overestimated.

Soil Exploitation

Root length density (L_V) is the most commonly quoted root attribute related to soil exploitation and nutrient uptake (Atkinson, 1990; Hoad et al., 2001). In onion, L_V was severely depressed by the reduction in N availability. In contrast, the effects of changes in N availability in the soil over L_V in *A. fistulosum* were mild and marginal (Table 3). Under regular circumstances, the importance of L_V for N uptake, especially in its nitrate form, is highly disputable (Robinson et al., 1991; Wiesler and Horst, 1994). Very low L_V values have been estimated for some species through modeling to be enough to deplete almost all nitrate present in the rooting zone (Burns, 1980; Barraclough, 1989). In maize, however, the threshold below which N-uptake was shown to be directly dependent on root length was 1.5 $cm.cm^{-3}$ (Wiesler and Horst, 1994), definitely not that low an higher to what was observed here in onion (Table 3). Actually, there is reasonable agreement that, as roots become scarcer and N availability in the soil is reduced, N uptake, also as nitrate, becomes more and more dependent on L_V (Atkinson, 1990; Sattelmacher et al., 1993; Robinson, 1994; Wiesler and Horst, 1994).

Taking this into account, together with the reduced size of onion plants grew at the low N availability level (Table 6), our results strongly suggest that onion suffered from N shortage when the N available in soil was reduced and that happened in

consequence of two mechanisms, one being direct and, the other, indirect. The decrease in soil N availability reduced the uptake and very likely led to N limitation, which, in its turn resulted in smaller plants (Table 6). Smaller plants developed fewer roots, which impacted the N uptake, feeding back the N limitation. Therefore, N shortage was caused primarily by the reduction in N availability in the soil, but it was also, at the same time, cause and consequence of the severe reduction in L_V experienced by onion (Table 3). In addition, as shown by the root density of fine roots (Table 5), onion failed to preserve most of its fine roots, usually the most efficient in uptake (Burns, 1980; Robinson et al., 1991; Rao and Ito, 1998; Tischner, 2000), as N availability in the soil was reduced. Reduction in lateral root development in association with low nutrient availability was also reported in onion by Neeraja et al. (2001), who observed, in addition to the reduction in fine root proliferation, decreases in N-, P- and K-uptake.

Such drastic reduction in the soil exploitation capacity might have affected the uptake of other nutrients as well. In our experiments, P could have been an additional limiting factor to plant development both above and below ground in onion. Firstly, in soil similar to the one used here, with the same contents of P available, but managed in a conventional way, Greenwood et al. (1992) applied 100 $kg.ha^{-1}$ P_2O_5 to secure high yields in onion. Secondly, P uptake, much more than N, is dependent on L_V (Römer et al., 1988; Krannitz et al., 1991; Sattelmacher et al., 1993), especially in species like onion, which has low P uptake efficiency (Föhse et al., 1988). In species with these characteristics, if L_V is not high enough, P uptake becomes insensitive to demand, dependent on P concentration in the soil solution and totally limited by P diffusion (Drew and Nye, 1970; Krannitz et al., 1991). In such circumstances, as P is depleted in the root absorbing surface, the soil becomes increasingly unable to replenish it at a rate compatible with demand and ultimately plants experience deficiency and growth limitation (Drew and Nye, 1970; Sattelmacher et al., 1993).

Stem-borne roots are important for both *A. fistulosum* and onion because they are the solely site for lateral roots development, since neither species keep the primary root and re-branching, is very uncommon (Weaver and Brunner, 1927; Pulgarin et al., 1988; Birdsall and MacLeod, 1990; Chapter 2). In our experiments, onion and *A. fistulosum* differed strikingly in number of stem-borne roots, as also observed previously (Chapter 2), but nutrient availability had no significant effect over the number of stem-borne roots in neither species (Table 6). Kumar et al. (1998) also reported that supplies as different as 0 and 130 $kg.ha^{-1}$ N had no effect over the number of roots in onions. Actually soil moisture is the most determinant stimulus to the development of stem-borne roots in onion, which will elongate only if moisture

reaches the stem at least periodically (Jones and Mann, 1963). In our experiments, due to a very wet season, moisture was never a limiting factor.

Aboveground Fresh and Dry Matter

The reduction in N availability in the soil did not affect *A. fistulosum*, but led to a reduced aboveground development in onion (Table 6). There are several reports of yield depression in onion related to the decrease in nutrient availability or in fertilization rates. Regarding nitrogen, in all cases high yields are achieved only when high amounts of fertilizers are applied, in a range of 100 to 300 $kg.ha^{-1}$ of N (Wiedenfeld, 1986; Greenwood et al., 1992; Patel and Vachhani, 1994; Neeraja et al., 2001). In case of phosphate, fertilization rates vary from as low as 25 $kg.ha^{-1}$ of P, where P available in soil is abundant, to as high as 350 $kg.ha^{-1}$ of P, for tropical soils, where P available in soil is negligible (Fontes and Nogueira, 1984; Galbiatti and Castellane, 1990; Brewster, 1994). Contrary to onion, there is little published work on *A. fistulosum* nutrient requirements. Brewster (1994) mentioned the application of fertilizers three to four times during the growing season, using in total 200 and 150 $kg.ha^{-1}$ of N and P, respectively. Lee et al. (1996), after 10 years of experiments, reviewed the N recommendation for *A. fistulosum* in Korea, reducing it from 250 to 75 $kg.ha^{-1}$. As discussed earlier, the rooting zone was much larger and root density was much higher in *A. fistulosum* than in onion (Table 3). In addition, the proportion of thinner (lateral) roots, which are considered to be more efficient in nutrient uptake (Burns, 1980; Robinson et al., 1991; Rao and Ito, 1998), was also higher in *A. fistulosum* than in onion (Table 5). The accumulation these differences certainly played a role in sustaining *A. fistulosum* aboveground fresh and dry matter production when the N availability in the soil was reduced.

Root : shoot dry weight and root length : shoot dry weight

Föhse et al. (1988) have already stressed how small the root : shoot ratio actually is in onion when compared to several other vegetables. In our experiments, onion had, in addition to a smaller root : shoot dry weight ratio, also a much smaller root length:plant dry weight ratio than *A. fistulosum* (Table 7). This means that onion needed less mass and less length of roots to produce the same amount of shoot dry matter than *A. fistulosum*. Phrased in this way, it sounded very efficient of onion. However, such efficiency is highly questionable. There is a functional equilibrium between root and shoot development (Brouwer, 1962). The amount of roots available for nutrient uptake depends on the amount of shoots available for CO_2 fixation and vice-versa. Within the limits of this equilibrium, plants can take the risk or the insurance strategy (Atkinson, 2000). Plants that assume the risk maximize shoot production in detriment of roots, but become intrinsically dependent on ideal growing

conditions, such as an adequate supply of nutrients and water. In our experiments, onions assumed the risk, and, although efficient in supporting a heavy aboveground production, failed to keep it at the same level when the N availability in the soil was reduced (Table 6). The insurance strategy, taken in our experiments by *A. fistulosum*, is characterized by plants that allocate a higher proportion of carbohydrates to roots when compared to plants that assume the risk strategy. This allocation pattern is rewarded by an improvement in soil exploitation and, many times, exploration pattern, which leads, under suboptimal conditions, to an improved tolerance to stress. Obviously, the insurance strategy is by far the most adequate for more sustainable crop systems, such as low-input and organic cultivation.

Independent of the strategy developed by each species, changes in N availability in soil did not affect any of the below- : above-ground ratios in either onion or *A. fistulosum* (Table 7). Instead, the change in the N availability in the soil induced coupled variations in above and below ground development in both species. Some crops, most of them slow-growing plants, such as the biennial onion, show indeed little flexibility in their root : shoot ratios and, in conditions of shortage, both components are reduced proportionally (reviewed by Clarkson, 1985). Our results concerning the ration between root and shoot dry weight and between root length and shoot dry weight (Table 7) point to the conservative nature of the ratios in onion. Further research is needed to conclude about *A. fistulosum*, since it is likely that it has not experienced stress in our experiments.

Conclusions

The results obtained in this study clearly showed that onion suffered from nutrient shortage, but *A. fistulosum* not, or, at least, not as severely as onion. N use efficiency was not assessed in this work. Even then, the disparity in root morphology and development between *A. fistulosum* and onion was by far too large to be denied a key role in the achieved results. The difference in the size of the rooting zone (soil exploration) and the enormous difference in root length density and in the density of fine roots (soil exploitation) between the two species conferred to *A. fistulosum* the ability to access a much higher amount of nutrients than onion. Such plus was surely crucial in sustaining both above and below ground development when the concentration of N available in the soil was reduced by a factor of 3. These are strong indications that *A. fistulosum* matches much better than onion with low-input agricultural systems, where keeping a luxurious N availability in the soil is not an alternative, either because it is not feasible or because it is not advisable. Consequently, the introgression into onion of the characteristics that give *A. fistulosum* this superior adaptation, aiming at ultimately develop an onion cultivar with an *A.*

fistulosum-like root system is certainly a rewarding approach towards a more sustainable onion cultivation.

Acknowledgments

We would like to thank Andries Siepel and Henk van Rein, from the Lovinkhoeve Experimental Farm, for their help in carrying out the experiments, as well as Bruno Ligthart, from the HAS – Den Bosch, who helped us in collecting and cleaning the root samples. Furthermore, we would like to thank Dr. Thom Kuyper, from Wageningen UR for his valuable comments on the manuscript.

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Chapter 4

Shoot and root growth of *A. fistulosum* L. cultivated in an organically managed soil with indigenous and introduced Arbuscular Mycorrhizal Fungi (AMF)

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Abstract

This work was aimed at studying *Allium fistulosum* L. response to Arbuscular Mycorrhizal Fungi (AMF) introduced through seedling inoculation when plants were grown in an organically managed soil, in the presence and absence of indigenous AMF, in the greenhouse. To this end, seeds were sown in trays filled with either solely sterile sand or sterile sand with *Glomus intraradices* inoculum distributed in the sowing rows. Seedlings were transplanted five weeks later to pots filled with soil collected in an organic farm, half of the pots filled with heat sterilized soil. Inoculation with *G. intraradices* resulted in 40- to 50%-increase in shoot dry biomass and root length in both sterilized and unsterilized soil. AMF inoculation increased also the number of stem-borne roots from 40, in uninoculated, to 60, in inoculated plants. Root colonization was significantly higher in inoculated than in uninoculated plants in both sterilized and unsterilized soil, although uninoculated plants grown in unsterilized soil had already 50% of root colonization, due to the activity of indigenous AMF. Arbuscules were found only in plants grown in unsterilized soil and thus were very likely produced exclusively by indigenous AMF. Vesicles were found in significantly higher number when plants were inoculated. This is the first report in *Allium* of plant inoculation with AMF followed by growth in an organically managed soil, with its indigenous AMF inoculum. These results suggest that plant inoculation with AMF can potentially improve *A. fistulosum* yield in conditions of organic agriculture.

Keywords: Japanese bunching onion, Welsh onion, plant breeding, organic agriculture, root length density, stem-borne roots, soil sterilization

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous in soil and are found colonizing roots of the vast majority of plants, including most *Allium* species. There is extensive literature on the association between AMF and onion, including the well-known series of papers from Mosse and co-workers (Mosse and Hayman, 1971; Mosse, 1973; and many more), an excellent review (Stribley, 1990) and more research reported after that (Sharma and Adholeya, 2000; Charron et al., 2001a; 2001b). There is also research on leeks (reviewed by Stribley, 1990) and on chives (Bååth and Spokes, 1989; Wininger et al., 2003). *A. fistulosum*, commonly known as the Japanese bunching onion or Welsh onion, has also been a subject of investigation. Being an important vegetable in the Far East, most, if not all, published research involving the association between *A. fistulosum* and AMF comes from Japan (Tawaraya et al., 1996; 1999; 2001; Matsubara et al., 2002). In western countries, *A. fistulosum* is also cultivated for the harvesting of its flavor-rich green leaves. The abovementioned *Allium* species are responsive to AMF, or, in other words, react to the association with AMF by increasing growth and ultimately yield under nearly all environmental conditions (Plenchette et al., 1983).

The impact of AMF on conventional agriculture has been relatively low (for reviews, see Creighton Miller et al., 1986; and Stribley, 1990). This is due to a number of reasons, from which the most important are the lack of a thorough understanding of AMF biology and ecology and, more pragmatically, the relatively cheap and efficient solution provided by mineral fertilizers to supply nutrients to plants in large scale (Stribley, 1990; Hamel, 1996; Mäder et al., 2000; Charron et al., 2001a; Ryan and Graham, 2002). The raising importance of organic agriculture worldwide relived the opportunities for the application of AMF in agriculture. In organic agriculture, synthetic fertilizers are not used and alternative solutions to give plants a proper nutrition gained evidence, such as the use of *Rhizobium*, AMF and animal manure (Lammerts van Bueren et al., 2002). In addition, there is generally a reduction in soil nutrient availability in organically managed fields as consequence of the discontinuation of the use of mineral fertilizer (Dekkers and van der Werff, 2001). This reduction in soil nutrient availability favors the activity of both indigenous and introduced AMF in colonizing roots (Sattelmacher et al., 1991; Ryan et al., 1994; Miller and Jackson, 1998; Mäder et al., 2000; and reviews of Hamel, 1996; and of Ryan and Graham, 2002).

The use of AMF in organic agriculture can be especially successful in improving plant growth if coupled with plant breeding initiatives, such as the selection or development of cultivars with root systems more efficient in nutrient acquisition and/or with enhanced responsiveness to AMF (Hetrick et al., 1995; Murphy et al.,

1997; Kaeppler et al., 2000; Ryan and Graham, 2002). We have been working into this direction in onions (*Allium cepa* L.). In a previous work, we have shown that onions have a very limited variation for root traits and that *A. fistulosum* root system was much better developed when compared to onion's (Chapter 2). In addition, when evaluated in the field under organic management, *A. fistulosum* could stand a three-fold reduction in nitrate availability in soil without decreasing above and below-ground development, while in the same conditions onion growth was severely depressed (Chapter 3).

AMF can be implemented into organic agriculture by management of indigenous AMF, by addition (inoculation) of alien species and by a combination of both. (Dodd and Thomson, 1994) present a very elegant decision-make diagram on what choice to make. In the present work, the aim was to investigate if introduced AMF would improve *A. fistulosum* growth when plants were cultivated in a soil managed in a typical organic way. Plant inoculation was chosen in place of soil inoculation because it fits well with the *A. fistulosum* production system, which includes a transplant step (Brewster, 1994). Therefore, the current study was focused mainly on the effects of inoculated AMF on plant growth, differing from most of the work carried out with AMF in organic agriculture-oriented research, usually more concerned with the management and effectiveness of indigenous AMF.

Material and Methods

Seeds of *Allium fistulosum* accession CGN 16.442 (CGN = Center for Genetic Resources, Wageningen, the Netherlands) were sown in trays filled with sterile sand and kept in a greenhouse with controlled temperature (minimum of 15, maximum of 25^{o}C) until transplant. In half of the trays, seeds were inoculated with AMF by sowing in rows, over a cushion of inoculum (spores, soil and roots colonized by *Glomus intraradices*), using in average 200 mg of inoculum per seed. Three to five plantlets per tray were checked for AMF root colonization following the procedure used by Mosse and Hayman (1971), three and five weeks after sowing. In the latter check, roots were satisfactorily colonized by *G. intraradices*. Plants from the uninoculated trays were also checked and did not show any root colonization.

Five-week old uniform plantlets were carefully transplanted to big 5-L pots (1 plant per pot) filled with soil collected from the Lovinkhoeve Experimental Farm, which is organically managed since 1995 (management from 1997 to 2001 is presented in table 1). Species composition of the indigenous AMF community has not been assessed. Half of the pots were filled with sterilized soil, while half were filled with soil in its natural status, without sterilization. Sterilization was carried out by heating the soil up to a 105^{o}C, twice, with 24-hour rest in between, 30 days before starting the

experiment. To restore the soil microflora, a week after sterilization pots received soil leachings, filtered twice in Whatman paper number 1. Soil samples were collected from sterilized and unsterilized soil, a week before transplanting, for determination of N and P soil content and pH (Table 2).

Table 1. Crop rotation and organic amendments supplied to the soil at the Lovinkhoeve Experimental Farm.

Date	Crop	Amendment[1]	Nutrient Content (% of fresh weight)		
			N-total	P_2O_5	K_2O
April, 1997	Lucerne/Rye-grass	Pig slurry	0.60	0.34	0.58
May, 1998	Sugar beet/carrot	Cattle slurry	4.13	1.85	6.12
Oct., 1998	Wheat	Goat manure + straw	2.20	1.30	4.39
May, 1999	Potato	Cattle slurry	0.46	0.18	0.47
May, 2000	Corn	Pig slurry	0.30	0.07	0.37
April, 2001	Onion	Cattle slurry	0.25	0.12	0.28

[1]/ Solid manure was distributed in autumn and then incorporate by plowing up to 25 cm deep. Liquid manure was applied by injection, in spring, at 10-15 cm deep.

Table 2. Nutrient content and pH in sterilized and unsterilized soil collected at the Lovinkhoeve Experimental Farm.

Nutrient	Sterilized	Unsterilized
NH_4^+ (mg.100g^{-1} dry soil)	0.01	0.01
NO_3^- (mg.100g^{-1} dry soil)	4.43	1.14
N-total (mg.100g^{-1} dry soil)	11.6	12.1
P-total (mg P_2O_5.100g^{-1} dry soil)	176	171
Pw (mg P_2O_5.100g^{-1} dry soil)	31	32
pH - KCl	7.3	7.2

The experiment was carried out in a greenhouse in a completely randomized experimental design. Treatments were distributed in a factorial, with the two factors (AMF inoculation and soil sterilization) of two levels each (yes and no) resulting in four treatments. Each treatment was replicated eight times, with four plants in each replication, each plant used in one of the four harvests. Stem and leaf dry weight, hereafter referred as shoot dry weight, was evaluated biweekly from 45 to 90 days after transplanting. Shoot dry weight was obtained after drying for 96 hours, in a stove with forced ventilation, at 60^{o}C. To evaluate below ground growth, stem-borne roots were counted and root samples were collected using the Auger method (Oliveira et al., 2000). A 5-cm diameter core, positioned halfway between the plant and the border of the pot, was used to collect the samples from ground surface to the bottom of the pot (15 cm high). Subsequently, samples were frozen (-18^{o}C), then washed. Washing was carried out by immersion for 15 minutes in bubbling running water, with roots being retained in a sieve. A final hand cleaning was necessary to remove debris due to the large amount of organic matter in the soil. Once cleaned, roots were stained overnight in a 500 ppm solution of safranin, at 4^{o}C. Next, the stained roots were rinsed in water

to remove the excess of dye, well spread in a transparent tray, and scanned in a flatbed scanner. Root images were analyzed using the software WinRhizo® to obtain the overall root length in the sample and the root length in 13 classes of root diameter, with 0.1-mm increments. The root length obtained in each sample was used to calculate the root length density (L_V, in cm of root per cm^3 of soil) and to estimate the total root length (L_V x volume of soil in the pot). Total root length was used to calculate the ratio between root length and shoot dry weight. The length of roots with diameter greater than or equal to 0.2 and smaller than 0.4 mm was assumed to originate mostly from lateral roots (Chapter 2) and were summed up to obtain root length for fine roots and then root length density for fine roots (L_{Vfine}).

To assess AMF root colonization, root samples were collected in the two last harvests, 75 and 90 days after transplanting, in eight plants per treatment, the same used to evaluate shoot and root growth. Root colonization was assessed as described by Brundrett et al. (1996). Briefly, roots were kept overnight in ethanol 50%, at 4^oC, and in the following day, cleared by autoclaving for 15 minutes at 121°C, in a 10% KOH solution. After rinsing, 1-cm root segments were acidified for one hour in a 2% HCl solution and then stained overnight in 0.01% trypan blue, in lactoglycerol. Before analysis, excess of dye was removed by keeping the fragments for 1 hour in 50% glycerol. AMF root colonization was assessed in 20 root segments per plant, using a light microscope and counting the number of fungal structures (hyphae, vesicles and arbuscules) crossed by 100 intersection lines per plant.

Data were analyzed using GenStat® 6.1., applying the standard analysis model for a complete randomized factorial experiment. Shoot dry weight, number of stem-borne roots, L_V, L_{Vfine} and the root length:shoot dry weight ratio were tested by a two-way analysis of variance. L_V and L_{Vfine} were transformed for the natural logarithm before analysis. Data on root colonization did not fit ANOVA assumptions, due to heterogeneous variance and too many zeroes (sterilized soil, uninoculated plants). Therefore, root colonization was tested by regression analysis over a Poisson distribution.

Results

Soil Sterilization

Soil sterilization resulted not only in the suppression of the indigenous AMF, but also in soils with a four-fold increase in nitrate content and, consequently, in N promptly available for plant uptake (Table 2). Therefore, due to confounding effects of nitrogen availability and elimination of soil biota in sterilized soils, the growth of

inoculated and non-inoculated plants was compared only within a given soil sterilization status.

Shoot and root growth

Plants inoculated with *Glomus intraradices* produced significantly more shoot dry-biomass than those that were not inoculated, both in sterilized and in unsterilized soil, in all evaluation dates (Table 3). Mycorrhizal responsiveness, considered here as the same as mycorrhizal dependency, as described by Plenchette et al. (1983), was, on average of all evaluation dates, 45 and 47% respectively in sterilized and unsterilized soil (Table 3). It shows that plant inoculation with AMF was effective in improving shoot growth. When shoot dry biomass was plotted against time, the curves for the different treatments leveled off between 75 and 90 days after transplanting. However, in inoculated plants, the decreasing trend in mycorrhizal responsiveness over time suggests that their growth was stabilized very likely before it happened in non-inoculated plants.

Table 3. Shoot (leaf and stem) dry weight and mycorrizhal responsiveness in *Allium fistulosum* accession CGN 16.442 grown in an organically managed soil, after plant inoculation or not with *G. intraradices*, at four evaluation dates, in the greenhouse.

