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

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

Keywords *Allium cepa* · *A. fistulosum* · *A. roylei* · *Fusarium oxysporum* f. sp. c*epae* · *F. proliferatum*

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Introduction

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

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

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

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

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

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

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

Materials and methods

Fungal collection

A collection of *Fusarium* isolates was set up by sampling onion fields and storage sheds in Uruguay in 2003, and The Netherlands in 2004, as well as by kind supply from researchers and institutes from various countries (Table 1). This collection includes 43 isolates from onion, three from garlic (*A. sativum*) and one from shallot (*A. cepa* common group *aggregatum*). In addition, *Fusarium* isolates from other crops were included as controls (Table 1).

Slices of basal plates or diseased roots, 5–6 cm in length, were surface disinfected by immersion for 1 min in 70% ethanol, 1 min in NaOCl (15 gl^{-1}), two times in sterile water, and incubated in Petri dishes on a blotter (25°C, 3–7 days). From rotten bulbs, pieces of mycelium were isolated with a needle from the borders of the lesions that appeared in rotten bulbs.

Table 1 Collection of *Fusarium* isolates from onion and other host species included in the study of genetic diversity of *Fusarium* using AFLP markers

Identification[a]	Pathogenicity[b]	Host	Date of collection	Country and place of collection[c]	Collector or provider
Fusarium oxysporum isolated from onion (f. sp. *cepae*) and *Allium* crops					
93.816	+	Onion	1993	The Netherlands	Plant Research international, Wageningen, The Netherlands
CBS 148.25	+	Onion	1925	n.i.	Centraalbureau voor Schimmelcultures, The Netherlands
CBS 192.35	+	Onion	1935	Germany	Centraalbureau voor Schimmelcultures, The Netherlands
CBS 193.35	+	Onion	1935	Germany	Centraalbureau voor Schimmelcultures, The Netherlands
DSM 62306	+	Onion	n.i.	USA, California	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany
EZA	+	Onion	2004	Australia	Dr. K. Posthuma, Enza Zaden, Enkhuizen, The Netherlands
Fo Ech	+	Shallot	n.i.	France	Dr. C. Alabouvette and Dr. N. Gautheron, C.M.S.E.- INRA Dijon, France
Foc 06	+	Onion	n.i.	Turkey	Dr. M. Özer, University of Trakya, Turkey
Hue-2	n.d.	Garlic	2004	Spain, Huelva	Plant Research international, Wageningen, The Netherlands
Hue-3	n.d.	Garlic	2004	Spain, Huelva	Plant Research international, Wageningen, The Netherlands
Hue-5	n.d.	Garlic	2004	Spain, Huelva	Plant Research international, Wageningen, The Netherlands
LJC 10081	+	Onion	2004	Argentina, Buenos Aires	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
LJC 10045	+	Onion	2004	Argentina	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
LJC 10164	+	Onion	n.i.	USA, Texas	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
LJC 10165	+	Onion	n.i.	USA, Texas	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
LJC 10159	+	Onion	n.i.	USA	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
NL 102-1	+	Onion	2004	The Netherlands, Zeeland, Schoondijke	Plant Research international, Wageningen, The Netherlands
NL 102-2	n.d.	Onion	2004	The Netherlands, Zeeland, Schoondijke	Plant Research international, Wageningen, The Netherlands
NL 104-2	+	Onion	2004	The Netherlands, Zeeland, Kerkwerve	Plant Research international, Wageningen, The Netherlands
NL 106-2	+	Onion	2004	The Netherlands, Zeeland, IJzendijke	Plant Research international, Wageningen, The Netherlands
NL 106-3	n.d.	Onion	2004	The Netherlands, Zeeland, IJzendijke	Plant Research international, Wageningen, The Netherlands
NL 106-4	+	Onion	2004	The Netherlands, Zeeland, IJzendijke	Plant Research international, Wageningen, The Netherlands
NL 109-2	+	Onion	2004	The Netherlands, Zeeland,Langeweg	Plant Research international, Wageningen, The Netherlands
NL 132	+	Onion	2004	The Netherlands, Wageningen	Plant Research international, Wageningen, The Netherlands
NM 1	+	Onion	1999	USA, New Mexico	Dr. C. Cramer and Dr. Muhyi, New Mexico State University, NM, USA
NM 2-4	+	Onion	2004	USA, New Mexico	Dr. C. Cramer and Dr. Muhyi, New Mexico State University, NM, USA
NM 2-5	+	Onion	2004	USA, New Mexico	Dr. C. Cramer and Dr. Muhyi, New Mexico State University, NM, USA

Table 1 (continued)