	Shoot Dry Weight (mg)			
	45 DAP[1]	**60 DAP**	**75 DAP**	**90 DAP**
Sterilized Soil[2]				
Inoculated	218 a[4]	387 a	402 a	439 a
Uninoculated	111 b	181 b	241 b	271 b
Responsiveness (%)[3]	49	53	40	38
Unsterilized Soil[2]				
Inoculated	120 a	173 a	253 a	339 a
Uninoculated	61 b	70 b	164 b	182 b
Responsiveness (%)[3]	49	59	35	46

[1]/ DAP = days after planting; [2]/ Soil sterilized by heating up to 105°C, twice; [3]/ Responsiveness = dependency (Plenchette et al., 1983); [4]/ Means followed by the same letter in the column within a given soil status do not differ significantly from each other ($p < 0.05$).

Number of stem-borne roots and root length density (L_V) were significantly altered by plant inoculation with *G. intraradices*, independent of the soil sterilization status (Table 4). Plants inoculated with *G. intraradices* produced more stem-borne roots and had a higher L_V than uninoculated plants, in a given soil status. L_V for fine roots (L_{Vfine}) was higher where L_V was also higher (Table 4). However, the fraction of fine roots was not altered by AMF inoculation (Table 4). This meant that inoculation affected root development as a whole and not particularly root branching and lateral root growth. Furthermore, the ratio between root length and shoot dry weight was not altered by plant inoculation with AMF either (Table 4).

Root Colonization

Plant inoculation with *G. intraradices* and cultivation in sterilized soil (only the introduced AMF present) had 66 and 79% of root colonization respectively 75 and 90 days after transplanting (Table 5). The fractional root colonization showed that hyphae were the most frequent structure found in the roots. Vesicles were also present, but arbuscules were not observed. Uninoculated plants grown in sterilized soil did not show any fungi in their roots. Therefore, soil sterilization was effective in suppressing indigenous AMF and there was no contamination with other AMF sources (Table 5).

Table 4. Root characteristics in *Allium fistulosum* accession CGN 16.442 grown in an organically managed soil, after plant inoculation or not with *G. intraradices*, in the greenhouse.

	Stem-Borne Roots	**Root Length Density**			**Root Length/Shoot Dry Weight ($cm.mg^{-1}$)**
		Total[2] $cm.cm^{-3}$	**Fine[2] $cm.cm^{-3}$**	**Fine/Total %**	
Sterilized Soil[1]					
Inoculated	59 a[3]	1.57 a	0.84 a	53.0 a	19.6 a
Uninoculated	39 b	0.89 b	0.46 b	51.0 a	18.9 a
Unsterilized Soil[1]					
Inoculated	55 a	1.27 a	0.60 a	48.1 a	22.7 a
Uninoculated	36 b	0.58 b	0.28 b	46.0 a	19.3 a

[1]/ Soil sterilized by heating up to 105°C, twice; [2]/ Total = root length density considering all roots; Fine = root length density considering only roots with diameter greater than or equal to 0.2 and smaller than 0.4 mm; [3]/ Means followed by the same letter in the column within a given soil status do not differ significantly from each other ($p < 0.05$).

Table 5. AMF root fractional colonization (%) in *A. fistulosum* accession CGN 16.442 grown in an organically managed soil, after plant inoculation or not with *G. intraradices*, 75 and 90 days after transplanting, in the greenhouse.

	Root		**Hyphal**		**Vesicular**		**Arbuscular**	
Sterilized Soil[2]	75 DAP[1]	90 DAP	75 DAP	90 DAP	75 DAP	90 DAP	75 DAP	90 DAP
Inoculated	66 a[3]	79 a	63 a	68 a	21 a	28 a	0	0
Uninoculated	0 b	0 b	0 b	0 b	0 b	0 b	0	0
Unsterilized Soil[2]								
Inoculated	86 a	85 a	89 a	80 a	32 a	27 a	9 a	9 a
Uninoculated	44 b	53 b	36 b	48 b	7 b	8 b	11 a	12 a

[1]/ DAP = days after planting; [2]/ Soil sterilized by heating up to 105°C, twice; [3]/ Means followed by the same letter in the column within a given soil status do not differ significantly from each other ($p < 0.05$).

Uninoculated plants grown in unsterilized soil, thus with indigenous AMF as the sole AMF source, had 44 and 53% of root colonization, respectively at 75 and 90 days after transplant (Table 5), showing that indigenous AMF were active. When inoculated (introduced and indigenous AMF) and uninoculated (indigenous AMF) plants grown in unsterilized soil were compared root colonization increased significantly to 86 and 85%, respectively at 75 and 90 days after transplanting, in the

former (Table 5). Hyphae were the most frequent structure found in colonized roots, both in inoculated and uninoculated plants, but significantly higher in the first (Table 5). Vesicles were present both in inoculated and uninoculated plants, but significantly more often in inoculated plants (Table 5). Arbuscular colonization was similar in inoculated and uninoculated plants (Table 5), suggesting that only indigenous AMF produced arbuscules when roots were collected. Pointing also into this direction is the fact arbuscules were never found where indigenous AMF were suppressed.

Discussion

Soil sterilization

In experiments involving AMF, soils have been systematically sterilized as a way of suppressing indigenous AMF. The methods most commonly used for soil sterilization are irradiation, fumigation and heat sterilization, using either dry- or steam-heat, and more recently soil solarization (Yost and Fox, 1979; Hale and Sanders, 1982; Jakobsen and Andersen, 1982; Bååth and Spokes, 1989; Bendavid-Val et al., 1997; Charron et al., 2001a; Tawaraya et al., 2001; Bressan and Vasconcellos, 2002). All these methods can favor plant growth for other reasons than AMF inoculation, such as elimination of harmful soil biota and increase on nutrient availability either through reducing the immobilization caused by soil biota or through the sterilization process itself (Yost and Fox, 1979; Plenchette et al., 1983; Bendavid-Val et al., 1997). In case of heat, especially in soils sterilized for the first time, as it was the case here, relatively large amounts of ammonium are formed as result of organic matter decomposition (Sonneveld, 1979). The soil used in this work is a mineral clay soil (de Vos, 1997), but with a content of organic matter higher than usual for mineral soils (81.5 $g.kg^{-1}$ soil in the top 30 cm), probably due to the organic management. However, sterilization caused an increase in nitrate, not in ammonium (Table 2). Jakobsen and Andersen (1982) also observed an increase of 2 to 3 times in the soil nitrate content following heat sterilization. Considering that nitrates are not especially affected by heat and nitrifying bacteria are eliminated during sterilization (Sonneveld, 1979), such significant changes should not occur. Nevertheless, in this experiment, after potting the sterilized soil, pots were irrigated with soil leachings to restore the microflora. The soil leachings were free from AMF, but full of the ever-present nitrifying bacteria. At the soil pH of 6.0 or above (Table 2), nitrifying bacteria are very efficient in quickly converting ammonium in nitrate (Silva and Vale, 2000). Plants grown in sterilized soil in this experiment could have benefited also from the elimination of the soil harmful biota. However, as there were no signs of damage or disease in the roots and shoots of plants growing in unsterilized soil, this benefit might have been limited.

Plant inoculation with AMF in organically managed soils

This is the first report of growing plants inoculated with AMF in an organically managed soil, in sterilized an unsterilized conditions. The work carried out so far with AMF using organically managed soils addressed the issue of soil infectivity and indigenous AMF management. No report could be found that dealt with the effects of AMF inoculation over shoot biomass, when plants, after inoculation, were cultivated in organically managed soils. Mäder et al. (2000), also in a greenhouse experiment using organically managed soil, found that inoculated AMF improved root colonization in comparison to indigenous AMF, but did not extend the evaluation to shoot biomass production. Scullion et al. (1998) observed significant yield increments in leek due to soil inoculation with AMF inocula collected in organically managed fields, but plants were grown in sterilized and then re-inoculated soil and not in a soil with its indigenous AMF and own inoculum potential. Gaur and Adholeya (2002) observed an impressive increase in dry biomass above and below ground in oat, corn and sorghum inoculated with AMF and grown in unsterilized field beds. However, the field beds were not made of genuine organically managed soil, but of a marginal soil amended exclusively with organic matter.

In our experiment, the effect of plant inoculation with AMF on shoot development was studied in a soil organically managed for several years (Table 1), with its indigenous AMF community and inoculum potential. It should be mentioned that disturbances on the soil natural status might have happen during the process of collecting and potting. In addition, the soil P content was moderate to high (Table 2). Although not very common in organically farmed system, such P levels can occur, as P remains in abundance in soil for a long time after fertilizer application has stopped (Dekkers and van der Werff, 2001). Under these conditions, *A. fistulosum* inoculation with *G. intraradices* resulted in a 40 to 50% increase in both shoot dry weight and root length (Tables 3 and 4). Such positive response of *A. fistulosum* to AMF inoculation was previously observed when plants were inoculated with other *Glomus* species and grown in conventional conditions (Tawaraya et al., 1996; 1999; 2001; Matsubara et al., 2002). In addition to the increase in biomass and root length, an increase in the number of stem-borne roots was also observed in the present experiment. The same reaction to AMF inoculation was reported by Berta et al. (1990) in leek cultivated in conventional conditions.

Tawaraya et al. (2001) found that, regarding shoot growth, *A. fistulosum* responsiveness to AMF inoculation (Plenchette et al., 1983) was 73% or higher, depending on the cultivar. In our experiment, *A. fistulosum* was less (40 to 50%, on average) but still highly responsive to AMF for shoot growth, in both sterilized and

unsterilized conditions (Table 3), in spite of the reasonably high soil P content. That plant inoculation benefited shoot and root growth in the sterilized soil was expected. However, that the magnitude of the benefit was the same in sterilized and unsterilized soil, especially when the indigenous AMF were quite active (Table 5), was intriguing. It is possible that inoculation increased responsiveness also in unsterilized soil because there was not enough inoculum in the soil and/or because the quality or efficiency of the introduced *G. intraradices* was superior to the indigenous AMF (Dodd and Thomson, 1994). It is likely also inoculation speeded up colonization and growth. Although uniform inoculated and uninoculated seedlings were transplanted, in the inoculated ones the association between plant and AMF was already established, while in uninoculated it was only about to start.

Root colonization

Plants inoculated and cultivated in sterilized soil, thus where indigenous AMF were eliminated, reached 79% of root colonization in the last harvest (Table 5). Such a high root colonization is common to *Allium* species after inoculation (Hayman and Mosse, 1971; Powell et al., 1982; Smith et al., 1986; Bååth and Spokes, 1989; Furlan and Bernier-Cardou, 1989; Wininger et al., 2003) and is reported also when *G. intraradices* was used (Charron et al., 2001a; Wininger et al., 2003). *A. fistulosum* was never before inoculated with *G. intraradices*, but when inoculated with *G. fasciculatum*, root colonization up to 93% were observed, although it varied markedly with cultivar (Tawaraya et al., 1999; 2001).

Not only the inoculated *G. intraradices* was colonizing roots in our experiment. Indigenous AMF had also an intense activity in the soil. In uninoculated plants grown in unsterilized soil, hence in the presence of exclusively indigenous AMF, 53% of root length was colonized 90 days after transplanting (Table 5). There are no other reports of natural colonization by indigenous AMF in *Allium* in organically managed soils. In rye and wheat, similar or even higher levels of natural root colonization by indigenous AMF were found in conditions of organic agriculture (Sattelmacher et al., 1991; Ryan et al., 1994).

Arbuscules were found only in unsterilized soil or hence where indigenous AMF were present (Table 5). However, since inoculation significantly improved *A. fistulosum* shoot and root growth (Tables 3 and 4), it is likely that somewhere in time the inoculated *G. intraradices* formed also arbuscules in the roots of *A. fistulosum*. It is possible that the association between plant and the inoculated fungi was in a later stage than the association between plant and indigenous AMF. A higher number of vesicles were found in inoculated than in uninoculated plants (Table 5). Vesicles are more commonly found in more advanced stages of the association between plant and AMF,

while arbuscules are more frequent in the phase of intense root colonization (Hayman and Mosse, 1971; Sutton, 1973; Brundrett et al., 1985; Creighton Miller et al., 1986; Ryan et al., 1994). In addition, inoculation took place five weeks before transplant, when the association between plants and indigenous AMF started. The moderate to high soil P content (Table 2) might have contributed also to shorten the period in which arbuscules were formed, since it is known that soil P availability interferes with the formation and abundance of arbuscules (Mosse, 1973; Dekkers and van der Werff, 2001).

The interaction between introduced and indigenous AMF

It is likely that roots of inoculated *A. fistulosum* plants grown in presence of active indigenous AMF, thus in unsterilized soil, were colonized both by the introduced *G. intraradices* and by the indigenous AMF (Table 5). The inoculated AMF were certainly present in the roots, since inoculated plants were checked for root colonization before transplant. In addition, the number of vesicles was significantly higher in inoculated than in uninoculated plants and vesicles were typically being produced by *G. intraradices* (Table 5). Assuming that only indigenous AMF were forming arbuscules (Table 5), the presence of arbuscules gave strong evidence that indigenous AMF were also active in roots that had contact with both AMF sources. Owusu-Bennoah and Mosse (1979), working with onions in a conventional field, were so far the only ones to confirm *in loco*, by checking the colonizing hyphae, that introduced and indigenous AMF could co-exist in the same root. For organic agriculture, in which the inoculated AMF are challenged by the improved activity or inoculum potential of the indigenous AMF (Sattelmacher et al., 1991; Ryan et al., 1994; Miller and Jackson, 1998), it is important to know if the introduced AMF succeeds in raising root colonization. No other work, except that of Mäder et al. (2000) and the present one addressed this point. In both cases, root colonization was much improved by inoculation. However, in the work of Mäder et al. (2000), indigenous AMF were less active than in the present study, since in the absence of the introduced AMF, Mäder et al. (2000) found only 12% of root colonization.

Conclusion and perspectives

Inoculation with *Glomus intraradices* significantly improved shoot and root growth in *Allium fistulosum* cultivated in an organically managed soil, in greenhouse conditions. Shoot growth was improved by 50% on average, even in the presence of active indigenous AMF in the soil. Therefore, plant inoculation with AMF can be an important ally of organic farmers in improving *A. fistulosum* yield. This finding is even more important if it is taken into account that in the context of organic agriculture there

are few alternatives for plant nutrition that can promote such a substantial increment in yield.

It is very likely that onions will be also responsive to AMF in organically managed soils, as it is largely known to happen in conventional conditions (reviewed by Stribley, 1990). This work also suggests that, if one consider that *A. fistulosum* root system is much better developed and more efficient in foraging than onion's (Chapters 2 and 3) and, even then, *A. fistulosum* responded to AMF. However, inoculation followed by transplant, although appropriated to *A. fistulosum*, is not an adequate system to integrate AMF into the onion production system, since onion is mostly directly sown in the field. Therefore, if AMF inoculation is to be integrated into the organic production of onions, the efficiency of either seed or field inoculation in improving growth has yet to be tested. In addition, research on developing simple and effective inoculation methods and economic systems to produce inoculum at large scale is still necessary. Starting points can be the on-farm production of inoculum (Sharma and Adholeya, 2000; Gaur and Adholeya, 2002) and the use of expanded clay as a vehicle for inoculation (Dehne and Backhaus, 1986; Baltruschat, 1987). Alternatively, AMF can be integrated into the onion organic production through management to improve indigenous AMF inoculum potential and efficiency (Dodd and Thomson, 1994). In either way, inoculation or soil management, breeding for more efficient root systems and/or enhanced responsiveness to AMF would be another important step in the direction of the sustainability of onion production.

Acknowledgements

We would like to thank Willeke van Tintelen for her help in assessing root fractional colonization; Paul Keizer, for his help with the statistical analysis; and Gerard Brouwer, for a big hand in washing and scanning the soil samples used for measuring root development.

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Chapter 5

In vitro propagation in onion, *Allium roylei* Stearn, *A. fistulosum* L. and derived populations using a multi-tissue approach and improved disinfection methods

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Abstract

Not much is known about *in vitro* propagation in *A. roylei* and *A. fistulosum*, both important species for onion introgression breeding. In this work, a reliable method to propagate both, their interspecific hybrid and tri-hybrid genotypes between onion and the latter is presented. The approach was to use distinct explant sources to spread the risk of non-response. Unripe umbels were successfully disinfected through surface flaming; ripe umbels, through immersion in sodium hypochlorite; and soil-grown basal plates, through immersion in ethanol, followed by 90 minutes immersion in acidified (pH 6.0) calcium chloride. Basal plates had the highest frequency of responsive explants and produced the most vigorous shoots, but receptacles were the only explant source to yield plantlets of all genotypes. Flower buds from unripe and ripe umbels produced plantlets in respectively 68% and 32% of the genotypes. When genotypes were responsive, flower buds from unripe umbels were the source of potentially more plantlets. Shoots regenerated from basal plates, receptacles and flower buds were transferred to rooting medium respectively after 4, 9 and 13 weeks of culture. *A. roylei* and *A. fistulosum* produced plantlets out of any explant source. This was the first report of a successful *A. roylei* propagation through direct organogenesis and also the first report of *A. fistulosum* plants obtained from shoots developed through direct organogenesis out of receptacles and flower buds.

Keywords: *A. cepa*, *A. roylei*, Japanese bunching onion, Welsh onion, introgression breeding, tissue culture, micro-propagation

Introduction

In vitro propagation of *Allium* has been used for several purposes, among them production of pathogen-free plants and maintenance and bulking of male-sterile lines, of fully or partially sterile interspecific hybrids and of accessions in germplasm banks, which is specially important for outbreeders (Hussey, 1978; Dunstan and Short, 1979; Hussey and Falavigna, 1980; Novák, 1990; Pike and Yoo, 1990; Rodrigues et al., 1997). For onions, *A. cepa* L., there is extensive literature and well-established methods for *in vitro* propagation. Usually, the best results are achieved using explants derived from bulb or set basal plates and from immature umbels (Dunstan and Short, 1977; Hussey, 1978; Matsubara and Hirara, 1978; Fujieda et al., 1979). Meristem tips (Havel and Novák, 1985; Phillips and Hubstenberger, 1987), flower buds (Pike and Yoo, 1990; Keller, 1990; Luthar and Bohanec, 1999) and ovaries (Keller, 1990; Luthar and Bohanec, 1999) also regenerated shoots through direct organogenesis, but the rate of success was lower and, in the case of ovaries, both haploid and diploid plants arose (Keller, 1990).

Reports of *in vitro* propagation of species closely related to onion, which are important for introgression breeding (for review, see Kik, 2002), particularly *A. fistulosum* and *A. roylei*, are scarce. In the case of *A. fistulosum*, *in vitro* propagation was successfully carried out using meristem tip and umbel explants (Fujieda et al., 1977; Phillips and Hubstenberger, 1987). Flower buds were also tested, but did not regenerated shoots directly, without previous callus formation (Keller, 1990). Attempts to propagate *A. roylei* through tissue culture were restricted to a single experiment, in which shoot tip explants were used, without satisfactory results (Phillips & Hubstenberger, 1987).

In breeding, especially in cross-pollinated species, a single seed represents a genotype, and difficult crosses, like interspecific ones and bridge-crosses, often yield few seeds. To maintain valuable and unique genotypes such as these in a reliable manner, it is important to obtain as many explants as possible from a single plant. In onion, innumerous plants can be obtained from a single plant using basal plates (Kahane et al., 1992) and basal plates have been a reliable source of responsive explants. However, there is always the chance that a given genotype or species does not behave like that. *A. roylei*, for instance, was considered as having low regeneration potential when basal discs derived from sets were used for *in vitro* multiplication (Phillips and Hubstenberger, 1987). In addition, when plants are grown in soil, basal plates are the most infected plant part (Lu et al., 1989), which raises the risk of loosing a genotype during *in vitro* culturing due to contamination. Thus, testing alternative explant sources becomes highly advisable.

In this work a multi-tissue approach for *in vitro* propagation of onions, *A. roylei*, *A. fistulosum* and derived populations is presented. In addition, efficient disinfection procedures for obtaining explants free of contamination are described.

Material and Methods

Plant material and explants

We used as stock plants *A. roylei* accession CGN 20.520 (CGN: Center for Genetic Resources, Wageningen, the Netherlands) and *A. fistulosum* accession CGN 14.763. The interspecific *A. roylei* x *A. fistulosum* hybrid genotype 91.021-08 was obtained by handcrossing the two accessions, after emasculating *A. roylei*. Tri-hybrid genotypes were obtained by crossing a male-sterile onion line to 91.021-08, using blow flies. For onion, two plants of the F_1 hybrid Accent were used as stock for basal plate explants and six plants of the male-sterile line 98.240 were used as stock for umbels and flower bud explants. Stock plants grew from vernalized bulbs and were kept in pots filled with a fertilized non-sterile mixture of soil and turf, in the greenhouse. Except for onions, basal plate, umbel and flower bud explants were taken from the same plant.

As soon as the first flower scapes were available (June), umbels and flower buds were collected. Unripe umbels (spathe closed) were sterilized by a brief dipping in 70% alcohol, followed by surface flaming. The environment inside the closed spathe is usually free of contamination. Ripe umbels (spathe opened) were sterilized by dipping in alcohol 70% for 1 minute, followed by bleaching (2% sodium hypochlorite, 5 minutes). After sterilization, umbels were rinsed three times in sterile distilled water. Unripe umbels larger than 1.5 cm in diameter were opened under sterile conditions and flower buds around 1 mm were collected and used as explants (A-type). Flower buds of the same size were collected also from ripe umbels (B-type explants). Flower buds were cultured with pedicels, sticking the pedicels to the medium. Unripe umbels smaller than 1.5 cm in diameter were opened under aseptic conditions and all flower buds were removed. The remaining flower disc or receptacle was quartered in cross shape to produce the explants (C-type). In total, 16 explants from receptacles (four umbels) and 25 flower buds of each explant type (A and B) were cultured per genotype.

Further in the growing season (October), plants were collected and leaves and roots were removed. To obtain the basal plate explants (D-type), outer layers of the bulbs were detached under running tap water and shoots were separated from each other, if more than one were present in each bulb. Individualized shoots, with their protecting scales, were then thoroughly washed in running tap water and reduced in

length and diameter to respectively 2 x 2 cm using a clean scalpel. Next, shoots were disinfected by immersion in ethanol 96% for 15 minutes, followed by immersion in $CaCl_2$ (37 g L^{-1}), pH 6.0, for 90 minutes. During disinfection, shoots were shaken every half-hour to expel the air present in the inner layers. When disinfected, shoots were rinsed three times in sterile distilled water, reduced to 1 cm in height and then each shoot was cut in cross-shape, over the basal stem to yield individual explants, each explant containing two to three scales joint by the basal plate (Fujieda et al., 1979). A minimum of six explants per genotype was used.

Culture, sub-culturing and rooting

The medium used to culture the flower bud explants was as described by Pike and Yoo (1990). Explants were transferred to sterile plastic Petri dishes, with 70 mm in diameter, five explants per dish. Petri dishes were placed at 24°C, with a 16-hour photoperiod, under white fluorescent light (72 µmol $m^{-2}.s^{-2}$). A month later, sprouting explants were transferred to 160 x 24 mm sterile glass tubes containing the same medium used for basal plate explants (see next paragraph), one shoot per tube, and were kept in the same conditions as those. Non-sprouting explants, including those developing callus, were transferred to new Petri dishes, with the same medium as before, and kept in the same conditions for another 30 days.

Receptacle and basal plate explants were cultured in MS medium (Murashige and Skoog, 1962) + vitamins, with 40 $g.L^{-1}$ sucrose, 2.0 $mg.L^{-1}$ BAP and 0.2 $mg.L^{-1}$ NAA. The pH was adjusted to 5.7 and 10.0 $g.L^{-1}$ of Bacto-agar were added before autoclaving (17 minutes, 112°C). After cooling down, 125 $mg.L^{-1}$ of Cefotaxime were added (only for basal plate explants). Thirty ml of medium were dispensed into 60 x 30 mm screw-topped sterile plastic vials, two explants per vial. Vials were placed at 18°C, with a 16-hour photoperiod, under white fluorescent light (72 µmol $m^{-2}.s^{-2}$).

Independent of the explant type, when shoots were at least 50 mm high, they were transferred to 160 x 24 mm sterile glass tubes and placed at 18°C, with a 16-hour photoperiod, under white fluorescent light (72 µmol $m^{-2}.s^{-2}$), for rooting. Medium for rooting was half-strength MS + vitamins, 40 $g.L^{-1}$ sucrose, without growth regulators or antibiotics. The pH was adjusted to 5.7 and 10 $g.L^{-1}$ Bacto-agar were added. Thirteen ml of medium were poured in each glass tube, which were then closed with cotton lids covered by aluminum foil and sterilized (autoclave, 17 minutes at 121°C).

Acclimatization

After root development, plants were kept *in vitro* during the winter, in the same conditions as described above, being subcultured once, when shoots and roots were trimmed. In February, explants were pulled out from the tubes and the medium around

roots was thoroughly washed away using tap water. Plantlets were transferred to trays filled with fertilized sand and placed in a greenhouse where temperature was kept between 15 and 25^{o}C. Following transplant, plantlets were thoroughly water-sprayed and trays were covered with 25-cm high light-permeable lids, forming a humid chamber. For three to four days after transplanting, lids were covered with cheesecloth to avoid sun burning. One week after transplanting, the lids were removed. Three to four weeks after transplanting, plantlets were transferred to pots containing fertilized compost + turf and placed in a greenhouse without controlled temperature, where they grew to maturity.

Evaluation and statistical analysis

A month after culturing and, for flower bud explants, also a month later, explants developing shoots through direct organogenesis in the absence of visible callus, referred here as responsive explants, were counted. The number of explants showing contamination and developing callus was also noted. In flower bud explants, explants with exposed stamen and swollen carpels were counted up to 30 days of culture, while for receptacle explants, the number of explants developing flower buds was observed. For receptacle and flower bud explants, when transferring shoots to growing medium, the number of shoots per explant was counted. For basal plate explants, only up to ten shoots per explant were counted. For practical reasons, a limit of ten shoots per explant type per genotype was established. The number of shoots that produced roots and survived transplant to greenhouse was also recorded. Data were analyzed using the Kruskal-Wallis one-way analysis of variance by ranks and the Mann-Whitney U test (Siegel, 1956), where appropriate.

Results

Disinfection

Despite growing the stock plants in a non-sterile mixture of soil and turf, the disinfecting procedures applied were successful in producing clean explants. In flower bud and receptacle explants, 100% of disinfection was achieved. On explants derived from the basal disc, in ten, out of the 22 genotypes used, disinfection also resulted in 100% of clean explants. In nine other genotypes, one explant per genotype developed contamination, while in three other genotypes, two explants per genotype were contaminated. In total, only 15 out of the 154 basal plate explants were contaminated.

Explant responsiveness

The first *in vitro* reaction we observed in both A- (flower buds extracted from unripe umbels) and B-type (flower buds extracted from ripe umbels) explants was the

opening of buds followed by stamen exposure. However, if A-type explants showed this response soon after being placed *in vitro*, B-type explants remained first as closed buds, opening and exposing the stamen later. All genotypes showed buds with exposed stamen, but in different frequencies, varying from 12 to 100%. Following, carpels started to enlarge. Onion had an extreme carpel swelling, with carpels turning yellowish. On contrary, in all other genotypes, even in those with excessive swelling, carpels remained green. Although all genotypes presented both stamen exposure and carpel swelling most of the times in the same flower bud, the frequency of flower buds with exposed stamen was higher than those with swollen carpels in most of the genotypes, for both explants types. Nevertheless, neither exposure of stamen, nor carpel swelling, were significantly correlated to shoot production through direct organogenesis. In C-type explants (obtained directly from the receptacle of immature umbels), the earliest visible response to *in vitro* culture was the growth of flower buds over the explants. This response was negatively correlated to both percentage of responsive explants ($R= -0.62$, $p< 0.01$) and total number of shoots ($R= -0.63$, $p< 0.01$). Shoot development was the first response of basal plate (D-type) explants to culture.

Shoots were produced through direct organogenesis in the absence of visible callus in all explant types. In flower bud explants, shoots were noticed after 49 days of culture, two weeks after explants were transferred to fresh medium. In these explants, carpels, after swelling or not, first seemed to dehydrate, before a shoot emerged adjacently to them. Two weeks later (63 days of culture), the existing shoots were transferred to growing medium, for further development. In receptacle explants, shoots emerged directly over the receptacle and were perfectly distinct after 28 days of culture. A week later, shoots were transferred to growing medium. In basal plate explants, shoots were observed even sooner, within 14 days of culture, emerging from the scales. Two weeks later, shoots were strong enough to be transferred to the rooting medium.

All genotypes were responsive when C- and D-type explants were used, but only 16 and 9 genotypes, out of the 22 tested, were responsive when respectively A- and B-type explants were placed *in vitro* (Table 1). *A. roylei*, *A. fistulosum* and the interspecific hybrid between them succeeded in regenerating shoots from all explant types, while onion and five and twelve tri-hybrid genotypes failed to produce shoots out of flower buds, from A- and B-type explants respectively. As result, the percentage of responsive explants varied significantly among all four explant types (Kruskal-Wallis, $N = 88$, $H = 69.36$, $p < 0.001$). The percentage of responsive explants was significantly the highest in D-type explants, while C-type was significantly more responsive than A-type and, that, significantly superior to B-type explants (Mann-Whitney U test; $n_A= n_B = n_C = n_D = 22$; $z_{AB} = 3.31$, $z_{AC} = 5.21$, $z_{CD} = 4.36$, $p < 0.01$). The only significant correlation when the

percentage of responsive explants was considered occurred between A and B-explants ($r = 0.42$, $p < 0.05$). The correlation was due to the lack of responsiveness in the same genotypes, since the significance of the correlation disappeared when only responsive genotypes were taken into account.

Table 1. Number and percentage of explants derived from flower buds collected in unripe (A-type) and ripe (B-type) umbels, from receptacles of unripe umbels (C-type) and from basal plates of mature bulbs (D-type) that developed shoots through direct organogenesis.

Genotype	Responsive explants (%)				Total number of shoots (TOT)				Shoots/explant (AV)		
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**
CC - *A. cepa*											
98.240	0.0	0.0	50.0				8				1.75
Accent				100.0				> 10[2]			
RR - *A. roylei*											
CGN 20.520[1]	32.0	12.0	68.7	100.0	12	3	10	> 10	1.50	1.00	2.30
FF - *A. fistulosum*											
CGN 14.763[1]	48.0	8.0	62.5	100.0	15	2	11	> 10	1.25	1.00	2.91
RR x FF											
91.021-08	32.0	4.0	75.0	100.0	12	1	12	> 10	1.50	1.00	2.75
CC x RF											
96.284-01	8.0	0.0	62.5	100.0	3		10	> 10	1.50		2.20
96.284-15	20.0	16.0	25.0	100.0	5	4	4	> 10	1.00	1.00	1.50
96.284-16	36.0	4.0	75.0	100.0	12	2	12	> 10	1.33	2.00	2.50
96.284-22	24.0	4.0	75.0	100.0	8	1	12	4	1.33	1.00	2.67
96.284-24	30.0	8.0	81.2	83.3	6	2	13	> 10	1.00	1.00	2.31
96.284-25	0.0	0.0	50.0	50.0			8	5			3.13
96.284-26	0.0	0.0	93.7	50.0			15	3			1.67
96.284-27	12.0	0.0	43.7	100.0	3		7	> 10	1.00		1.86
96.284-29	40.0	0.0	62.5	33.3	14		10	4	1.40		3.50
96.284-36	8.0	0.0	50.0	100.0	3		8	> 10	1.50		2.75
96.284-42	36.0	4.0	50.0	100.0	12	1	8	> 10	1.33	1.00	2.25
96.284-47	0.0	0.0	62.5	100.0			10	> 10	-		2.30
96.284-49	24.0	0.0	87.5	100.0	6		14	> 10	1.00		2.71
96.284-54	8.0	0.0	50.0	100.0	2		8	7	1.00		1.50
96.284-55	40.0	0.0	68.7	100.0	12		11	> 10	1.20		1.18
96.284-63	20.0	12.0	75.0	66.7	6	3	12	> 10	1.20	1.00	2.08
96.284-64	0.0	0.0	37.5	100.0			6	> 10			2.50
96.284-68	0.0	0.0	12.5	100.0			2	> 10			2.50

[1]/ CGN: Center for Genetic Resources, Wageningen, the Netherlands; [2]/ >10: ten or more shoots.

Callus was formed in all genotypes when flower bud explants were employed, although the number of explants that developed callus was extremely variable among genotypes (data not shown). Callus started developing after two weeks of culture, around the basis of stamen and carpels. In onion, callus grew to cover the carpel completely, while in the other genotypes, the carpel remained always above it. *A. fistulosum*, *A. roylei*, the interspecific hybrid between both and most of the tri-hybrid

genotypes developed much less pronounced callus than onion. Sprouts came out from callus in many genotypes, including onion, *A. roylei*, *A. fistulosum* and the interspecific hybrid between the latter. In receptacle explants there was almost no callus development. For this sort of explant, calli were observed only in explants from onion, *A. fistulosum*, *A. roylei* and from the tri-hybrid 96.284-55 and even then, much less developed than those observed in flower bud explants. No callus was observed when basal plate explants were cultured.

Shoot development

In umbel and flower bud explants, the number of shoots could easily be determined, whereas in basal plate explants, it was less simple because explants produced many sprouts. When subculturing D-type explants, the practical limit established to subculture (10 shoots per genotype) was very quickly reached. However, a few genotypes were less prolific and in those shoots could still be accurately counted (Table 1). Thus, total number of shoots (TOT) produced through direct organogenesis, as well as the average number of shoots per explant (AV), were analyzed only for A-, B- and C-type explants. To avoid having the effects of explant type over the number of shoots per explant shadowed by the lack of responsiveness of some genotypes in A- and B-type explants, number of shoots per explant was studied considering only the responsive genotypes. Both TOT and AV varied significantly among A-, B- and C-type explants (Kruskal-Wallis, N = 47, H_{TOT} = 17.8; H_{AV} = 67.5, $p < 0.01$). TOT in A- and C-type explants did not differed from each other (Mann-Whitney U test, n_A= 16, n_c = 22, z_{AC} = 0.9) and in both it was significantly higher than in B-type explants (Mann-Whitney U test, n_A= 16, n_B = 9, n_c = 22, U_{AB} = 10.5, z_{BC} = 4.1, $p < 0.01$). AV was significantly higher in C- than in A-type explants, which, in their turn, performed significantly better than B-type explants (Mann-Whitney U test, n_A= 16, n_B = 9, n_c = 22, U_{AB} = 36.0, $p < 0.05$, z_{AC} = 8.67, $p < 0.01$). There was a significant correlation for TOT and AV between A- and C-type explants (R_{TOT} = 0.54, R_{AV} = 0.50, $p < 0.05$), which meant that prolific genotypes tended to be the same in both explant types. No significant correlation was found for B-type explants.

Rooting and acclimatization

Shoots developed from basal plates, receptacles and flower buds could be transferred to rooting medium respectively 4, 9 and 13 weeks after explants were placed *in vitro*. Up to ten shoots per explant type per genotype were transferred and most of the shoots rooted fairly well (Table 2). On average, 93.4% of the shoots rooted, with the rooting rate per explant type varying from 77.8% (B-type explants) to 97.4% (C-type explants). There was no root formation in shoots derived from basal plate in genotypes 96.284-22 and 96.284-54 (Table 2). Strong shoots, most of the

times derived from C- or D-type explants, rooted first and could have been easily transplanted to the greenhouse as early as three weeks after being transferred to the rooting media. Most of the plantlets transplanted to greenhouse survived (Table 2). The average survival rate was 95.2%, varying from 78.2 (B-type explants) to 98.2% (D-type explants).

Table 2. Number of shoots in explants derived from flower buds collected in unripe (A-type) and ripe (B-type) umbels, from receptacles of unripe umbels (C-type) and from basal plates of mature bulbs (D-type) that were transferred to rooting media, developed roots and survived the transplant to the greenhouse.

Genotype	Shoots Transferred to Rooting Medium				Rooted Shoots				Surviving Plants in Greenhouse			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
CC - *A. cepa*												
98.240			8				7				6	
Accent				10				10				10
RR - *A. roylei*												
CGN 20.520[1]	10	3	10	10	10	3	9	10	7	2	9	10
FF - *A. fistulosum*												
CGN 14.763[1]	10	2	10	10	9	2	9	8	9	2	8	8
RR x FF												
91.021-08	10	1	10	10	10	1	10	8	10	0	9	8
CC x RF												
96.284-01	3		10	10	3		10	10	3		9	10
96.284-15	5	4	4	10	5	2	4	10	5	2	4	10
96.284-16	10	2	10	10	10	1	10	10	9	1	10	10
96.284-22	8	1	10	4	8	1	10	0	7	1	10	0
96.284-24	6	2	10	10	6	2	10	10	6	1	10	10
96.284-25			8	5			8	5			6	2
96.284-26			10	3			10	3			10	3
96.284-27	3		7	8	3		7	8	2		7	8
96.284-29	10		10	4	10		10	4	10		9	4
96.284-36	3		8	10	2		8	10	2		7	10
96.284-42	10	1	8	10	10	0	8	10	10	0	8	10
96.284-47			10	10			10	8			10	8
96.284-49	6		10	10	5		10	10	5		10	10
96.284-54	2		8	7	2		8	0	0		6	0
96.284-55	10		10	10	10		10	9	10		10	9
96.284-63	6	3	10	10	6	3	9	10	6	2	9	10
96.284-64			6	10			5	8			5	8
96.284-68			2	10			2	10			2	10

[1]/ CGN: Center for Genetic Resources, Wageningen, the Netherlands.

Discussion

Disinfection

The use of flaming instead of hypochlorite to disinfect unripe umbels is presented here for the first time. Flaming was quicker and more practical than using hypochlorite and as efficient in eliminating contamination in explants derived from

receptacles and from flower buds collected from unripe umbels. Onion inflorescences are indeed very clean, especially the environment inside the closed spathe (Phillips and Hubstenberger, 1987; Lu et al., 1989; Pike and Yoo, 1990).

On the contrary, basal plate explants are heavily contaminated. Lu et al. (1989), although not mentioning numbers, found contamination of basal plates to be the highest when plants were not in intense growing stages. Rodrigues et al. (1997) reported from 12 to 80% of explants derived from onion basal plates showing contamination, depending on the disinfection procedure used. Capote-Rodriguéz and Pérez-Díaz (1997) stated that they needed a repeated disinfecting procedure when using basal plate explants. In the present work, a single disinfecting round was applied to basal plates collected from plants that have passed the most intense growing period and yet 90% of disinfection was achieved. The use of ethanol was combined with a long-immersion (90 minutes) in a pH-reduced hypochlorite solution as main steps in disinfecting the basal plates. A long immersion in an acidified hypochlorite solution was tested before (Rodrigues et al., 1997) and proved to be efficient in reducing contamination in onion basal plates. However, Rodrigues et al. (1997) did not precede it by an ethanol step. Ethanol improves the penetration of the disinfection solution in onion bulbs (Novák, 1990). Lu et al. (1989) recommended a method to disinfect basal plates of F_1 hybrids between onion and *A. fistulosum* with nine short (up to 10 minutes) steps using water, ethanol and hypochlorite, followed by an overnight rest before obtaining the explants. Here, water, ethanol and acidified hypochlorite were used in only five steps, with no need for an overnight rest. Instead, explants were obtained straight after disinfection.

Explant responsiveness

Receptacles of unripe umbels were the only explant source to produce rooted shoots and thus new plants from all genotypes. This low genotype-specificity is vital to deal with unfamiliar species or new genotypes, as it is in an introgression breeding program. When compared to basal plates, receptacles were, in addition, easier to disinfect and allowed obtaining explants without destroying the stock plant, a precious advantage in case of unique genotypes. However, the frequency of responsive explants was higher when basal plates were used as the explant source and shoots regenerated out of basal plate explants developed fast and were more vigorous. Flower buds from unripe umbels, for responsive genotypes, could have potentially generated more plants than any other explant source. In addition, flower buds were by far the easiest explants to obtain and to manipulate. However, they were the latest in regenerating shoots and are more prone to develop callus (Matsubara and Hirara, 1978). Flower buds from ripe umbels produced too few shoots and were the most genotype-dependent explant

source. Shoots came out of basal plate, receptacles and flower bud explants after respectively two, four and seven weeks of culture. Although important for planning sequential activities, especially when the *in vitro* propagation step is included in a breeding program, not so many authors refer to this aspect in their papers. When it was mentioned, our results were in agreement to what was reported mainly in onion, to the different explant sources (Fujieda et al., 1977; Dunstan and Short, 1979; Pyke and Yoo, 1990; Mohamed-Yasseen et al., 1993; Luthar and Bohanec, 1999).

Explant potential in generating shoots

When only responsive genotypes were considered, receptacles and flower buds collected from unripe umbels yielded the same amount of shoots. Hence, the bottleneck in using flower buds as explants lied at first place at responsiveness. It is true that more flower buds (25) than receptacle (16) explants were cultured per genotype. However, 16 receptacle explants, or four umbels, were the maximum most of the genotypes could produced, while 25 flower buds were just a fraction of what was possible from a single umbel. In addition, it was more laborious to prepare explants from receptacles than use flower buds. Pike and Yoo (1990) had already realized the potential of using flower buds to propagate onion. They estimated that on average an onion umbel would generate 650 plants through direct organogenesis. We prefer to be more conservative about figures. Not all cultured florets were able of regenerate shoots. Moreover, florets were prone to develop callus. It was evident also that older flower buds had limited shoot production and were even more genotype-sensitive than younger ones.

If on the one hand flower buds from unripe umbels were potentially a more prolific source of shoots than receptacles in responsive genotypes, on the other hand receptacles were a more efficient explant source, since they produced more shoots per explant than flower buds. Dunstan and Short (1977, 1979) obtained from 10 to 15 shoots per receptacle, when they used young umbels and less than 5 shoots per receptacle, when they used more developed umbels, in what they classified as meiotic stage. In the present work, umbels of around 1.5 cm in diameter and with an already elongated scape, then more to the mature stage, were employed and yet a satisfactory multiplication rate was achieved, varying from 5 to 14 shoots per receptacle, depending on the genotype (Table 1). In contrast, on average 1.3 and 1.1 shoots per explant were obtained using flower buds from respectively unripe and ripe umbels, lower than the average of 5 shoots per flower bud reported by Pyke and Yoo (1990).

In vitro *culture of* A. roylei *and* A. fistulosum

Phillips and Hubstenberger (1987), in the single attempt of propagating *A. roylei* through tissue culture reported so far, considered it as having a low regeneration potential when compared to onion. In the current study, *A. roylei* was responsive and

prolific, showing unquestionably a high regeneration potential. A. *fistulosum* was already considered highly regenerative when propagated through basal plates and umbels (Fujieda et al., 1979; Phillips and Hubstenberger, 1987), but failed to regenerate shoots through direct organogenesis out of flower buds (Keller, 1990). In our work, *A. fistulosum* was also highly regenerative, including when flower buds were used as explants. Phillips and Hubstenberger (1987) did not mention rooting and plantlet development for both *A. roylei* and *A. fistulosum*. Here, shoots from both species, independent of explant type, had high rooting and survival rates when transplanted to the greenhouse. Thus, this work is the first to report the successful *in vitro* propagation of *A. roylei* through direct organogenesis. In the case of *A. fistulosum*, the development of mature plants out of shoots obtained through direct organogenesis from receptacles of unripe umbels, as well as from flower buds, is presented also for the first time.

Conclusions

It is a significant move forward to add an *in vitro* propagation step to an onion introgression breeding program involving *A. roylei* and *A. fistulosum*, as a measure to safeguard unique genotypes. To accomplish this, the best choice in case of genotypes whose response to *in vitro* culturing is unknown is to use receptacles from immature umbels as the explant source. Receptacles of immature umbels did not show genotype specificity, were easy to disinfect and are a non-destructive explant source. If genotypes are responsive to the *in vitro* culturing of flower buds, then flower buds from unripe umbels should be preferred, because they are the easiest explant to manipulate and can potentially generate the largest number of plants. When needed, bulb basal plates can also be used as an additional explant source.