Identification[a]	Pathogenicity[b]	Host	Date of collection	Country and place of collection[c]	Collector or provider
NM 2-7	+	Onion	2004	USA, New Mexico	Dr. C. Cramer and Dr. Muhyi, New Mexico State University, NM, USA
UR 07	n.d.	Onion	2003	Uruguay, Canelones, La Paloma	Facultad de Agronomía, Universidad de la República, Uruguay
UR 16	+	Onion	2003	Uruguay, Canelones, Progreso	Facultad de Agronomía, Universidad de la República, Uruguay
UR 17-3	+	Onion	2004	Uruguay, Canelones, Canelón Grande	Facultad de Agronomía, Universidad de la República, Uruguay
UR 17-5	+	Onion	2004	Uruguay, Canelones, Canelón Grande	Facultad de Agronomía, Universidad de la República, Uruguay
UR 17-8	+	Onion	2004	Uruguay, Canelones, Canelón Grande	Facultad de Agronomía, Universidad de la República, Uruguay
F. oxysporum f. sp. *lilii*					
Fol 11	n.d.	Lily	n.i.	The Netherlands	Plant Research international, Wageningen, The Netherlands
Fol 4	n.d.	Lily	n.i.	The Netherlands	Plant Research international, Wageningen, The Netherlands
F. oxysporum f. sp. *tulipae*					
Fot 10	n.d.	Tulip	2003	The Netherlands	Plant Research international, Wageningen, The Netherlands
Fot 13	n.d.	Tulip	2003	The Netherlands	Plant Research international, Wageningen, The Netherlands
Fot 47	n.d.	Tulip	2003	The Netherlands	Plant Research international, Wageningen, The Netherlands
Fot 67	n.d.	Tulip	2003	The Netherlands	Plant Research international, Wageningen, The Netherlands
Fot Yoko3	n.d.	Tulip	2003	The Netherlands	Plant Research international, Wageningen, The Netherlands
F. oxysporum f. sp. *lagenaria*					
UR 13	n.d.	Pumpkin	n.i.	Uruguay	Facultad de Agronomía, Universidad de la República, Uruguay
F. oxysporum f. sp. *loti*					
UR 15	n.d.	Birds-foot trefoil	n.i.	Uruguay	Facultad de Agronomía, Universidad de la República, Uruguay
Fusarium proliferatum					
LJC 10013	+	Onion	2004	Argentina, San Juan, Pocito	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
LJC 10023	+	Onion	2004	Argentina, San Juan, Pocito	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
LJC 10033	+	Onion	2004	Argentina, Mendoza, Maipú	Dr. C.R. Galmarini and Dr. J. Valdez, INTA La Consulta, Mendoza, Argentina
NL 109-1	n.d.	Onion	2004	The Netherlands, Zeeland, IJzendijke	Plant Research international, Wageningen, The Netherlands
NL 131-1	+	Onion	2004	The Netherlands, Wageningen	Plant Research international, Wageningen, The Netherlands
NL 131-2	+	Onion	2004	The Netherlands, Wageningen	Plant Research international, Wageningen, The Netherlands
NL 131-3	+	Onion	2004	The Netherlands, Wageningen	Plant Research international, Wageningen, The Netherlands
UR 01	+	Onion	2003	Uruguay, Canelones, Las Piedras	Facultad de Agronomía, Universidad de la República, Uruguay
UR 03	n.d.	Onion	2003	Uruguay, Canelones, Villa Nueva Sauce	Facultad de Agronomía, Universidad de la República, Uruguay
UR 06	n.d.	Onion	2003	Uruguay, Canelones, Villa Nueva Sauce	Facultad de Agronomía, Universidad de la República, Uruguay

Table 1 (continued)

Identification[a]	Pathogenicity[b]	Host	Date of collection	Country and place of collection[c]	Collector or provider
Fusarium equiseti					
UR 09	−	Pumpkin	2003	Uruguay, Canelones, Progreso	Facultad de Agronomía, Universidad de la República, Uruguay
Fusarium verticillioides					
MRC 826	n.d.	Maize	n.d.	South Africa	Dr. W. Marasas, South African Medical Research Council, South Africa
Fusarium avenaceum					
UR 04	+	Onion	2003	Uruguay, Canelones, Canelón Grande	Laboratorio de Fitopatología, F. de Agronomía, Univ. de la República, Uruguay
UR 10	n.d.	Pumpkin	2003	Uruguay, Canelones, Progreso	Laboratorio de Fitopatología, F. de Agronomía, Univ. de la República, Uruguay
Fusarium graminearum					
Fg 820	n.d.	Wheat	n.i.	The Netherlands	Plant Research international, Wageningen, The Netherlands
Fusarium culmorum					
IPO39	+	Wheat	n.i.	The Netherlands	Plant Research international, Wageningen, The Netherlands
NL 110-1	+	Onion	2004	The Netherlands, Zeeland, Stroodorp	Plant Research international, Wageningen, The Netherlands
NL 110-2	+	Onion	2004	The Netherlands, Zeeland, Stroodorp	Plant Research international, Wageningen, The Netherlands
NL 110-3	+	Onion	2004	The Netherlands, Zeeland, Stroodorp	Plant Research international, Wageningen, The Netherlands

[a] In the identification codes, a figure behind a slash distinguishes isolates obtained from different plants or bulbs in a single field or storage shed.