Acknowledgments

We would like to thank Betty Henken, Greetje Kuiper and Bernadette van Kronenburg for their technical assistance in the *in vitro* work. We wish to thank also Dick Geurtsen and Jaap van den Berg for watching over the plants in the greenhouse. Furthermore, we thank Rémi Kahane (CIRAD, France) for giving us valuable advice for the *in vitro* culturing of our material and our colleague, Dr. Sjaak van Heusden, for his careful revision of the manuscript.

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Chapter 6

Mapping of quantitative trait loci for root morphology in the cross *Allium cepa* L. x (*A. roylei* Stearn x *A. fistulosum* L.)

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Abstract

The low number of stem-borne and lateral roots makes onion very inefficient in exploring and exploiting the soil reserves. *Allium fistulosum* develops substantially more roots and crosses of *A. fistulosum* with onion can produce a fertile offspring if *A. roylei* is used as a bridge-cross species. Forty-nine individuals from the cross *A. cepa* x (*A. roylei* x *A. fistulosum*) were evaluated for root traits in a greenhouse experiment. A genetic linkage map for the male parent, *i.e.* the *A. roylei* x *A. fistulosum* hybrid, was constructed based on Amplified Fragment Length Polymorphisms (AFLPs). The map included 450 AFLP markers and covered 661 cM of an expected length of 700 - 800 cM. The map was divided in eight linkage groups that could be assigned to the eight chromosomes. Distorted segregation was found for 26% of the markers and, interestingly, under-represented markers (too few plants having a marker) were 3.5 times more frequent than over-represented. The genetic linkage map and the segregation of root and bulb traits were used for a Quantitative Trait Locus (QTL) analysis. All traits, evaluated at full vegetative growth, showed a broad variation. Numbers of bulbs and stem-borne roots were highly correlated and the two QTLs found for the latter coincided with the (putative) QTLs for the former. One QTL was pinpointed for the number of lateral roots per stem-borne root and another one for the relative root length of fine and thick roots. The results showed the feasibility of (marker assisted) breeding for improved root systems in onion using the interspecific hybrid between *A. roylei* and *A. fistulosum* as crossing parent.

Keywords: onion, Japanese bunching onion, linkage map, QTL, segregation distortion, stem-borne roots, lateral roots, root length, introgression breeding, bridge-cross

Introduction

Onions have a shallow and poor laterally spread root system, made up almost exclusively of stem-borne roots (Jones and Mann, 1963; Portas, 1973). Lateral roots occur in a very low number, are usually short and rarely re-branch (Weaver and Brunner, 1927; Pulgarin et al., 1988; Chapter 2), resulting in onion's typical low root density (Greenwood et al., 1982; Chapter 3). In addition, root hairs are mostly missing (Föhse et al., 1991). Such a combination of unfavorable characteristics severely restricts nutrient uptake. As consequence, a large excess of nutrients must be available, this in spite of the not especially high needs of onion plants (Brewster, 1994). So far, high yields have been made possible in onion due to the massive application of fertilizers (Fontes and Nogueira, 1984; Patel and Vachhani, 1994). For nitrogen fertilization, not more than 37% of what is applied is estimated to be recovered by the crop (Greenwood et al., 1992), while for example in cabbage the N-recovery is nearly 100% (Thorup-Kristensen and Sorensen, 1999). However, the costs of fertilizers and regulations restricting their use are making it more and more difficult to maintain high-input production systems (Sattelmacher et al., 1994; Matson et al., 1997).

The need for plants with high nutrient use efficiency comes also from alternative agricultural systems such as organic cultivation. In organic agriculture, ecological interactions within the soil-plant system are favored and synthetic fertilizers are replaced by more complex sources of nutrients, like animal manure (van Bruggen, 1995; Lammerts van Bueren et al., 2002). Although in the long term this approach results in enhanced soil fertility, the availability of nutrients in the soil solution is always lower than when synthetic fertilizers are used (Matson et al., 1997; Mäder et al., 2002). For plants with such a meager root system as onions it is hard to cope with these conditions.

Root characteristics can be changed through breeding (Clarke and McCaig, 1993). However, breeding relies on variation and not much variation in root morphology was found in *Allium cepa* (Chapter 2). Fortunately, increased root development was found in *A. fistulosum* L., a close relative of onion, commonly known as the Japanese bunching onion or Welsh onion, which is an important vegetable in China and Japan (Jones and Mann, 1963; Brewster, 1994). Not only does *A. fistulosum* develop many more stem-borne roots than onion, it also branches far more often, resulting in two- to three-fold higher root densities than in onion (Chapters 2 and 3). *A. fistulosum* has always been a potentially important gene reservoir for onion breeding, although its use was severely hampered by the high level of sterility of its hybrids with onion (reviewed by Kik, 2002). However, it has been shown that the use of *A. roylei* as a bridge-species between onion and *A. fistulosum* successfully

circumvents the sterility barrier (Khrustaleva and Kik, 1998; 2000). So it is possible to use the bridge-cross to breed onions with improved root systems.

Direct breeding for root traits is possible, but by no means easy. Firstly, root evaluation is complex and tedious (Atkinson, 2000), secondly, the relation between root morphology and function is not fully understood (Clarke and McCaig, 1993) and finally plants display an enormous phenotypic plasticity for root traits in response to various soil conditions (Lynch and Brown, 2001). All these reasons make direct selection difficult and somewhat inefficient. Indirect selection through Marker Assisted Breeding (MAB) can be of great help. If a molecular marker is associated to a root trait, it can complement or even replace phenotypic selection. In rice, where mapping studies focussed on root traits are abundant, marker assisted breeding was shown to be effective for root traits (Champoux et al., 1995; Ray et al., 1996; Price and Tomos, 1997; Yadav et al., 1997; Zheng et al., 2000; Kamoshita et al., 2002). Important traits for drought avoidance, like the maximum rooting depth and the weight of deep roots, were significantly improved using MAB (Shen et al., 2001). In maize, Quantitative Trait Loci (QTLs) were found for number and thickness of adventitious roots and also for other root traits (Landi et al., 2002). In lettuce and its putative wild ancestor, *Lactuca serriola* L., QTLs were found, amongst other root traits, for number of lateral roots (Johnson et al., 2000). In onion or any other *Allium*, there are no reports of QTL mapping for any root traits. To date, the only QTLs reported in *Allium* were for characteristics related to the onion bulb flavor (Galmarini et al., 2001).

The objective of this investigation was to study the segregation of root traits in the progeny of the cross *A. cepa* x (*A. roylei* x *A. fistulosum*) and to map quantitative trait loci (QTLs) for these traits on an AFLP map. The root characteristics studied here have consistently differentiated onion from *A. fistulosum* (Chapter 2) and, in experiments carried out at fields with an organic management, they have conferred yield stability to *A. fistulosum* in conditions in which onion showed reduction in productivity (Chapter 3). The identification of QTLs for these root traits will ultimately be a valuable tool in the development of onion cultivars with improved root systems and a step forward into the sustainability of onion production.

Material and Methods

Plant Material

Allium roylei Stearn accession CGN 20.520 (CGN = Center of Genetic Resources, Wageningen, the Netherlands) was crossed to *A. fistulosum* L. accession CGN 14.763. An interspecific hybrid genotype (91.021-08), hereafter referred to as RF-hybrid, derived from this cross was used as pollen donor in a cross with a

cytoplasmic male-sterile onion line from the Rijnsburger group. A progeny of 49 plants of this cross was the mapping population used in this study.

Plant and root evaluation

Four *in vitro* propagated plants of each of the 49 genotypes were used. These plants were analyzed in a completely randomized design together with *A. cepa*, the RF-hybrid and the *A. roylei* and *A. fistulosum* parents of the RF-hybrid. Regrettably *A. roylei* plant grew too slowly and could not be evaluated. In the greenhouse, plants were placed in 12-cm diameter x 30-cm high PVC pipes, closed in the bottom with root-proof plastic. Pipes were filled with a 3:1 mixture of sand and soil collected in a field cultivated with onions in the previous year. The mixture of sand and soil was fertilized with Hoagland solution ½ strength. Weeds were eliminated regularly by hand.

Plants were harvested at full vegetative grown (75 to 85 days after planting). The pipes were placed over a sieve and carefully removed, while the sand and soil mixture was being washed away. Once the root system was clean, the number of bulbs per plant and the number of stem-borne roots per bulb were counted. Roots were then cut off from the bulbs and the root system of each plant was flattened over a surface using a gentle water stream. Following, the root mass was divided longitudinally in two parts. From one part, three stem-borne roots were randomly taken and the lateral roots emerging from them were counted. From the other part, roots were cut into 5-cm segments, well stirred in water and, pinched five times with a forceps, to form the sample used for measuring root length. These samples were stained overnight (500 ppm safranin), at 4^{o}C. The next day, stained roots were rinsed in water to remove the excess of dye, spread in a transparent tray and scanned with a table flatbed scanner.

Total root length and root length in 11 classes of root diameter, from 0 to 1.0 mm or thicker, with 0.1-mm increasing steps, were obtained from root images using the software WinRhizo®. All roots, including the ones used for counting root branching, were dried for 72 hours in an oven with forced ventilation, at 70^{o}C. The sample used for measuring root length was dried separately and its weight was added to the rest to obtain the total root dry weight. Total root length was estimated by multiplying the root length of the scanned sample by the factor obtained by dividing total root dry weight by the root dry weight in the scanned sample. Root length was expressed as root length density (L_V), in cm of root per cm^3 of soil (Atkinson, 2000).

AFLP procedure

DNA was isolated from young leaves from the tri-hybrid genotypes, from both the onion and the RF-hybrid parent, and from the *A. roylei* and *A. fistulosum* accessions used to produce the RF-hybrid. DNA was extracted using the miniprep

protocol described by van Heusden et al. (2000a) and AFLP reactions were carried out according to Vos et al. (1995). Two restriction enzyme combinations, namely *Eco*RI/*Mse*I and *Pst*I/*Mse*I, and a total of 23 primer combinations were used. Selective nucleotides were used at the pre-amplification step (+1, +2) and at the final amplification. For the *Eco*RI/*Mse*I enzyme combination, seven (+3, +4) selective nucleotides were used in the final amplification, while for the *Pst*I/*Mse*I combination, six (+3, +3) were used. AFLP fragments that were exclusive for either *A. roylei* or *A. fistulosum* were scored. AFLP fragments were named as described by van Heusden et al. (2000b). For instance, E35M52A-302F stands for the restriction enzymes *Eco*I and *Mse*I, primers E35 and M52, A identifies the additional 7th selective base, 302 is the length of the fragment and F or R is whether the marker is specific for *A. roylei* or *A. fistulosum*. A list of all primer combinations used is presented in table 1.

Linkage analysis

A paternal (*A. roylei* x *A. fistulosum*) map was calculated using JoinMap® 3.0 (van Ooijen and Voorrips, 2001). Linkage groups were separated with a LOD threshold of 4.0, with an occasional subdivision of a linkage group by raising the LOD threshold. To calculate the map positions in the individual groups pairwise recombination estimates below 0.49, LOD scores higher than 0.001 and Kosambi's mapping function were used. Markers which clearly belong to a certain linkage group but were not placed in the first two mapping rounds of JoinMap® 3.0, were forced into the linkage group by using the map 3 option.

QTL Mapping

QTLs were identified using MapQTL® 4.0 (van Ooijen et al., 2002) and were considered significant at a LOD threshold of 2.65, estimated on the basis of population type and of number and average length of linkage groups in the map (van Ooijen, 1999).

Results

Molecular map

It was possible to score 396 and 333 fragments in the parents and progeny that were unique to *A. roylei* and to *A. fistulosum* respectively (Table 1). Because we used 23 AFLP primer combinations this summed up to an average of 17.2 *A. roylei* specific fragments and 14.5 *A. fistulosum* specific fragments per primer combination. From this total, 450 markers were distributed in the 8 largest linkage groups, producing a 661 cM map (Figure 1). Forty-six of the presently mapped *A. roylei* markers corresponded to markers mapped in a previous linkage study in *Allium* based on a progeny originated

from a selfing of an interspecific hybrid between *A. cepa* and *A. roylei* (van Heusden et al., 2000a). Most of the linkage groups of this *A. cepa* x *A. roylei* map were already assigned with the help of monosomic addition lines to the physical *A. cepa* chromosomes (van Heusden et al., 2000b). This knowledge made it possible to indirectly determine the correspondence between chromosomes and the eight linkage groups obtained in the present study.

Table 1. Number of *A. roylei* and *A. fistulosum* (between brackets) species-specific AFLPs detected in the parents and in the mapping population by a combination of (1) *Eco*RI-primers with three selective nucleotides and *Mse*I-primers with four selective nucleotides and (2) *Pst*I-primer in combination with *Mse*I-primers both with three selective nucleotides.

	M52A			**M52T**		**M52C**		**M52G**	
E35	20 (16)			22 (16)		19 (14)			
E36	24 (23)					23 (20)			
E37						15 (15)		27 (15)	
E38	33 (20)			14 (23)		18 (16)		17 (16)	
	M32	**M33**	**M34**	**M35**	**M36**	**M47**	**M48**	**M50**	**M51**
P31		28 (26)		20 (13)					
P35	16 (15)	12 (12)	11(10)	15 (09)	11 (13)			16 (10)	
P38						09 (09)	08 (03)		
P43					08 (06)				10 (13)

From the total number of scored AFLP fragments, 191 were significantly skewed, 74 in the direction of the presence of the *A. roylei* allele, and 117 in the direction of the presence of the *A. fistulosum* allele. Eighty-four (44%) of the skewed segregating markers could still be mapped. The chromosomal areas with skewed markers, either in the direction of *A. roylei* or *A. fistulosum*, are indicated in figure 1. Extreme skewness was also observed. Eight fragments, two from *A. roylei* and six from *A. fistulosum* were never found in the progeny. Of all *A. roylei* or *A. fistulosum* specific fragments only one was present in every progeny plant (this fragment was an *A. roylei* fragment).

Number of bulbs

Onion and *A. fistulosum* plants developed a single bulb or a single pseudo-stem respectively, while plants from the RF-hybrid developed on average three pseudo-stems per plant (Table 2). *A. roylei* plants grew too slowly in this experiment to be fairly evaluated. The tri-hybrid population mean was 2.6 bulbs per plant, varying from 1 to 6 bulbs per plant (Table 2). A putative QTL was found on chromosome 5 for the number of bulbs (Table 3, Figure 2). Tri-hybrid individuals that inherited this chromosomal region from *A. roylei* had on average 3.2 bulbs, while those carrying the *A. fistulosum* alleles had in average 1.7 bulbs (Table 3).

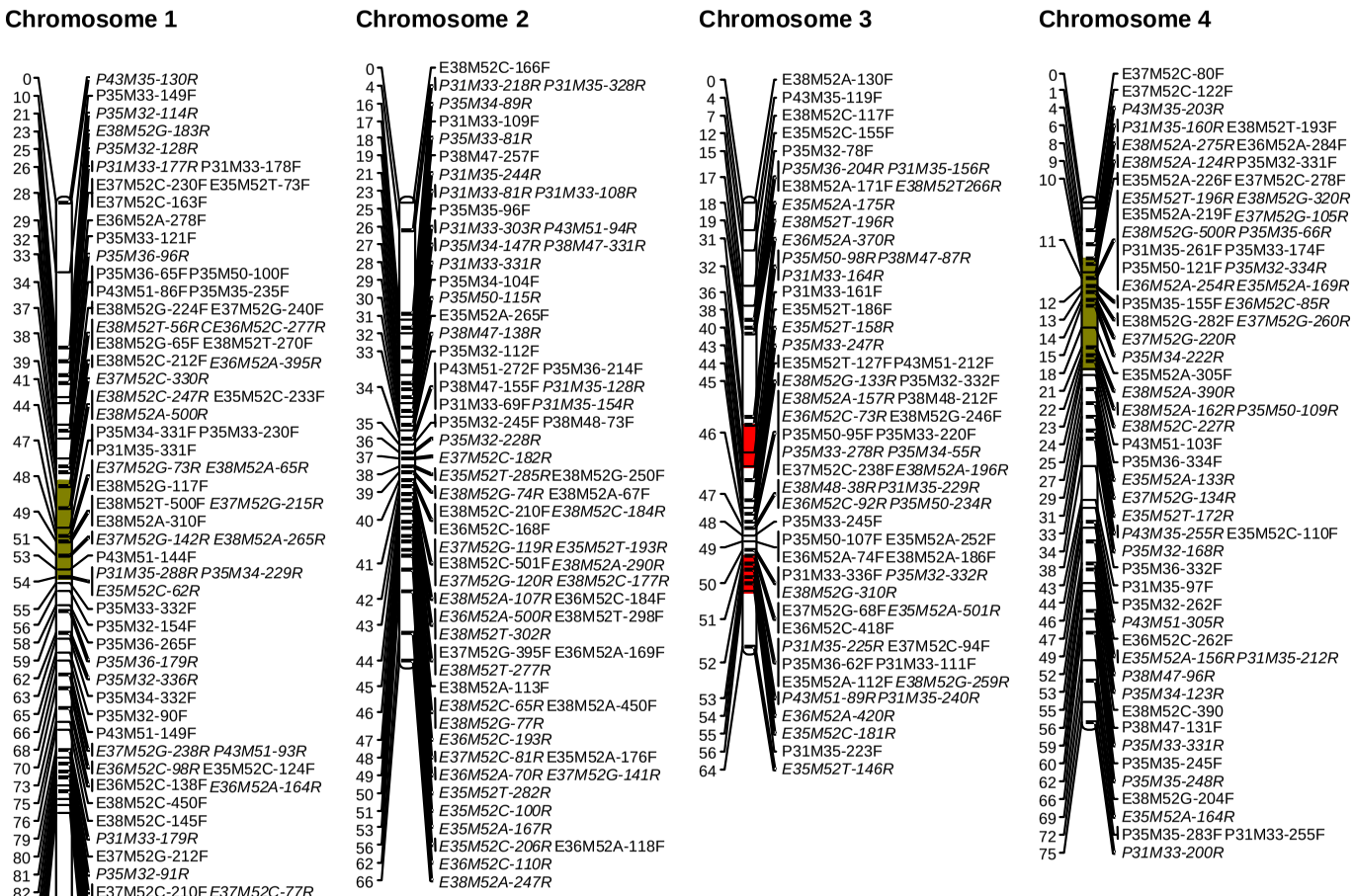

Figure 1. Paternal linkage map based on the progeny of the cross *A. cepa x (A. roylei* x *A. fistulosum)*. AFLP-marker names ending with an R (in italics) are specific for *A. roylei*, the ones ending on an F are *A. fistulosum*-specific. Some chromosomal regions were highly skewed and are shown in the figure by a filled bar (e.g. chromosome 4, 11-20 cM)

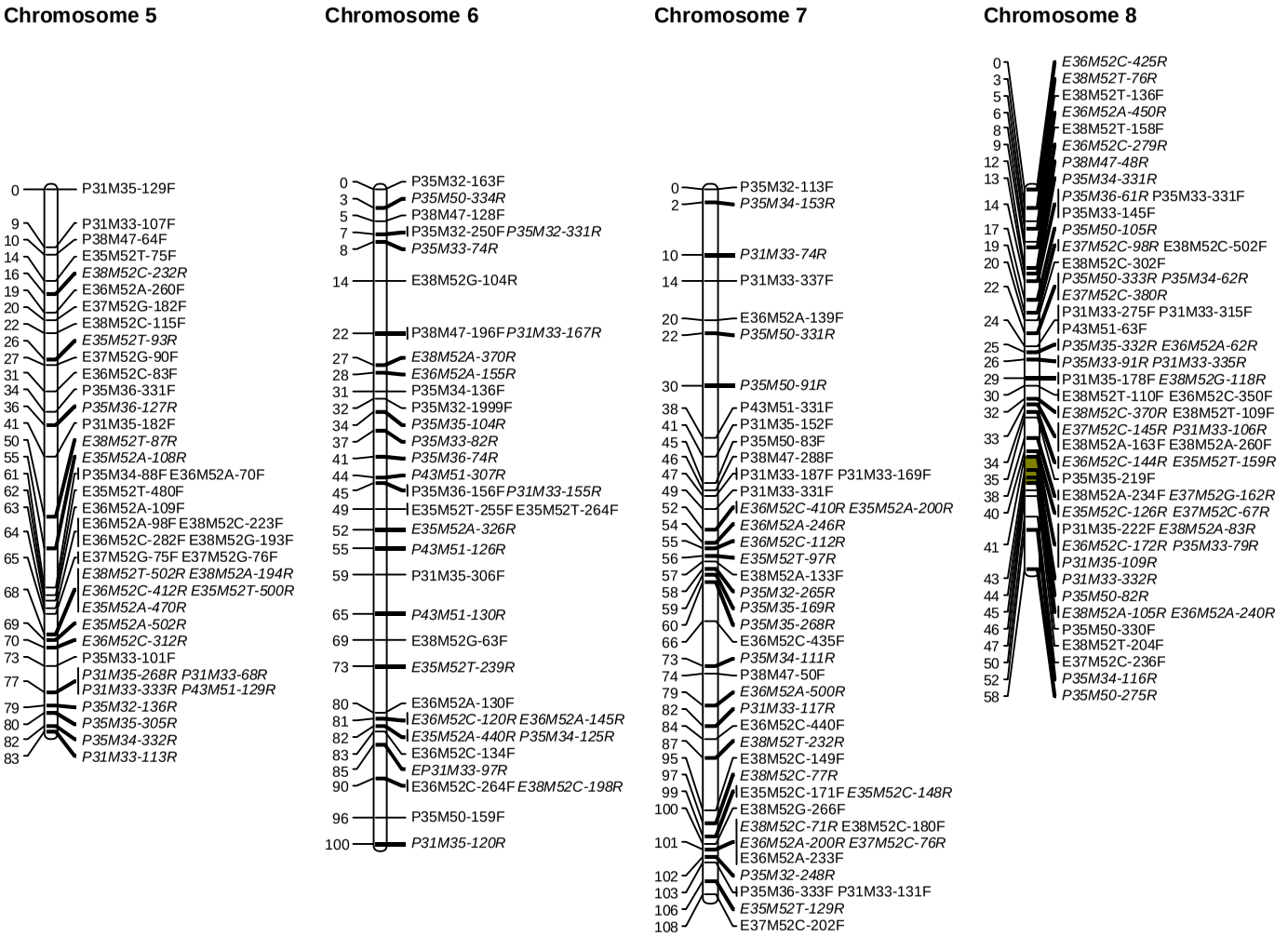

Figure 1. Continuation

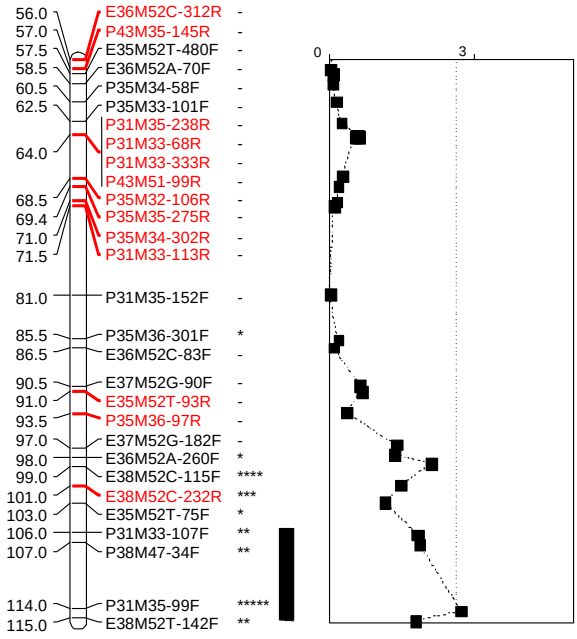

Figure 2. LOD-graph after interval mapping of part of chromosome 5. The figure shows the position of the QTL for number of bulbs. The LOD 1 interval is given by the solid bar and the level of significance in a Kruskal-Wallis analysis is in asterisks (*:0.1 **:0.05 ***:0.01 ****:0.005 *****:0.001 ******:0.0005).

Stem-borne and lateral roots

The numbers of stem-borne roots and laterals per stem-borne root were significantly higher in *A. fistulosum* and in the RF-hybrid than in onion. For the tri-hybrid individuals, the population mean for stem-borne roots either calculated for the whole plant or for only the main bulb was also higher than in onion (Table 2). This was also seen for the mean number of laterals per stem-borne root (Table 2). Nevertheless, in the tri-hybrid population there was a broad variation for both types of roots. When all roots of a plant were considered, the number of stem-borne roots varied from 16 to 135 (Table 2). Taking only the main bulb, the variation of the number of stem-borne roots in the tri-hybrid individuals was narrowed to 11 to 48 roots. The number of laterals per stem-borne root varied from 9 to 32 (Table 2).

Both the interval mapping and the restricted MQM option of MapQTL® 4.0 gave two putative QTLs for stem-borne roots per plant, one on chromosome 5 (LOD with MQM = 2.2) and another one on chromosome 7 (LOD with MQM = 2.9) (Table 3). However, number of stem-borne roots was not unexpectedly positively correlated to the number of bulbs, since each bulb had its own stem-borne roots. Therefore, QTLs for number of bulbs and for number of stem-borne roots were likely to coincide. On chromosome 5 this was observed. The *A. fistulosum* alleles of this QTL contributed to the reduction in number of bulbs and to the reduction of stem-borne roots (Table 3). For the putative QTL on chromosome 7, it was not so obvious. Yet, this region

influenced, although less significantly, the number of bulbs; plants with the *A. roylei* alleles had on average one bulb more than the plants with the *A. fistulosum* alleles (3 vs. 2). No markers were significantly associated with number of stem-borne roots in the main bulb. This indicates that very likely the number of stem-borne roots was only dependent on the number of bulbs and not influenced by other factors.

Table 2. The number of bulbs and some root traits of the mapping population and all involved parental genotypes[1], evaluated 75-85 days after planting.

	A. cepa	*A. roylei* x *A. fistulosum*	*A. fistulosum*	Tri-hybrids	
				Average	Range
Bulbs (number)	1	3	1	2.6	1 - 6
Stem-borne roots (number)					
per plant	9	75	68	59	16 - 135
main bulb	9	41	68	29	11 - 48
Laterals / stem-borne root (number)	9	25	27	20	9 - 32
Root length density ($cm.cm^{-3}$)	0.51	2.41	3.01	1.94	0.62 - 3.25
Relative length for fine roots (%)[2]	10.3	34.5	43.3	20.7	9.8 – 34.5
Relative length for thick roots (%)[3]	3.1	17.9	18.7	14.8	5.5 – 23.7

[1]/ *A. roylei* plants grew very slowly in this experiment and therefore could not be evaluated; [2]/ Root length in the class of fine roots (diameter greater than or equal to 0.2 and smaller than 0.4 mm) in relation to total root length; [3]/ Root length in the class of thick roots (diameter > 1.0 mm) in relation to total root length.

Table 3. The detected QTLs for number of bulbs and for some root traits in the tri-hybrid progeny.

	LOD	Chromosome and position	Progeny average	Allele effect[1]		Variance (%)
				roylei	*fistulos*	
Bulbs (number)	2.7	5 (0 cM)	2.6	3.2	1.7	22.4
Stem-borne roots (number)						
per plant	2.6	5 (0 cM)	59	71	44	21.7
	2.8	7 (46 cM)		72	41	23.1
Laterals/stem-borne root (number)	6.0	1 (101 cM)	20	13	22	43.8
Relative length for fine roots (%)[2]	4.2	6 (72 cM)	20.7	25.0	18.7	34.4
Relative length for thick roots (%)[3]	4.3	6 (72 cM)	14.8	12.3	16.6	35.3

[1]/ Average of the individuals possessing the allele with the highest LOD score in the cluster; [2]/ Root length in the class of fine roots (diameter greater than or equal to 0.2 and smaller than 0.4 mm) in relation to total root length; [3]/ Root length in the class of thick roots (diameter greater than 1.0 mm) in relation to total root length.

For number of lateral roots a QTL was found on chromosome 1 (Table 3, Figure 3). Markers in this QTL-region showed a skewed segregation: on average for every three individuals carrying the *A. fistulosum* alleles there was one individual with the *A. roylei* alleles. When the marker with the highest LOD in the cluster was considered, the average number of lateral roots emerging in the stem-borne roots of plants carrying the *A. fistulosum* allele was nearly twice the number of lateral roots in plants carrying the *A. roylei* allele (Table 3, Figure 4).

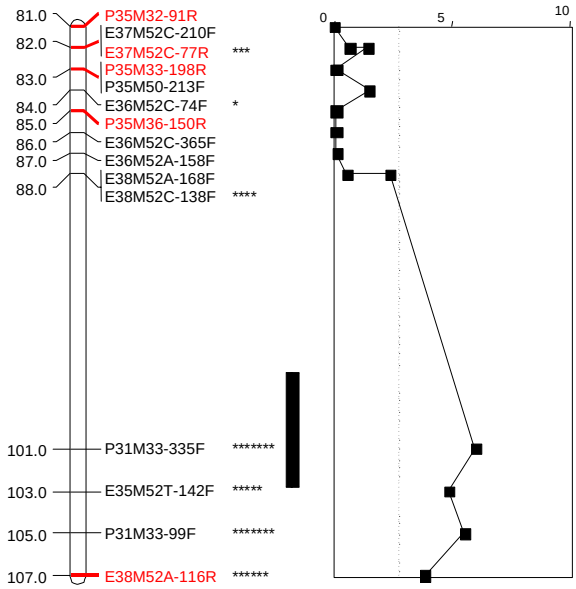

Figure 3. LOD-graph after interval mapping of part of chromosome 1. The figure shows the position on the linkage map of the QTL for lateral roots. The LOD 1 interval is given by the solid bar and the level of significance in a Kruskal-Wallis analysis is in asterisks (*:0.1 **:0.05 ***:0.01 ****:0.005 *****:0.001 ******:0.0005).

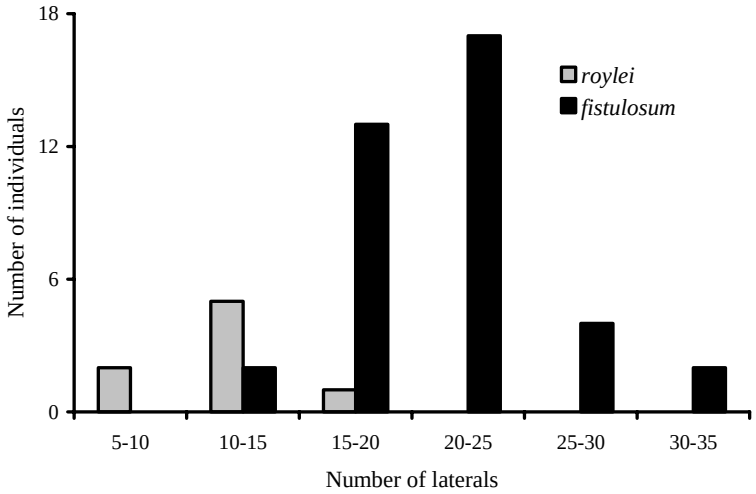

Figure 4. The distribution of the individuals in the mapping population *A. cepa* x (*A. roylei* x *A. fistulosum*) for number of lateral roots per stem-borne root. In gray are the individuals with the *A. roylei* allele and in black the individuals with the *A. fistulosum* allele of the AFLP-marker P31M35-99F.

Root length density (L_V) and relative root length in classes of root diameter

L_V in the RF-hybrid and in *A. fistulosum* was respectively five and six fold higher than in onion (Table 2). The L_V mean in the progeny of the tri hybrid cross was

four times higher than in onion with some individuals showing L_V values higher than the RF-parent and similar to *A. fistulosum* (Figure 5). In spite of this broad variation, no QTLs were found for L_V in the tri-hybrid mapping population.

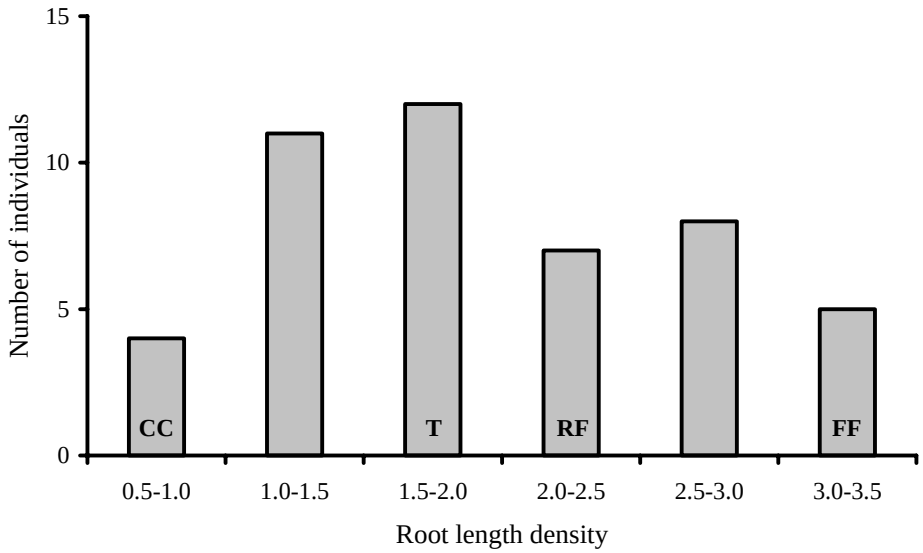

Figure 5. Distribution of tri-hybrid individuals in classes for root length density ($cm.cm^{-3}$), measured 75-85 days after planting. Capital letters in the columns give the average values of most parentals and for the tri-hybrid population (CC = *Allium cepa*, T = the tri-hybrid progeny, RF = hybrid between *A. roylei* and *A. fistulosum*, FF = *A. fistulosum*).

There were also clear differences in root diameter among onion, the RF-hybrid and *A. fistulosum*. In onion, most of the root system was made up of roots with a diameter ranging from 0.4 to 0.7 mm, while in the RF-hybrid and especially in *A. fistulosum* most of the roots were thinner than in onion, with a diameter from 0.2 to 0.5 mm (Figure 6). In addition, thick roots, with more than 1.0 mm in diameter, constituted an important class of roots in the RF-hybrid and in *A. fistulosum*, but not in onion. The tri-hybrid progeny had mixed values between those of onion and the RF-hybrid parent, with most of the roots showing a diameter between 0.3 and 0.7 mm. A number of tri-hybrid plants had a high proportion of thick roots, like it was observed in the RF-hybrid parent and in *A. fistulosum.* A QTL was found on chromosome 6 for the relative root length of fine (root diameter greater than or equal to 0.2 and smaller than 0.4 mm) and thick (root diameter greater than 1.0 mm) roots in the plants. The *A. fistulosum* alleles of this QTL raised the proportion thick roots and lowered the proportion fine roots (Table 3).

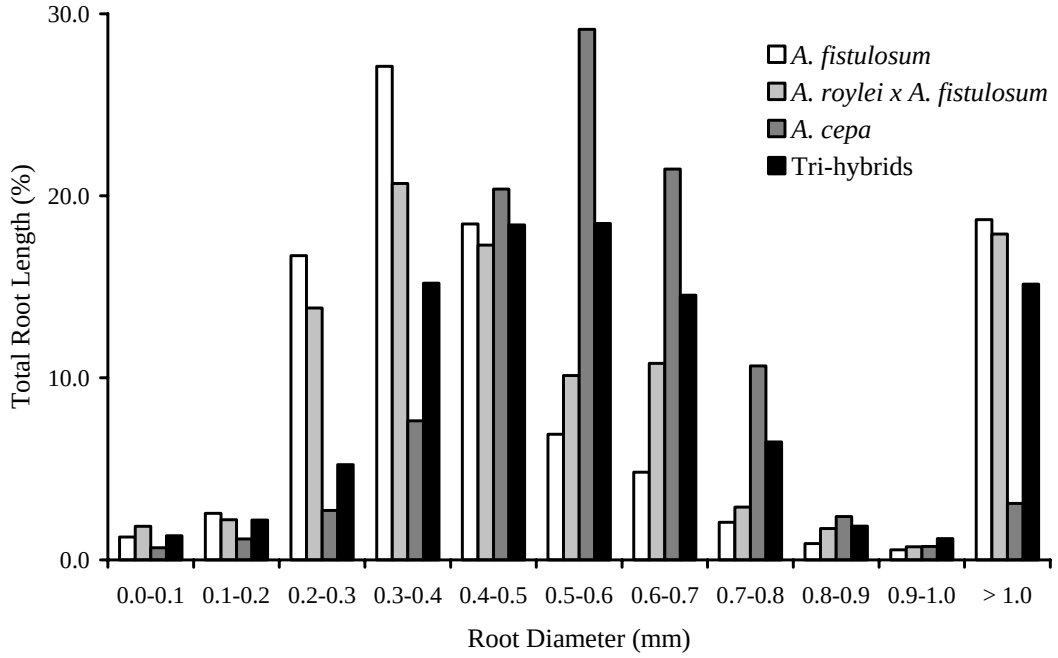

Figure 6. Average percentage of total root length represented in the different classes of root diameter. The distribution is given for: *A. fistulosum* CGN 14.763, the interspecific hybrid *A. roylei* x *A. fistulosum*, the onion male-sterile line and for the average of the mapping population, 75-85 days after planting.

Discussion

Map coverage and marker integration

Based on a chiasmata frequency of 14.2 in an *A. roylei* x *A. fistulosum* hybrid (de Vries et al., 1992), a map of 710 cM could be expected. Our map covers about 661 cM, over 90% of the expected length. This coverage is in the same range as the other two existing *Allium* maps (King et al., 1998; van Heusden et al., 2000a). In addition, in the presented map, the linkage groups could be assigned to the eight chromosomes.

In spite of the broad coverage and the absence of large gaps (no gaps > 13 cM and only 7 gaps > 10cM) between markers, still 38% of the originally scored markers remained unlinked in our map. In the onion and *A. roylei* AFLP-based maps, 32 and 20% respectively of the markers remained unlinked (van Heusden et al., 2000a). There can be several reasons for this, such as the relatively small population and skewness of some chromosomal regions. Also only a few mistakes or missing cases can make the mapping of some loci troublesome. This was particularly the case for the enzyme combination *Eco*RI/*Mse*I that, even with the addition of a 7th selective base in the primers, frequently resulted in banding patterns which were difficult to analyze. Nearly 43% of *Eco*RI/*Mse*I bands could not be mapped, while the rate of unmapped *Pst*I/*Mse*I-markers was much lower (32%). Even then, still more *Eco*RI/*Mse*I- than *Pst*I/*Mse*I-

markers ended up in the map, on average respectively 22.2 and 17.2 per primer pair in each enzyme combination.

Markers remain also unmapped when they reveal simultaneously polymorphisms on different chromosomal regions. In complete sets of *A. fistulosum – A. cepa* monosomic addition lines, approximately 10% of *A. cepa*-specific AFLP-markers could be assigned to more than one chromosome (van Heusden et al., 2000b). King et al. (1998) observed, also in onion, that 21% of the cDNA probes used revealed segregating RFLPs in different positions. In these cases, there is a chance that unlinked or loosely linked loci are analyzed as if they were one, resulting in deviating segregation ratios that prevent successful mapping. Finally, the estimation of the map length based on the frequency of chiasmata might be too conservative (Sybenga, 1996). Therefore, in the present work it is also possible that a fraction of the unmapped markers belong to parts of the genome still not integrated into the map. A larger population size and the use of other markers can fill up gaps and may identify markers in regions with a relatively low number of AFLP fragments.

Segregation distortion

Distortion from the expected segregation rate of 1:1 was observed for 26% of the markers. Distorted segregation is widespread in genetic maps, especially when involving interspecific crosses (e.g. Haanstra et al., 1999; Chetelat and Meglic, 2000; Bliss et al., 2002; Chani et al., 2002). In *Allium*, van Heusden et al. (2000a) found in their AFLP mapping study that 26 and 51% of respectively *A. cepa* and *A. roylei* markers were distorted. Distorted segregation occurs due to the presence of alleles that favor gamete and zygote formation, such as a faster pollen tube growth rate, as classically demonstrated by Mangelsdorf and Jones (1926) or of alleles linked to fitness and lethality (Rick, 1966; Luo and Xu, 2003). In mapping studies, distorted marker segregation appears when markers are in chromosomal regions associated with these alleles.

Distortion can be due to over or under presence of a marker. In our map, because only one complement of the diploid genome was analyzed, one would expect equal numbers of both. The under presence of *A. roylei* markers in a given region implies the over presence of *A. fistulosum* markers in the same region and vice-versa. However under presence occurred 3.5 times more than over presence. A possible explanation for this unbalanced ratio is that in case of under presence of an *A. fistulosum* marker for example, there was a preference for the *A. roylei* counter part during fertilization, which might have occurred due to higher similarity with *A. cepa*. More similarity reduces chances of finding fragments that reveal polymorphism between *A. roylei* and *A. cepa*. The same goes for under represented *A. roylei*.

Number of bulbs

Onion develops a single bulb, *i.e.* a single round-thickened pseudo-stem (Jones and Mann, 1963). The *A. fistulosum* type used in this work is a vigorous plant that develops a single, long and only slightly enlarged pseudo-stem (Brewster, 1994). *A. roylei* is a slow-growing *Allium* species that produces small bulbs (van Raamsdonk and de Vries, 1992b). The RF-hybrid used in this investigation resembles *A. fistulosum*, except for having multi-stems, a characteristic from *A. roylei* (van Raamsdonk and de Vries, 1992a) (Table 2, Figure 7). Multi-stems are undesirable in onion, in which a plant has to produce, for commercial reasons, a single and heavy bulb.

Figure 7. *A. fistulosum* CGN 14.763 (left) and the interspecific hybrid between *A. roylei* and *A. fistulosum* (middle) with their long pseudo-stems. *A. roylei* CGN 20.520 (right) shows the narrow and short leaves and the multi-stem habit.

Most of the tri-hybrids developed round-thickened pseudo-stems, similar to onion bulbs. Multi-bulbs were observed (Table 2), but nearly 35% of the tri-hybrid individuals had predominantly a single bulb. To our knowledge no research has been published about the genetic basis of the number of bulbs in onion or shallots (*A. cepa* L. group Aggregratum). In this investigation, a single QTL was found for the number of bulbs (Table 3, Figure 2). This QTL showed that it makes a difference for the number of bulbs whether the QTL-region originated from *A. fistulosum* or from *A. roylei* (Table 3). However, it is likely that after further backcrossing of the tri-hybrids with *A. cepa*, most of the plants will tend to have a single bulb and, therefore, this QTL will be of little practical use in breeding.

Stem-borne and lateral roots

The broad segregation and the higher mean of the tri-hybrid population for stem-borne and lateral roots (Table 2) showed the potential of the tri-hybrid cross to

introgress both root traits into onion. Some tri-hybrid genotypes produced as much as 5 times more stem-borne roots on the main bulb and 3.5 times more lateral roots than the onion parent did. There is no information concerning root morphology in *A. roylei*, but *A. fistulosum* has shown to have much more stem-borne and lateral roots than onion (Chapter 2). Both characteristics were also higher in the *A. roylei* x *A. fistulosum* hybrid than in onion (Table 2). The importance of stem-borne and lateral roots was discussed in length in chapter 2. Briefly, in plants with adventitious root systems like the *Alliaceae*, stem-borne roots are responsible for expanding the rooting zone to allow access to resources further away and for overcoming eventual soil mechanical resistance. Lateral roots, in their turn, are essential to maximize nutrient uptake and to allow the plant to quickly respond to localized resource availability. The enhancement of plant ability to explore (stem-borne roots) and exploit (lateral roots) the soil is especially important in conditions of non-abundant nutrient supply, as it is in organic agriculture.

In this study, two putative QTLs were found for the total number of stem-borne roots produced by a plant (Table 3). Rice and maize are the only two other crops in which mapping studies for this trait were carried out. In rice, as much as 19 and as few as 2 QTLs were found for number of adventitious roots in two different populations (Ray et al., 1996; Zheng et al., 2000; Kamoshita et al., 2002). In maize, 1 or 2 distinct QTLs were detected (Lebreton et al., 1995). However in our experiment the number of stem-borne roots and of the number bulbs were highly correlated. Therefore, these QTLs might be of limited use in raising the number of stem-borne roots in onion. In rice, number of roots and number of tillers were also found to be consistently correlated (Ray et al., 1996; Shen et al., 2001).

Concerning the number of laterals per stem-borne root, a major QTL was found at one end of chromosome 1 (Figure 3), with *A. fistulosum* alleles increasing the number of laterals (Table 3, Figure 4). This is the second report of finding markers significantly associated with lateral roots. In lettuce, one major QTL, responsible for 72% of the variation, was found for the number of laterals per cm of the main root, a trait quite similar to the one assessed here (Johnson et al., 2000). The presence of a single and strong QTL in lettuce and now in *Allium* suggests that major genes govern this particular trait. Such QTLs with a high impact are favorable for breeding.

Root length density (L_V) and relative root length in classes of root diameter

Similar as for the other traits, L_V showed a wide segregation in the progeny of the tri-hybrid cross, with onion and *A. fistulosum* placed respectively at the lowest and the highest classes in the distribution (Figure 5). This indicates that gains in onion L_V are possible by means of selecting in the tri-hybrid cross. However no QTL above the

threshold level was found for this trait. Root length is an aggregate measurement of root growth (Zheng et al., 2000; Lynch and Brown, 2001). In *Allium*, it will depend on number, length and degree of branching of stem-borne roots and also in the length of lateral roots (Weaver and Brunner, 1927); Chapter 2). This complex nature, in combination with the small size of the mapping population and the unknown influence of the onion homologues hampered the detection of QTLs for L_V.

When fine (diameter greater than 0.2 and smaller than 0.4 mm) and thick (diameter greater than 1.0 mm) roots are considered (RL_{fine} and RL_{thick}), it was evident that they are important components of the root system in *A. fistulosum* and in the RF-hybrid (Figure 6). This bi-modal distribution of root length along root diameter was consistently found in *A. fistulosum* (Chapters 2 and 3). RL_{fine} and RL_{thick} varied broadly in the tri-hybrids (Table 2) and a QTL was mapped for both characteristics on chromosome 6 (Table 3). In one of our previous studies (Chapter 2), it was shown that fine and thick roots corresponded well to number of respectively lateral and stem-borne roots. However, in the present investigation, there was no correlation between RL_{fine} and the number of lateral roots and only a very weak correlation between RL_{thick} and number of stem borne roots. Moreover, QTLs for both RL_{fine} and RL_{thick} were found in the same chromosomal region, with alleles from one or the other species having opposite effects over fine and thick roots (Table 3). No other, but stem-borne roots can be thicker than 1.0 mm, therefore this class surely represent the thick roots regularly observed in *A. fistulosum* (Chapters 2, 3 and 4). Indeed, alleles from *A. fistulosum* contributed to raise RL_{thick} (Table 3). Not all stem-borne roots were that thick, in plants with more than one bulb, stem-borne roots were often long and thin and thick roots were rare. These thin roots had evidently thinner distal halves that could have perfectly well fitted into the class of fine roots. Therefore, selecting for RL_{thick} can certainly raise the proportion of thick stem-borne roots, without necessarily reducing the proportion of lateral roots. However, as a safeguard, it should be combined with selection for number of lateral roots.

Perspectives

The small population size used in this study (49 individuals) certainly reduced the chance of finding QTLs. Therefore, the tri-hybrid individuals were cloned (Chapter 5) and evaluated in a replicated experiment, since replication enhances the power of finding QTLs in small populations (Cowen, 1988; Soller and Beckmann, 1990). More individuals would have been used in the present study, if available. However, considering the needs of mapping studies, the *A. cepa* x *(A. roylei* x *A. fistulosum*) tri-hybrid cross was not exactly generous in seed set and germination.

In addition to the population size, another uncontrolled factor interfered with QTL detection in the present study: the influence of the ever-present *A. cepa* homologous chromosomes. This has certainly contributed to reduce the significance of any possible association between chromosomal regions of the RF-hybrid and a complex trait like, for example, root length. Nevertheless, QTLs were identified for number of bulbs and lateral roots, and for the relative root length of fine and thick roots. It is likely that these traits are less complex than root length. The QTLs for number of laterals and for the relative importance of thick roots are of particular interest in breeding onion with improved root systems. However, before being applied in Molecular Assisted Breeding, all identified QTLs need to be confirmed in an independent population and under several environmental conditions, including organically managed fields (Lande and Thompson, 1990; Young, 1999).

The tri-hybrid plants composed of onion, *A. roylei* and *A. fistulosum* are unquestionably a further step towards the use of *A. fistulosum* in onion breeding. *A. roylei* works efficiently as a bridge-species to mediate the introgression of *A. fistulosum* genes into *A. cepa* (Khrustaleva and Kik, 2000) and this makes it possible to effectively use the QTLs described here in breeding for improved root systems. In addition, the tri-hybrid cross has the property of simultaneously bringing into *A. cepa* introgressions of two highly valuable gene reservoirs (for review, see Kik, 2002).

Acknowledgments

We would like to thank Dr. Oene Dolstra and Prof. E. Jacobsen, from Wageningen UR, for careful and critically reading the manuscript.

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Chapter 7

General discussion

This thesis deals with the interface between plant breeding and organic agriculture, fields whose crossing points have been scarcely explored from an experimental point of view. Recently, in a more conceptual study on this subject, plant breeding strategies for organic agriculture were outlined and the concepts of cultivar and plant health were redesigned (Lammerts van Bueren, 2002). In this context, the main requirements for a cultivar oriented to organic agriculture were proposed, including the adaptation to the properties of soil fertility in organic farming (Lammerts van Bueren et al., 2002). In organic agriculture, nutrients are supplied using complex sources, such as animal manure and composts (van Bruggen, 1995; Atkinson, 2000). In the long term, these fertilizers will contribute to the overall improvement of soil fertility (Matson et al., 1997). However, even in well-established systems, after years of organic management, nutrients are less promptly available than where synthetic fertilizers are used (Lengnick and King, 1986; Mäder et al., 2002). Therefore, cultivars oriented to organic farming are expected to be efficient nutrient scavengers and able to cope well with unpredictable and not rarely stressful nutrient availability levels (Lammerts van Bueren et al., 2002).

Onion (*Allium cepa* L.) was chosen to be studied here because (1) it is, also in organic agriculture, an important crop and (2) it has few, shallow and poorly superficially spread roots, which, in addition, lack root hairs (Weaver and Brunner, 1927; Greenwood et al., 1982; Föhse et al., 1991). The co-occurrence of these root characteristics result in a root system of very limited ability to forage for enough nutrients to supply plant demands (Baligar and Barber, 1978; Föhse et al., 1988). Until now, this limited ability has been compensated by a massive application of fertilizers (Greenwood et al., 1980; Fontes and Nogueira, 1984; Greenwood et al., 1992). In view of this, it can be easily foreseen that the completion of a successful onion cultivation cycle, including the realization of an economically sustainable yield, can be very problematic in organic agricultural systems.

Given the aforementioned, the challenge was to take the first steps towards the improvement of onion's root system and, hence, towards the (ecological and economical) sustainability of production. The work described in this thesis was as follows: firstly a study of the variation present in root morphology in old and modern onion populations and in its close relative *A. fistulosum*; secondly to understand the functionality of this variation in an organic agriculture context; and finally to search

for molecular markers associated to root traits. Furthermore, an *in vitro* multiplication step was introduced and a preliminary study on the interaction between arbuscular mycorrhizal fungi (AMF) and *A. fistulosum* in an organically managed soil was performed.

Variation in root systems of onion and *A. fistulosum*

The development of organic cultivars, meant as those selected under and specifically for organic farming conditions, is an important issue in organic agriculture. However, as has been stated in the general introduction of this thesis (Chapter 1), breeding companies are not willing to take the financial risk of developing these cultivars. The small market for organic cultivars and the difficulty of producing high quality seeds in organic conditions would make the price of organic seeds very high compared to conventional ones.

The development of an onion cultivar with an improved root system will only take place if such a cultivar will have an added value in both organic and conventional systems. Three arguments for developing onions with improved roots for conventional circumstances are: the raising costs of fertilization, the increasing legislative regulation restricting the use of fertilizers in many countries and a crescent environmental awareness (Sattelmacher et al., 1994; Matson et al., 1997). If an onion cultivar with an improved root system has to be developed, what would be the best strategy? One way to go is to screen the onion germplasm for accessions with better root traits or, alternatively, to transfer genes with known effects on roots from closely related species.

The screening made on the germplasm of onion did not reveal much variation. An evaluation of onion cultivars from two distinct geographic origins (Eastern Europe and old and new cultivars from the Netherlands) revealed that all of them had a poor rooting system (Chapter 2). However within the small differences, an interesting phenomenon was observed: Dutch onion cultivars released in the 1940s and 1950s had a significantly higher root length density (cm of root per cm^3 of soil) than their modern counterparts. This might have been caused by selection for improvement of above ground traits at the expense of the root system. A poorer root system must then be compensated by a higher external input. Onions are not unique in this respect, because the same was observed in crops like barley (Brown et al., 1987) and wheat (reviewed by Hoad et al., 2001). Onion cultivars bred for use in high input systems are not adequate to organic farming, where plants have to develop and maintain a large and active root system to sustain a reasonable yield (Lammerts van Bueren et al., 2002). Therefore, the use of ‘organic seed’ from modern cultivars is not enough to the organic production of onions.

Without enough variability to improve onion roots in *Allium cepa*, one can extent the horizon and examines allied species. *A. fistulosum* L., a close relative of onion, showed consistently a higher root length density than onion, both in the greenhouse and at an organically managed field (Chapter 2). Moreover, the improved root density in *A. fistulosum* was mainly due to the massive presence of fine roots, mostly lateral roots (Chapter 2). In *A. fistulosum* the number and thickness of stem-born roots was also higher than in onion (Chapter 2).

The functionality of the variation in root traits in organic agriculture

It is known that in monocots, that have adventitious or fibrous root systems, stem-borne (adventitious) roots are responsible for enlarging the rooting zone and for overcoming eventual mechanical resistance in the soil while foraging for resources (Materechera et al., 1992; Sattelmacher et al., 1994). In the case of the *Alliaceae*, which lose the seminal root shortly after seed germination, stem-borne roots are also the only possible site for lateral root development (Jones and Mann, 1963; Pulgarin et al., 1988; Birdsall and MacLeod, 1990). Lateral roots are more efficient in uptake than stem-borne roots and are responsible to raise the root density, which intensifies the soil exploitation and allows the plant to quickly respond to the localization of nutrients (Krannitz et al., 1991; Robinson, 1996; Malamy and Benfey, 1997; Lynch and Brown, 2001). The enhancement of plant ability to explore (stem-borne roots) and to exploit (lateral roots) the soil is of course especially important in conditions of non-abundant nutrient supply, as it is in organic agriculture (Lammerts van Bueren et al., 2002; Mäder et al., 2002).

Therefore, the soil exploration and exploitation patterns of onion and *A. fistulosum* roots were studied (Chapter 3). The experiments were carried in two locations on an organically managed farm. Between the two locations there was a three-fold difference in nitrate availability in the soil. The results showed that *A. fistulosum* explored always a larger volume of soil than onion did. Moreover, *A. fistulosum* responded to the decrease in nitrate availability with deeper rooting, a reaction not seen in onion. For access to soil resources, the ability of *A. fistulosum* to explore larger volumes of soil is very relevant. If a radial symmetric root distribution around the plant is assumed (van Noordwijk et al., 1985), *A. fistulosum* had a rooting zone 30% larger than onion. In case of organic farming, the exploration of a larger volume of soil can be even more decisive. In organically managed soils, nutrients are often dispersed in patches as a result of the time- and space-localized decomposition of organic matter (van Vuuren et al., 1996). The larger the rooting zone, the higher the chances of accessing more nutrient patches.

To be able to efficiently exploit the soil in general and not only nutrient patches, plants need to establish an adequate root density, which is mostly achieved through the development of lateral roots (Drew, 1975; Burns, 1991; Robinson, 1994). In organically managed soils, due to a more intense competition with weeds (Lammerts van Bueren et al., 2002), the (quick) development of lateral root is especially important. Root length density (L_V) is the most commonly used root attribute for soil exploitation and nutrient uptake (Atkinson, 1990; Hoad et al., 2001), especially for less mobile nutrients, like phosphorus (Römer et al., 1988). In case of low L_V, especially if combined with nutrient shortage, this holds also for mobile nutrients, such as nitrogen (Robinson et al., 1991; Sattelmacher et al., 1993; Wiesler and Horst, 1994). In our experiments, L_V was always higher in *A. fistulosum* than in onion, it was not lowered by the reduction in nitrate availability and, therefore, there was no change in aboveground development. Onion, which had a L_V already low when nitrate was abundant, had an even lower L_V in the reduced nitrate level and failed to sustain its aboveground development.

Root growth in *A. fistulosum* was higher than in onion also when it was compared to shoot growth (root length : shoot dry weight and root : shoot dry weight). High root: shoot ratios characterize a developmental strategy that chooses insurance instead of risk (Atkinson, 2000) and rewards the larger allocation of photosynthates to roots with enhanced tolerance to water and nutrient stress. Yet able to respond well to a wealthy environment, plants that follow the insurance pathway are well prepared to display stability when under stress. Such plasticity is of high importance in organic agriculture.

Molecular markers and the variation in root traits

Onion and *A. fistulosum* differed enormously in relation to root traits (Chapter 2). These differences formed the basis of the superior soil exploration and exploitation abilities in *A. fistulosum* in relation to onion (Chapter 3). Actually, the pattern displayed by *A. fistulosum* matched the expectations of organic breeding for root development. Therefore, the introgression from *A. fistulosum* to onion of the root traits responsible for the difference and ultimately the development of an onion cultivar with an *A. fistulosum*-like root system would be an important step towards the sustainability of onion production.

Nevertheless, the use of *A. fistulosum* in onion breeding has been fraught with difficulties in the past, due especially to the sterility barrier between the two species (Albini and Jones, 1990; van der Valk et al., 1991; Ulloa et al., 1994). Fortunately, it was recently shown that this obstacle could be successfully circumvented if *A. roylei* is used as a bridge-species (Khrustaleva and Kik, 1998; 2000). In addition, the bridge-

cross (tri-hybrid) strategy allows the simultaneous exploitation of two very rich gene reservoirs for the breeding of onions (for review see Kik, 2002). The bridge-cross approach is a realistic option to exploit the *A. fistulosum* germplasm in the direction of improving the onion root system. Molecular markers were used to analyze the progeny of the tri-hybrid cross *A. cepa* x (*A. roylei* x *A. fistulosum*), with the objective of locating QTLs that could be used in Marker Assisted Breeding (MAB).

As a first step, an AFLP genetic map was made for the parental *A. roylei* x *A. fistulosum* interspecific hybrid (Chapter 6). The map assembled 450 markers in eight linkage groups, covering 660 cM, more than 90% of the expected length. Forty-six of the mapped *A. roylei* markers corresponded to markers mapped in a previous linkage study in *Allium*, in which most of the linkage groups were assigned to the physical *A. cepa* chromosomes (van Heusden et al., 2000a; 2000b). This knowledge made it possible to indirectly determine the correspondence between chromosomes and the eight linkage groups obtained in the *A. roylei* x *A. fistulosum* map.

To perform large scale mapping studies, the tri-hybrid cross was not generous enough in seed set and germination. Only a limited number of tri-hybrid genotypes (49) was available. Considering the impossibility of obtaining more tri-hybrid genotypes in the short term, the linkage analysis was strengthened by evaluating the 49 phenotypes in a replicated experiment (Cowen, 1988). To be able to do this, all genotypes were cloned using *in vitro* techniques. Few information was available for *in vitro* multiplication of *A. fistulosum* and, especially, of *A. roylei*. To spread the risk of non-response and to avoid losing any of the tri-hybrids, explants were extracted from different plant organs (Chapter 5). Explants were produced using the basal stem and the receptacle of closed umbels. Also flower buds from unripe (spathe closed) and ripe (spathe opened) umbels were used. Results showed that the use of receptacles is advisable when there is no previous information about the *in vitro* multiplication behavior of a given genotype. All genotypes developed plants when receptacles were used, receptacles were easy to disinfect and the method is non-destructive. If genotypes respond to the *in vitro* culturing of flower buds, then flower buds from unripe umbels should be preferred. They are the easiest to manipulate and can potentially generate the largest number of plants. When needed, bulb basal plates can also be used as an explant source, regarding that they are properly disinfected.

After successful cloning of the tri-hybrids and their parents, the plants were evaluated in a four-replication experiment (Chapter 6). Most of the tri-hybrids developed bulbs, *i.e.* round-thickened pseudo-stems (Jones and Mann, 1963). Multi-bulbs, a characteristic likely to be inherited from *A. roylei* (van Raamsdonk and de Vries, 1992) were observed, but nearly 35% of the tri-hybrid individuals developed a single bulb, which is the behavior expected from an onion plant. A single QTL was

found for number of bulbs on chromosome 5 and showed that it made a difference whether the QTL-region originated from *A. fistulosum* (fewer bulbs) or from *A. roylei* (more bulbs).

Number of stem-borne and lateral roots, as well as for root length density had a broad segregation in the tri-hybrid progeny, showing the high potential of the cross to allow the introgression of these valuable root traits into onion (Chapter 6). Some tri-hybrids produced as much as 5 times more stem-borne roots and 3.5 times more lateral roots than the onion parent did. A quarter of the tri-hybrids had a root length density at least 5 timer higher than onion. However, in spite of the replications, the small population size indeed reduced the chances of finding QTLs. In addition, the ever present *A. cepa* chromosomes shadowed the effect of their *A. roylei* x *A. fistulosum* homologues over more complex and aggregate traits, like root length (Lynch and Brown, 2001). Yet, QTLs were identified for number of stem-borne and lateral roots, and for the relative root length of thick roots. In the case of stem-borne roots, the trait was highly correlated to the number of bulbs, which limits the value of these QTLs in onion breeding.

Nevertheless, the single and strong QTLs found for number of laterals and for the relative importance of thick roots are of particular interest. This was only the second report ever of a QTL for lateral roots. The pioneer work was carried out in lettuce and found also only one major QTL for number of laterals per cm of the main root, a trait quite similar to the one assessed here (Johnson et al., 2000). The presence of single and strong QTLs in lettuce and now in *Allium* suggests that the trait is very likely governed by a few major genes, quite an advantage for breeding. The same goes for the relative importance of thick roots. In both cases, *A. fistulosum* was the donor-species of the favorable alleles.

Allium fistulosum and Arbuscular Mycorrhizal Fungi (AMF)

Allium species clearly benefit from the interaction with AMF (Creighton Miller et al., 1986; Stribley, 1990). In this thesis, research was conducted to study the effect of AMF over the performance of an *Allium* species, in this case *Allium fistulosum* (Chapter 4). This work had two particularities. First, the soil used was collected from an organic farm and had a moderate to high level of P available (P_w of ± 30 mg P_2O_5.100 g^{-1} of dry soil). Second, the aim was not restricted to studying the effect of indigenous AMF over root colonization and plant growth. The contribution given to plant development by exogenous AMF (*Glomus intraradices*), introduced through seedling inoculation was also investigated. This was quite a distinct approach from what is commonly done when the work involving AMF and organic agriculture is

considered (Sattelmacher et al., 1991; Ryan et al., 1994; Scullion et al., 1998; Mäder et al., 2000).

Indigenous AMF and the inoculated *Glomus intraradices* were both found colonizing the roots. Indigenous AMF alone reached 53% of root colonization and this was the first report of root colonization by uninoculated indigenous AMF in *Allium* in conditions of organic agriculture. Inoculation with *G. intraradices* resulted in 79 and 85% of root colonization respectively in the absence and presence of indigenous AMF. Inoculation promoted on average a 50% increment on shoot dry weight (50% dependence on AMF, as defined by Plenchette et al., 1983). In addition, total and fine root length density and the number of stem-borne roots were remarkably higher in inoculated plants. Therefore, in spite of the high activity of indigenous AMF and of the moderate levels of available P in the soil, plant inoculation with AMF was very beneficial. This result is even more relevant in the context of organic agriculture, where there are not many alternatives to promote such a substantial increment in yield.

Conclusions and perspectives

The results described in this thesis are solid evidence of the tri-hybrid cross *A. cepa* x *(A. roylei* x *A. fistulosum*) potential to improve the root system of onion. Considering that fertile tri-hybrid individuals can be obtained and that the three genomes recombine (Khrustaleva and Kik, 2000), the road is open for the introgression into onion of the genes that give *A. fistulosum* a profuse root system. Marker Assisted Breeding (MAB) is particularly of use in breeding for root traits, as those traits are laborious to assess and show a phenotypic plasticity that reduces gains through direct selection (Lynch and Brown, 2001). QTLs were found for the relative root length of thick (stem-borne) roots and for the number of lateral (fine) roots. Strong and numerous stem-borne roots can efficiently explore the soil in the chase for nutrients and water (Materechera et al., 1992; Sattelmacher et al., 1994). A large amount of lateral roots will quickly and efficiently exploit the available resources (Krannitz et al., 1991; Robinson, 1996; Lynch and Brown, 2001). An onion cultivar with such a root system would be beneficial for organic farmers (Lammerts van Bueren et al., 2002) and would certainly have also an added value for onion growers in general.

Before any of these QTLs can effectively be applied in MAB, they need to be confirmed in an independent population and/or in further backcross material and in different environments (Lande and Thompson, 1990; Young, 1999), including organically managed fields. To achieve this aim, new crosses involving the three species have to be done. The know-how acquired in cloning the tri-hybrids using *in vitro* tools will be especially useful to multiply the genotypes for multi-location trials.

The integration of Arbuscular Mycorrhizal Fungi (AMF) to the organic production in *Allium* is definitely appealing. In this thesis, in a preliminary evaluation, *A. fistulosum* showed to react to the inoculation with AMF with a 50%-increase in dry weight, even when cultivated in an organically managed soil with quite active indigenous AMF. Considering that the *A. fistulosum* root system is much better developed than onion's and that onion is also a mycorrhizal crop (Stribley, 1990), it is plausible to believe that onions would have an ever larger benefit from inoculation with AMF. However, the system used for *A. fistulosum*, inoculation followed by transplant, is not applicable to onions. Onion fields are mostly established through direct sowing. Thus, before integrating AMF inoculation into the organic production of onions, the efficiency of either seed or field AMF addition in improving plant growth has to be tested. In addition, research on developing simple and effective inoculation methods and economic systems to produce AMF inoculum in scale is still necessary. Starting points could be the on-farm production of inoculum and the use of expanded clay as a vehicle for inoculation (Dehne and Backhaus, 1986; Baltruschat, 1987; Sharma and Adholeya, 2000; Gaur and Adholeya, 2002)

The breeding of onion (and *A. fistulosum*) cultivars for an optimum use of the association with AMF is also an issue. But, what are the perspectives for that? It is known from wheat, barley and maize that, at least on the plant side, the interaction with AMF has a genetic component (Hetrick et al., 1995; Murphy et al., 1997; Kaeppler et al., 2000). In onion and *A. fistulosum*, cultivars have been shown to react differently to inoculation with AMF (Powell and Verberne, 1982; Sharma and Adholeya, 2000; Tawaraya et al., 2001). Thus, there is basis for selection. In addition, onion is reported to profit from the association with AMF even in rather high soil contents of phosphate, over 120 $mg.100g^{-1}$ dry soil (P_{Olsen}), equivalent to approximately 60 $mg.100^{-1}$ P_W (Plenchette et al., 1983; Charron et al., 2001). The positive response from *A. fistulosum* to the inoculation with AMF reported in this thesis took place in a soil with 30 $mg.100g^{-1}$ (P_W). This is a P level common for Dutch mineral clay soils, from where most of the Dutch onion production comes. All things considered, there is no other conclusion to draw than that there is plenty of 'room' for (research on) AMF in the organic production of onions.

Finally, we conclude that introgression breeding for root improvement in onion with the use of the tri-hybrid cross *A. cepa* x (*A. roylei* x *A. fistulosum*) is not only feasible, but also promising and fascinating. It opens the perspective for a new paradigm: onion as a crop of efficient roots and well-integrated into sustainable production systems.

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Summary

Onion (*Allium cepa* L.) is one of the most important vegetable crops and it is grown worldwide. The reason for such a global distribution is the universal acceptance of onion as food and condiment, but certainly not the simplicity of growing. Actually, onion is a difficult crop to grow and one of the main challenges is to give onion plants enough nutrients. Onions have a rooting zone of not more than 20 cm and, in addition, roots are scarce, seldom branching and lack root hairs. The limited soil exploration and exploitation abilities make onion very vulnerable to drought and to nutrient deficiency. To date, this problem has been solved with a massive use of fertilizers throughout the growing season. In addition, to achieve high yields, irrigation is often used. This high-input production model is becoming more difficult to sustain and, at the same time, alternative agricultural systems, such as organic agriculture, are becoming more widespread. However, the particularities of the soil fertility management in organic agriculture and the avoidance of synthetic fertilizers demand plants that are good nutrient scavengers. Thus, it can be envisaged that problems related to onion nutrition might become a strong obstacle to productivity and stability of production in organic farming systems. It is possible to breed for improved root systems, but it is not easy. Root evaluation is laborious and root traits display a large phenotypic plasticity in response to various soil conditions, which reduces the efficiency of direct selection. In this context, Marker Assisted Breeding can be of great help in breeding for onions with improved root systems. But breeding relies on variation and there was no information in onions, in particular, or in *Allium*, in general, about genetic variation of root traits.

This thesis explored the interface between plant breeding and organic agriculture. The research was carried out to study the variation present in root morphology and development in onion and in the allied species *A. fistulosum* L. and to study its functionality in the framework of organic agriculture. Attention was paid on the behavior of the root systems of both species on organically managed soils with contrasting contents of nitrogen and on the interaction between *A. fistulosum* and arbuscular mycorrhiza fungi. Furthermore, a reliable *in vitro* method to secure the propagation of valuable *Allium* accessions and interspecific hybrids was developed. A molecular marker-based genetic map was developed for the progeny of an *Allium* interspecific tri-hybrid cross and genomic regions involved in root development were identified.

Chapter 1 of the thesis describes in a bird's eye view the general concepts of organic agriculture and their implication to plant breeding for organic environments. It summarizes the constraints related to the onion poor rooting habit and situates it in the

perspective of organic farming. It finishes with the aim of this thesis and an outline of its chapters.

In **chapter 2** an account is given of the variation in root morphology in onion and in *A. fistulosum* germplasm. The experiments were carried out in organic soil and in a greenhouse. The results obtained in both conditions coincided to a large extent, an important finding for the reliability of future greenhouse experiments. Not much variation related to root traits was found in the onion germplasm analyzed. However, of interest was the fact that relatively old onion cultivars had a higher root length density than recently developed cultivars. This points into the direction that the increase in onion yield achieved through breeding might have happened to some extent at the expense of reducing the root system, a trend also observed in other crops. Large variation was observed between onion and *A. fistulosum*, the latter species being an interesting gene reservoir for onion, as it has a much larger root system and more stem-borne and lateral roots.

The analysis of the interaction between the soil nitrogen availability and both onion and *A. fistulosum* root systems was the challenge in **chapter 3**. Experiments were carried out in an organic soil, at two locations, where a contrasting supply of organic amendments produced a three-fold difference in the content of nitrate in the soil. Auger sampling was used to obtain the root samples. Onion and *A. fistulosum* reacted in a different way upon the difference in N availability. Onion significantly reduced its root density and yield in response to the decrease in N availability, whereas *A. fistulosum* suffered no or only a slight depression. *A. fistulosum* had a larger exploration capacity than onion, as its roots were found further away from the plant. In addition, only *A. fistulosum* was able to increase its rooting depth when N became scarce. The ratio in onion between root and aboveground development was half of that of *A. fistulosum*. This indicated that onion privileged aboveground development more than *A. fistulosum*. Taking this into account, one could say that onion followed a risk, in opposition to an insurance strategy, pursued by *A. fistulosum*, which is, by far, more adequate for organic cultivation.

Chapter 4 deals with the influence of arbuscular mycorrhiza fungi (AMF) over *A. fistulosum* development. The investigation was carried out in the greenhouse, using soil collected from an organic farm, either sterilized or kept in its natural status. The most important finding here was that above and below ground biomass increased significantly (50-60%) in both sterilized and unsterilized soil when plants were inoculated with *Glomus intraradices*. The root colonization rate by indigenous AMF was 53% and increased significantly to 86% following plant inoculation with AMF. All these results point into the direction of the potential benefits AMF can bring to *A. fistulosum* and, possibly, to onion organic cultivation as well. The results described in

this chapter were promising and clearly warrant further research, both from fundamental and applied points of view.

In **chapter 5**, onion, *A. fistulosum* and a third allied species, *A. roylei* Stearn, as well as genotypes derived by crosses involving the three species were propagated *in vitro* using a multi-tissue approach. *A. roylei* was introduced because it allows a successful introgression of genes from *A. fistulosum* to onion, generating a fertile progeny when it is used in a bridge-cross. On contrary, the progeny of the direct cross between onion and *A. fistulosum* is highly sterile. Onion vegetative propagation is usually carried out using basal plates as the explant source. Although this is in principle a reliable method, not all genotypes can be propagated using this method. This is an obstacle when unique genotypes, which should not get lost, have to be multiplied. Therefore, research was carried out to test other plant organs as alternative explant sources. Explants derived from basal plates indeed regenerated plantlets for most of the genotypes, but inflorescence receptacles were the only explant source to yield plantlets of all genotypes. If genotypes are responsive, and in our study 68% were, flower buds from unripe inflorescences were the explant source with the potential to regenerate the largest number of plantlets. Flower buds collected in ripe inflorescences were also tested, but response was very poor.

Chapter 6 deals with the identification in a genetic map of genomic regions associated to root morphology in two onion allied species. An AFLP-based molecular marker map was constructed for the parental *A. roylei* x *A. fistulosum* complement, based on a population of 49 hybrid individuals of the cross *A. cepa* x (*A. roylei* x *A. fistulosum*). The map included 450 AFLP markers and was divided in eight linkage groups that could be assigned to the eight chromosomes. The genetic linkage map and the segregation of root and bulb traits were used for a Quantitative Trait Locus (QTL) analysis. Phenotypes were assessed in the greenhouse, in a four-replication experiment, using *in vitro* cloned plants. All traits, evaluated at full vegetative growth, showed a broad segregation. Numbers of bulbs and of stem-borne roots were correlated and the two QTLs found for the latter coincided with the (putative) QTLs for the former. One QTL was pinpointed for the number of lateral roots per stem-borne root and another one for the relative root length of fine and thick roots. These results showed the feasibility of breeding for onions with improved root systems using the interspecific hybrid between *A. roylei* and *A. fistulosum* as a genetic source of root traits.

In **chapter 7**, the general discussion, the work carried out in this thesis was summarized. The results of the different chapters are connected and give a comprehensive view of the possibilities of breeding onions for improved root systems. Some important perspectives on the use of cultivars carrying such root traits in onion organic production are highlighted.

Samenvatting

Ui (*Allium cepa* L.) is één van de belangrijkste groentegewassen ter wereld en wordt overal verbouwd. De reden voor de wereldwijde verspreiding is de algemene waardering voor ui als voedsel en smaakmaker, maar het is zeker niet de eenvoud waarop uien geteeld kunnen worden. Ui is namelijk een moeilijk gewas en één van de grootste uitdagingen is te zorgen dat uienplanten genoeg voedingsstoffen op nemen. Ui heeft een wortelstelsel dat niet dieper gaat dan ongeveer 20 cm, en er zijn ook maar weinig wortels die bovendien ook nog zo goed als niet vertakt zijn en geen wortelharen hebben. Een dergelijk wortelstelsel maakt dat de uienplant tijdens de groei in de bodem maar geringe mogelijkheden heeft te zoeken naar de aanwezigheid van water en voedingsstoffen. Deze eigenschap maakt uien erg kwetsbaar voor gebrek aan voldoende voedingsstoffen en voor droogte. Dit probleem is altijd opgelost door gedurende het gehele groeiseizoen grote hoeveelheden kunstmest te gebruiken. Dit, samen met irrigatie, maakt hoge opbrengsten mogelijk. Door regelgeving en ook kostenaspecten wordt het steeds moeilijker een dergelijk productiemodel, met kunstmest en irrigatie, te handhaven. Tegenwoordig worden alternatieve landbouwsystemen, zoals de biologische landbouw, steeds algemener. In deze systemen, met hun andere manier van omgaan met bodemvruchtbaarheid, zijn rassen nodig die efficiënt voedingsstoffen kunnen zoeken. Omdat uienplanten dit niet kunnen is het te verwachten dat in de biologische landbouw de nu aanwezige uienrassen niet voldoen. Het is mogelijk om ui te gaan veredelen met een beter functionerend wortelsysteem, maar dit zal zeker niet gemakkelijk zijn. Het evalueren van worteleigenschappen is arbeidsintensief en bovendien hebben wortels een grote mate van phenotypische plasticiteit als antwoord op verschillende omstandigheden in de bodem. Deze omstandigheden reduceren de efficiëntie van directe selectie tijdens het veredelen. Merker gestuurde veredeling is een buitengewoon waardevolle toevoeging aan het veredelingsproces. Om een veredelingsprogramma, met als doel uien met een verbeterd wortelsysteem, op te zetten is het essentieel de aanwezige genetische variatie van de eigenschap te onderzoeken. Voor *Allium*, maar ook in het algemeen, waren er maar weinig studies gedaan naar de genetische variatie van worteleigenschappen.

Dit proefschrift beschrijft onderzoek op het grensgebied tussen plantenveredeling en biologische landbouw. Het doel van het onderzoek was het bestuderen van de variatie in wortelontwikkeling in ui (*Allium cepa* L.) en zijn verwante soort *A. fistulosum* L. en deze gevonden variatie tegen het licht te houden van de biologische landbouw. Aandacht werd besteed aan het gedrag van de wortelstelsels van beide soorten onder invloed van een veranderend stikstofaanbod in de grond en

ook aan de interactie tussen *A. fistulosum* en arbusculaire mycorrhiza schimmels. De genetische basis van wortel- en bolontwikkeling is gedeeltelijk ontrafeld met behulp van een moleculaire merkerkaart. Om genoeg uitgangs materiaal te hebben voor alle experimenten werd ook een betrouwbare *in vitro* methode ontwikkeld voor de vegetatieve vermeerdering van *Allium* genotypen.

Hoofdstuk 1 beschrijft in vogelvlucht de algemene concepten van de biologische landbouw en de biologische plantenveredeling en behandelt de opzet van het onderzoek aan de wortelstelsels van ui en zijn verwante soort *A. fistulosum*.

In **hoofdstuk 2** wordt de gevonden variatie in wortelstelsel eigenschappen beschreven tussen ui en *A. fistulosum*. Experimenten werden zowel onder biologische omstandigheden in het veld als in de kas uitgevoerd. De behaalde resultaten onder beide milieuomstandigheden kwamen goed overeen, hetgeen essentieel was voor de vertaalbaarheid van kasexperimenten naar de biologische praktijk. Er werd weinig variatie binnen ui gevonden voor worteleigenschappen. Een interessant aspect was dat relatief oude uienrassen een significant hogere wortellengte per bodemvolume hadden vergeleken met recent ontwikkelde rassen. Dit wijst in de richting van selectie op bovengrondse opbrengst ten koste van het wortelstelsel: een trend die al eerder beschreven was voor andere gewassen. Grote verschillen in het wortelstelsel werden gevonden tussen ui en *A. fistulosum*: laatstgenoemde soort had een veel langer wortelstelsel met meer fijnere wortels en meer hoofdwortels die afkomstig zijn van de basale plaat.

De uitdaging in **hoofdstuk 3** was om veranderingen in het wortelstelsel van ui en *A. fistulosum*, veroorzaakt door variatie in de hoeveelheid stikstof in de bodem, te beschrijven en te verklaren. De experimenten werden uitgevoerd in een biologisch beheerd perceel. In dit perceel werden twee locaties gekozen die een factor drie van elkaar verschilden met betrekking tot de hoeveelheid beschikbare stikstof in de bodem. Met behulp van een grondboor (Auger) werden bodemmonsters verzameld en er werd aangetoond dat de wortelstelsels van ui en *A. fistulosum* sterk van elkaar verschilden in hun reactie op verschillen in bodemvruchtbaarheid. Bij ui werd worteldichtheid en totale biomassa opbrengst sterk gereduceerd bij een mindere beschikbaarheid van stikstof, terwijl bij *A. fistulosum* weinig tot geen verschillen aan te tonen waren. *A. fistulosum* had een grotere exploratie capaciteit vergeleken met ui doordat de wortels verder weg van de plant doorgroeiden en in geval van een verlaagd stikstofniveau bleken de wortels van *A. fistulosum* ook dieper in de grond door te groeien. Verder werd gevonden dat de verhouding tussen totale wortellengte en bovengrondse biomassa bij ui de helft was in vergelijking met *A. fistulosum*. Dit suggereert dat de verdeling van koolhydraten over wortel en spruit bij ui meer in de richting van bovengrondse ontwikkeling gaat als we het vergelijken met *A. fistulosum*. Het lijkt er

op dat ui meer een risicostrategie volgt en *A. fistulosum* meer een verzekeringsstrategie. Duidelijk zal zijn dat laatstgenoemde strategie een voordeel kan zijn onder biologische teeltomstandigheden. Daarom is het zeker aan te bevelen om biologische uienrassen te ontwikkelen met een *A. fistulosum* type wortelstelsel.

Hoofdstuk 4 gaat over de invloed van arbusculaire mycorrhiza schimmels (AMS) op bovengrondse- en worteleigenschappen van *A. fistulosum*. Het experiment werd uitgevoerd in de kas met biologische grond, die al dan niet met stoom was gesteriliseerd. Veruit de belangrijkste vondst was dat de totale boven- en ondergrondse biomassa significant toenam (50-60%) wanneer planten werden geinoculeerd met de AMS *Glomus intraradices*. Dit gold zowel voor planten in gesteriliseerde grond als in niet gesteriliseerde grond. Ook werd gevonden dat de AMS kolonisatie van de wortels sterk toenam (van 44% naar 86%) en dat ook de totale lengte van het wortelstelsel toenam. Deze waarnemingen, verricht aan *A. fistulosum*, kunnen ook zeer interessant voor de biologische teelt in Nederland zijn. Vandaar dat het aanbeveling verdient om hier meer onderzoek aan te doen en te kijken in welke mate AMS kunnen bijdragen aan het verhogen van de opbrengst en het verbeteren van de oogststabiliteit van gewassen onder biologische omstandigheden.

In **hoofdstuk 5** is een nieuwe *in vitro* vermeerderingsmethode ontwikkeld voor ui, *A. fistulosum* en een derde verwante soort *A. roylei* Stearn gebaseerd op het simultaan gebruik van meerdere weefseltypes. *A. roylei* is een belangrijke brugsoort om terugkruising in de soortskruising tussen ui en *A. fistulosum* mogelijk te maken. *In vitro* vermeerdering bij ui geschiedt o.a. via het gebruik van basale plaatweefsel als start-explantaat. Alhoewel dit in principe een betrouwbare methode is, kunnen niet alle genotypen via deze methode vermeerderd worden en dit is natuurlijk een probleem wanneer het gaat om unieke genotypen die niet verloren mogen gaan. Daarom is onderzoek verricht om alternatieve explantaat types te vinden. Gevonden werd dat via explantaten van de basale plaat bijna alle genotypen *in vitro* konden worden vermeerderd, en dat via het gebruik van de bodem van de bloeiwijze alle genotypen *in vitro* konden worden vermeerderd. Uit bloemknopjes van nog niet volledig ontwikkelde bloeiwijzen konden, als deze responsief waren, de meeste regeneranten worden verkregen. Bloemknopjes uit volledige ontwikkelde bloeiwijzen werden ook getest, maar dit was niet erg succesvol.

Hoofdstuk 6 behandelt een gedeeltelijke ontrafeling van de genetische basis van worteleigenschappen uit twee wilde verwanten van ui. Gebruikmakend van een kruising tussen ui (CC) en een interspecifieke hybride tussen *A. roylei* en *A. fistulosum* (RF) is een AFLP merkerkaart geconstrueerd van de RF ouder. De kaart bevatte 450 merkers en was opgesplitst in acht koppelingsgroepen die toegewezen konden worden aan de acht chromosomen. De genetische kaart en de segregatie van wortel- en

boleigenschappen zijn gebruikt voor een koppelingsanalyse (*Quantitative Trait Locus, QTL*). Een significante associatie werd gevonden voor het aantal bollen per plant en drie significante associaties voor de hoeveelheden en vorm van de verschillende worteltypes. Deze resultaten laten duidelijk zien dat het haalbaar is om met behulp van merker gestuurde selectie en de wilde verwant *A. fistulosum*, uien te veredelen met een verbeterd wortelsysteem voor enerzijds biologische teelten maar anderzijds ook zeker voor de conventionele uienteelt.

In hoofdstuk 7, de algemene discussie, zijn de behaalde resultaten samengevat en bediscussieerd in de context van biologische teelt. Verder worden een aantal belangrijke aspecten uit dit proefschrift nader toegelicht.

Resumo

A cebola (*Allium cepa* L.) é uma hortaliça de grande importância em todo o mundo. A razão para essa distribuição global é seguramente a sua aceitação generalizada como alimento e condimento e não a facilidade de cultivo. Ao contrário, a cebola é uma planta de cultivo complexo. Um dos maiores desafios enfrentados pelos produtores é o fornecimento às lavouras de uma nutrição adequada, uma vez que a cebola desenvolve poucas raízes que dificilmente se ramificam e são desprovidas de pêlos radiculares. Soma-se a isso o fato de suas raízes explorarem um limitado volume de solo, não indo além de um raio de 20 cm ao redor da planta. Em consequência, as plantas de cebola, inaptas a utilizar eficientemente os recursos existentes no solo, são extremamente suscetíveis a estresses hídricos e nutricionais. Esse desenvolvimento radicular deficiente tem sido compensado pela utilização maciça de fertilizantes, aplicados no plantio e em cobertura. A irrigação também é frequentemente utilizada para garantir produtividades elevadas. Porém, a sustentabilidade deste modelo de produção, baseado em intensa aplicação de insumos, é cada vez mais frágil. Ao mesmo tempo, modelos alternativos como, por exemplo, sistemas orgânicos, vão se tornando mais populares e, consequentemente, mais importantes. Entretanto, as particularidades do manejo da fertilidade do solo em sistemas orgânicos de cultivo e o abandono do uso de adubos sintéticos convertem-se, no caso da cebola, devido às peculiaridades de seu sistema radicular, em grandes entraves à produção.

É possível utilizar o melhoramento genético para o desenvolvimento de genótipos de cebola com sistemas radiculares abundantes, mas esta não é uma tarefa fácil. A avaliação de características radiculares é trabalhosa e, como a maioria delas apresenta grande plasticidade fenotípica em resposta às variadas condições de solo, a eficiência da seleção direta fica comprometida. Neste aspecto, ferramentas como o Melhoramento Assistido por Marcadores Moleculares podem ser de grande valia no melhoramento para desenvolvimento radicular. Porém, o melhoramento depende de variabilidade genética e, no caso da cebola, em particular, ou em *Allium*, em geral, não existem informações a este respeito.

Esta tese foi desenvolvida na interface entre melhoramento de plantas e agricultura orgânica. O trabalho foi realizado com o objetivo de estudar a variabilidade genética existente em cebola e em *Allium fistulosum* L. em relação a características morfológicas e ao desenvolvimento de raízes, assim como para avaliar a funcionalidade desses diferentes sistemas radiculares em sistemas orgânicos de produção. Especial atenção foi dada ao comportamento do sistema radicular em ambas as espécies quando cultivadas em níveis contrastantes de nitrogênio. A interação entre

A. fistulosum e fungos micorrízicos arbusculares também foi objeto de estudo. Estabeleceu-se, ainda, um método seguro para propagação *in vitro* de valiosos genótipos e híbridos interespecíficos em *Allium*. Ao final, foi construído um mapa genético baseado em marcadores moleculares para a progênie de um cruzamento interespecífico envolvendo três diferentes espécies do gênero *Allium*. Neste mapa, foram identificadas as regiões genômicas significativamente associadas ao desenvolvimento radicular.

O **capítulo 1** desta tese descreve, de forma suscinta, conceitos gerais relacionados à agricultura orgânica e suas implicações sobre as práticas de melhoramento no que diz respeito ao desenvolvimento de genótipos para ambientes orgânicos de produção. Neste capítulo são apresentados também os principais obstáculos impostos à produção orgânica de cebola pelo seu escasso sistema radicular. O **capítulo 1** apresenta ainda o objetivo geral desta tese e os temas tratados em cada um de seus diferentes capítulos.

No **capítulo 2** é discutida a variação morfológica encontrada em raízes de cebola e de *A. fistulosum*. Os experimentos foram conduzidos em casa-de-vegetação e em campo, em um sistema orgânico de produção. Os resultados obtidos nos dois ambientes coincidiram em larga escala, uma observação importante para assegurar a confiabilidade de futuros experimentos em ambientes controlados. A variação morfológica das raízes dos diversos genótipos de cebola avaliados foi bastante restrita, embora cultivares relativamente antigas tivessem apresentado maior densidade radicular que híbridos modernos. Este fato indica que o aumento na produtividade de cebola alcançado através do melhoramento de plantas pode ter acontecido, até certo ponto, em detrimento da alocação de fotossintatos para o desenvolvimento das raízes, tendência observada também em outras espécies. Uma grande variação na morfologia das raízes foi observada entre *A. fistulosum* e cebola. O sistema radicular é mais abundante em *A. fistulosum* que em cebola, em virtude não só do desenvolvimento de um número muito maior de raízes adventícias naquele, mas também do fato dessas raízes se ramificarem com frequência. *A. fistulosum* constitui-se, portanto, em uma fonte de variabilidade para o melhoramento de cebola visando a obtenção de genótipos com bom desenvolvimento radicular.

A análise da interação entre sistema radicular e disponibilidade de nitrogênio no solo foi o tema do **capítulo 3**. Os experimentos foram conduzidos em solo manejado de acordo com os princípios da agricultura orgânica, porém em faixas com distintos níveis de nitrogênio disponível às plantas, obtidas através de um fornecimento contrastante, por vários anos, de adubos verdes e animais. As amostras de raízes foram coletadas através do método de Auger. Cebola e *A. fistulosum* reagiram de forma distinta à diferença nos teores de nitrogênio disponível no solo. Em cebola, houve uma

redução significativa na densidade radicular e no rendimento de matéria seca na parte aérea onde a disponibilidade de nitrogênio era menor, enquanto em *A. fistulosum* não houve redução em nenhuma das duas características. As raízes de *A. fistulosum* exploraram uma área de solo maior que as de cebola. Além disso, apenas *A. fistulosum* apresentou aprofundamento da zona de enraizamento em resposta ao decréscimo da disponibilidade de nitrogênio. A razão entre desenvolvimento de raízes e da parte aérea em cebola foi a metade da encontrada em *A. fistulosum*, o que demonstra que as plantas de cebola privilegiam menos o desenvolvimento radicular que as plantas de *A. fistulosum*. Portanto, *A. fistulosum*, por investir relativamente maior quantidade de carbohidratos no desenvolvimento radicular, assegura-se mais contra a redução nos níveis de água e/ou de nutrientes no solo que cebola, adequando-se melhor a sistemas de produção onde o uso de insumos é reduzido.

O **capítulo 4** trata da influência de fungos micorrízicos arbusculares (AMF) sobre o desenvolvimento de *A. fistulosum*. O experimento foi conduzido utilizando solo manejado de forma orgânica. Em metade do experimento foi utilizado solo esterilizado, enquanto na outra metade o solo foi mantido em seu estado natural. As plantas inoculadas com *Glomus intraradices*, independentemente da esterilização ou não do solo, apresentaram aumento significativo da biomassa aérea e radicular (50-60%) quando comparadas às plantas não inoculadas. A taxa de colonização das raízes por AMF autóctone, aumentou significativamente de 53% para 86% após a inoculação com *G. intraradices*. Estes resultados indicam o benefício potencial que a utilização de AMF pode trazer ao cultivo orgânico de *A. fistulosum* e, muito possivelmente, também ao de cebola. Os resultados obtidos neste capítulo foram muito promissores e apontam para a necessidade de um aprofundamento da pesquisa em relação à associação entre *Allium* e AMF em ambientes orgânicos.

O **capítulo 5** relata a propagação *in vitro* de cebola, *A. fistulosum* e uma terceira espécie, *A. roylei* Stearn, assim como dos genótipos derivados dos cruzamentos envolvendo essas três espécies. A propagação vegetativa de cebola é feita normalmente a partir de explantes obtidos do prato do bulbo. Porém, nem todos os genótipos podem ser propagados com sucesso desta forma, o que representa um risco à manutenção de genótipos raros. Por essa razão, neste trabalho, foram avaliadas também fontes alternativas de explantes. Explantes derivados do prato basal regeneraram plântulas na maioria dos genótipos, porém o receptáculo das inflorescências foi a única fonte de explantes a regenerar plântulas em todos os genótipos. Para genótipos responsivos, e nesse estudo 68% o foram, botões florais coletados em inflorescências imaturas, ainda protegidas pela espátula fechada, foram a fonte de explante com potencial para regenerar o maior número de plântulas. Botões florais coletados em inflorescências maduras, já com a espátula aberta, também foram

testados, mas pouco genótipos foram responsivos e, nestes, poucas plântulas foram obtidas.

A construção de um mapa molecular e a identificação neste mapa das regiões genômicas associadas à morfologia das raízes em *A. roylei* e em *A. fistulosum* é o assunto apresentado e discutido no **capítulo 6**. Utilizando um cruzamento entre cebola (CC) e o híbrido interespecífico entre *A. roylei* e *A. fistulosum* (RF), um mapa molecular foi desenvolvido para o complemento parental RF, baseado na segregação de marcadores AFLP em 49 indivíduos tri-híbridos (CC x RF). O mapa incluiu 450 marcadores, divididos em oito grupos de ligação, cuja identidade cromossômica pôde ser determinada com sucesso. A segregação de características relativas aos bulbos e às raízes foi avaliada nestes mesmos indivíduos e sua associação com o mapa genético foi determinada. Todas as características, avaliadas no pico do desenvolvimento vegetativo das plantas, mostraram segregação contínua, típico de características quantitativas. O número de bulbos por planta correlacionou-se significativamento com o número de raízes adventícias e os dois *loci* para características quantitativas (QTLs) encontrados para número de raízes coincidiram com os (prováveis) QTLs encontrados para número de bulbos. Um QTL foi identificado para número de raízes laterais, oriundas da ramificação das raízes adventícias, e um outro para o comprimento relativo de raízes finas (laterais) e grossas (adventícias). Estes resultados indicam que o uso do híbrido interespecífico entre *A. roylei* e *A. fistulosum* no melhoramento genético de cebola visando desenvolvimento abundante de raízes é bastante promissor.

No **capítulo 7**, discussão geral, o trabalho conduzido para a elaboração desta tese foi reunido, sintetizado e colocado em perspectiva. Os resultados obtidos nos vários capítulos foram interligados, mostrando as possibilidades do melhoramento genético para o desenvolvimento de cebola com sistemas radiculares mais abundantes e a aplicabilidade de tal característica em sistemas orgânicos de produção.

Acknowledgements, Agradecimentos, Nawoord

Several times I thought that these words would never be written. Fortunately, and due some extraordinary people, I was wrong. Many might think that there are too many thanks in here. This is a Brazilian thing: we feel that every special one deserves a word. I just cannot help it!

First things first, I would like to thank my attentive and loving mother (*in memoriam*) and father and my grandparents. From and with them, I learnt the power of work, ethics, family, love and faith, which are absolutely not ranked here, since I am totally unable of ranking such values (*Muito obrigado não só por me habilitarem a seguir um bom caminho, mas também por me ensinarem a reconhecê-lo*). Professionally, I was also very lucky, first to have such nice fellows at college and later, very talented colleagues at the Brazilian Agricultural Research Corporation, in short Embrapa. I will not name everybody, but I would like to thank you all. I would not be a dedicated agronomist and researcher without your contribution. I am also deeply grateful to the Brazilian people, who have been sponsoring my studies since college and, through Embrapa, funded most of my graduation period abroad.

Doing a Ph.D. abroad might sound simple. However, crossing the Atlantic to come back home only a few years later is quite an operation. In our case, it was possible only because we had ample encouragement and support of our families (*Dna. Christina; Pai, Sônia e Ana Carolina*; *Marcelo, Ana Cristina e Isabela; Joca, Rosângela, Júlia, Paulo Augusto e Maria Eduarda; Gute, Keyla, Alice e querido(a) nenenzinho: muito obrigado, do fundo do coração*) and because our dearest friend, Sieglinde Brune, accepted the "invitation" to look after our matters in Brazil (*muito obrigado, Linde, pela sua amizade sincera e por todo os abacaxis tão bem descascados durante esses anos. Obrigado também pela visita do ano passado*). I have to thank also Dr. José Amauri Buso, who not only pushed me and gave me confidence to apply for a Ph.D. abroad, but also accepted being my scientific representative at Embrapa, during the graduation period (*obrigado, Amauri, pela amizade, por ler todos aqueles intermináveis relatórios e pela sua enorme ajuda quando nós mais precisamos*). In Brazil, at Embrapa, I could also count with the friendship and support of Gorete Medeiros, Leonardo Giordano and Ruy Fontes, who did their best when we most needed (*Obrigado. Muito obrigado mesmo*!).

Once in Wageningen, I met my director of studies (*promotor*) Prof. Evert Jacobsen. Although we never actually worked side-by-side and in spite of his always busy agenda, Prof. Jacobsen managed to follow my Ph.D. in a very reasonable pace. It

is true that we had to re-schedule many of our previously scheduled meetings, but eventually he always found enough time to contribute to my progress (*Bedankt, Evert*).

Some time later, at Plant Research International (PRI), I met Dr. Chris Kik and Dr. Sjaak van Heusden, my supervisors (*co-promotoren*). These two are surely the supervisors every Ph.D. student would like to have. Chris' door was always opened and, while we were planning our work, he was truly interested in my ideas and listening to me. Not once or twice, but many times, he was out in the field and in the greenhouses, with his hands-at-work. In addition, he contributed decisively in improving the writing in every chapter of this thesis. Of course, he was also taking care that the necessary means to perform experiments and analyses were provided (*I do not know how you manage enough time to everything, Chris, but you certainly do. Thanks a lot for taking me into your group. Thanks also for providing the funds for my last year here*). I was lucky enough to have also Dr. Sjaak van Heusden as a supervisor. From Sjaak I learnt a lot (and I mean it) about molecular markers, mapping, mapping software. Sjaak had always a special ability to reveal the simplicity of apparently complex things and had always a positive and encouraging word to keep moods up (*Thanks, Sjaak, also for the big help on placing "all bricks in the wall" on chapter 6*).

Cytogenetics was planned to be an important component in this thesis. And indeed it was during the whole Ph.D., although, due to matters of time and deadlines, it did not ended in the thesis. Owing to the involvement with cytogenetics, I happened to know two outstanding people: Dr. Hans de Jong and Dr. Ludmila Khrustaleva. Hans supervised me for a short period just after I arrived in Wageningen. In his lab, where I had the pleasure of working with Henny Verhaar and Jannie Wennekes, I learnt a lot about chromosome preparations, meiosis, microscopes and scientific writing and artwork. I am also much in debt with Hans for his decisive support in the most difficult period of my Ph.D. (*Dank je wel, Hans*). Ludmila introduced me to the colorful world of chromosome *in situ* hybridization. She is extremely skilled, meticulous, and enthusiastic about her work. I learnt a lot from that and also from her broad knowledge on cytogenetics. Together we obtained beautiful blue-red-and-green metaphase preparations and produced nice pictures that soon will show up in a fine paper (*Ogromnoe spasibo, Ludmila*).

This thesis resulted also from the work and dedication of many other people. Karin Burger was a constant colleague in all experiments. Without her help, we would certainly have fewer results to report. Gerard Brouwer was essential for all the root work and trained me on root evaluation and image analysis. Ria Vrielink taught me, from A to Z and with a remarkable patience, how to work with AFLPs (even when the bubbles formed while pouring the polyacrylamide were driving me crazy). Claire Boddington and Dr. Thom Kuyper gave a major contribution to our work with

arbuscular mycorrhiza fungi. Paul Keizer created always some time to help with the statistics (*Karin, Gerard, Ria, Claire, Thom and Paul, thanks a lot*). I would like to thank also Gloria Ramirez for the big hand she gave in the mycorrhiza experiments (*Gracias, Gloria*) and Bruno Ligthart for helping in collecting and cleaning so many roots (*Dank je wel, Bruno*). But none of our experiments would have lasted long if it were not for the aid of Jaap van den Berg, Alex van Silfhout and Dick Geurtsen, in the greenhouses at "De Goor", and Andries Siepel and Henk van Rein, at the Lovinkhoeve (*Bedankt!*).

I would like also to thank some other people at PRI that contributed to this thesis either directly or indirectly. Thanks Elisa, Koen, Marleen, Reni, Rodrigo, Ronald Snijder and Si-Jun; Bernadette, Betty, Greetje, Linda and Richard (at the lab); Mariame, Petra and Ellen (at the secretary); Ilse (at Evert's secretary) and Peter and Jan (at the helpdesk). In the Laboratory of Genetics, I would like to thank Song-Bin, Liuda, Olga, Oxana, and Natalia; and, in the Laboratory of Plant Breeding, Luisa, Carlota, Carolina, Ronald Oomen, Marjan Bergervoet, Dirkjan, Annie Marshall and Dr. Ramanna (*Thank you all*).

But life was not only working (although in this last year it was mostly it!). Outside the PRI "walls", some people contributed definitely to make our years in Wageningen much easier and pleasant. Ankie Lamberts assisted us in finding a place to live (*Bedankt, Ankie. I consider the fact that you are not doing this anymore a major drawback in the relation between the university and its international students*). We are much and deeply grateful also to the crew (currently Dorien, Linda and Mijke) at the crèche " De Kleine Wereld", where our son Arthur had a lot of care and fun (*Hartelijk bedankt!*). Annemiek de Wit and Jeltje Osse, and their respective families, were very special people who we had the pleasure of meeting here (*Wij verwachten jullie spoedig in Brasilië te zien!*). René and John, at the Sport Center, were good friends on our Friday Happy Hours (*Dank je wel*).

I can not leave out all of our relatives and friends who crossed the Atlantic to visit us or who were so kind to spend part of their time with us when for whatever reason they came to Europe. Thanks Adrienne, Lílian, *tia* Diva (who celebrated her 90th birthday on the flight that brought her here), *vó* Iria, Myriam, Billy and Paula, Rogério and Maristerra, Tite, *tio* Carlos and *tia* Cida, *tia* Maria Lúcia and Fernanda, Juarez, Daniel and Verónica, *Dna.* Christina and Janilda. I would like to thank also Sandra, Ralf, Lukas and Maria Luíza, for their kindness when we visited them in Berlin.

Our time in Wageningen would not have been a tenth as nice if it were not for the large Brazilian family that we had the pleasure to belong to here. Our Christmas

and New-Year celebrations, *Festas Juninas*, *Festa Brega* and uncountable *feijoadas, vacas atoladas, quibadas* or simply getting together to eat were the sunshine we, tropical people, missed in the "wet'n cold" autumn, winter and (at least half of) spring days. Of course, eating was just an excuse to be together. Actually, a very nice one when you have such superb "chefs", as some turned out to be. Those that do not know the Brazilian way would not believe how long a dinner can last and how many different dishes and delicacies can be named and discussed while we are having something totally different to eat! This is another of those Brazilian manias that we just cannot help!

It was very nice to see some faces we already knew from Brazil in Wageningen. The first person I met when I arrived was André, a friend from Embrapa. He picked me up in Schiphol (another Brazilian thing: it does not matter how easy it is to go from any airport, train or bus station to wherever you want, if you have a Brazilian friend, you have a ride – *obrigado André, Diva, Laura e Luiza, pela acolhida*!) Next day, I met Tatsuya, another good friend from Embrapa. Very soon I was meeting Madelaine (*foi muito legal encontrar e conviver com você e poder conhecer o Ângelo, sô! Agora, falta ainda conhecer o Bruno*). Martien and Ineke Beek were also good friends we knew from Brazil and with whom we had always a pleasant time every time we met (*Dank je wel, muito obrigado*). Later Simone and Cristiano also came from Brazil (*que bom que vocês vieram! Foi muito bom ter vocês por perto*). Thank you all for the friendship and care.

But we were very successful to add several new faces (and hearts) to those already known. Some are still around: Marta, Sílvia and Tetê (*e também vovó Neide – por melhor que seja a sensação de dever cumprido e por mais feliz que estejamos por voltar para casa, não vai ser fácil deixar vocês. Mas em julho de 2004 a gente se encontra, combinado?*); Antônio, Cristiane and a baby coming soon (*três pessoas muito especiais. Desejamos a você, Cristiane, e a você também, Antônio, uma boa hora*); Vagner Benedito (*bom amigo e também companheiro de tantos almoços e de tantos sábados e domingos enclausurados no PRI*); Gilma (*parabéns pela tese!*) and Luis (*que embora já tenha voltado ao Brasil, estará em Wageningen no dia 29 de outubro – lembranças à Cristiane e Guilherme*); Eduardo (*obrigado pelas aulas de squash e de "formatação de tese"*) and Isabela; Jane, Bert and Thijs (*que gente boa!*); Zé Márcio (*para sempre capitão do Ranca Toco de Futebol e Regatas*), Regiane and Alissa; Joana; Danny and Priscila (*e um bebezinho já pronto para nascer*); André (*e Raquel e Lucas*); Isabella; Francisco; and Bia, Roberto, Kim and Anabel.

Among those that had already made the journey back home, very special ones were Rômulo, Flávia and now Isabela (*caros amigos, vocês podem sempre contar conosco*); Débora (*tia Dedé*) and Kostas (next stop: Curitiba! We will meet you there.

You can count on that!); Alan, Ivete, “Deiego” and later we knew Mariana (*a gente se vê em Brasília*); Amaral (*tá gelada, tem algum problema?*), Cláudia and Samuel (*e também a Gina*); Gustavo, Sandra, Pedro and Nina (*muito obrigado pela amizade e por toda a ajuda*); Irene and Arne; Américo and Cláudia; Flávia Alcântara; Tarcísio, Franciene and Marina; Raul, Viviane, Bruno, Olívia and Victor; Fábio, Susan and Brenda (*ou seria Ângela?*); Wellington, Denise, Jessika and, recently, Guilherme; Brandão, Adriana and later the little Talita; Renato and Denise; Rodrigo and Ana; Passarinho, Simone, Alissa, Arielle and Jonas; and Jorge and Flora. Of course, the Portuguese dearest contribution to our large family would not be forgotten: *Sara e Graça, muito obrigadinho pela amizade.*

But, be sure, not a word in this whole thesis would have been written if it were not for my wife. Véri was my first supporter to go abroad and while here she proved to be really tough. She wrote her own thesis, went back to Brazil to (very successfully) defend it and, when back here, she faced the disappointment of not being allowed to work due to the extremely restrictive labor policy for foreigners, even when scientific positions she could easily take were available. She did not give up and stood by my side until the very end (*Véri, muito obrigado por todo o carinho, pelo amor, pela dedicação. Eu sempre soube que poderia contar com você. E você sabe que pode contar comigo. Nada teria sentido se não fosse por você*). And as if this was not enough, she made me co-author in an undeniably fully successful genetic experience: our son, Arthur. Arthur was a gift and a piece of good fortune to us. In his short life up to now, he has already showed that he is great pal (*Filhinho, eu gosto demais de você! Obrigado por ajudar a conferir as referências bibliográficas da tese*).

More than finishing a Ph.D. we are closing a stage on our lives. There were some difficult moments, some very difficult flashes, but we had also a lot of pleasant time. Wageningen was and will always remain as a valuable life experience to us.

Thanks!

The author

Paulo Eduardo de Melo was born in February 07, 1964, in Belo Horizonte, a beautiful city, cradled by the mountains of “Serra do Curral”, in the state of Minas Gerais (or just Minas). In Belo Horizonte, where I did the primary and half of the secondary school, I gained a fondness for the mountains and I learnt to be proud of those that once, in the streets of our splendid baroque towns, insurrected against the Portuguese Crown to transform us into a free nation (*Libertas quae sera tamen* - Freedom, although late - the motto in Latin in the flag of Minas). I completed the secondary school and attended the high school in Goiânia, state of Goiás, 900 km Northwest of Belo Horizonte, where I developed a keen interest in agriculture. As waters run to the see, I attended the Agronomy College, at the Federal University of Goiás and, in 1986, I received a B.Sc. degree in Agronomy. In the following year, I worked as a trainee in the Brazilian Agricultural Research Corporation, at the National Center for Rice and Beans Research (in short, Embrapa Rice & Beans), with resistance of common beans to the bacterial blight. Next, I went back to the same university to receive a M.Sc. degree in Plant Breeding, with a thesis dealing with the use of cytoplasmic male-sterility in the production of cabbage hybrids. In July 1989, I started working at Embrapa Vegetables, in Brasília, the avant-garde capital of Brazil, 200 km Northeast of Goiânia, in the breeding of Brassica crops and potato. Some years later, Embrapa granted me a fellowship to pursue a Ph.D. abroad. In July 1st, 2000, I started working at Plant Research International, in Wageningen, in the research project that resulted in this thesis. Now, this stage is finished and the happy day of going home is close. In Brazil, I will resume my duties as a plant breeder at Embrapa.

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