[b] (+) pathogenic; (−) non-pathogenic; (n.d.) not determined.

[c] (n.i.) no information available.

Hyphal-tip colonies from root lesions, basal rot, or bulb rot, were first isolated in water agar (34 g agar l^{-1}), and then maintained on potato dextrose agar (PDA, Oxoid Ltd, UK).

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

Amplified fragment length polymorphism (AFLP) analysis

Fungal isolates were multiplied in 100 ml flasks containing 50 ml of potato dextrose broth (PDB, Difco, Madison, USA), and grown for five to seven days in a shaker rotating at 100 rpm in the dark, at 18°C. Fungal tissue was harvested, dried by vacuum filtration through a nylon membrane, and lyophilized overnight. For AFLP analysis, fungal DNA was isolated from 10 mg of lyophilized mycelium ground in 2 ml tubes. Cells were lysed by incubation with 450 µl Puregene Cell Lysis Solution D5002 (Gentra Sys., Minneapolis, USA) for 1 h at 65°C, followed by addition of 150 µl of Puregene Protein Precipitation Solution D-5003, and centrifugation at 14,000 rpm for 5 min. DNA was precipitated by mixing the supernatant with 300 µl chilled isopropanol, and centrifugation at 14,000 rpm for 10 min at 4°C. DNA pellets were washed with 300 µl 70% ethanol, dried and dissolved in 100 µl Tris-EDTA. RNA was degraded with 5 µl of RNAse A solution (37°C, 30 min). Four hundred nanograms of DNA suspension was used for AFLP reactions.

AFLP® fingerprinting (Keygene B.V., The Netherlands) was done as described by Vos et al. (1995)

using two combinations of restriction enzymes: *Eco*RI–*Mse*I and *Eco*RI–*Pst*I. Pre-amplification was performed in a volume of 20 µl using primers without extra-nucleotides: E_{00} (5′-GACTGCGTACCAATTC-3′)–P_{00}L (5′-GTAGACTGCGTACATGCAG-3′) and E_{00}–M_{00} (5′-GATGAGTCCTGAGTAA-3′). For selective amplification three primer combinations were used: E01 (E_{00}+A)–P16 (P_{00}L+CC); E15 (E_{00}+CA)–M14 (M_{00}+AT); and E15–M23 (M_{00}+TA). The primers P16 and E15 were fluorochrome-labelled IRD700 (Westburg BV, Leusden, The Netherlands). Reactions were performed in 10 µl containing 5 µl aliquot of the pre-amp template (*v*/*v* 1/20), 50 ng unlabelled primer, 0.5 pmol labelled primer, 0.2 mM of all four dNTPs, and 0.2 U Taq polymerase (SuperTaq, Enzym Tech., The Netherlands) in PCR buffer (Superbuffer, Enzym Tech., The Netherlands). From each sample, 0.5 µl was loaded on a 5.5% denaturing polyacrylamide gel (5.5% Gel Matrix, KB Plus, Westburg, The Netherlands), and gel electrophoresis was performed in a Li-Cor DNA Analysis System (Li-Cor Biosc., Lincoln, USA). Images were scored manually for the presence or absence of bands, mainly in the range of 100–500 bp, and the data were transformed into binary matrices.

Cluster analyses were performed in TREECON 1.3b (Van der Peer and De Watcher 1994) using Nei and Li's (1979) dissimilarity coefficient and UPGMA (unweighted pair-group method using arithmetic averages). In comparative studies, the combination of these methods resulted in the best fit of the tree to the distance matrix, expressed by the highest cophenetic correlation (Mace et al. 1999; Koopman et al. 2001). Support values for the nodes in the trees were calculated in 1,000 bootstrap replicates. In each replicate, the original data set is resampled, and a new tree is constructed based on the resampled data set. Subsequently, the bootstrap value for a certain node in the original tree is calculated as the percentage of trees from the resampled data sets that show that particular node (Felsenstein 1985). The data matrices were analyzed separately for each primer combination, and combined for all three primer combinations.

Determination of fungal species

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

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

Screening for resistance in onion and related *Allium* species

Three *Fusarium* isolates were selected to study the response of five onion cultivars and nine accessions from six related *Allium* species (Table 2). The *Fusarium* isolates were selected on the basis of the AFLP phenetic tree. One isolate was taken from each of the major groups, namely UR17-5 and NM2-4 belonging to each clade of *F. oxysporum*, and isolate NL131-2 belonging to *F. proliferatum* (Fig. 1). *Allium* species belong to Section *Cepa*, except *A. schoenoprasum*, which belongs to the closely related Section *Schoenoprasum* (van Raamsdonk et al. 2003). Because of the limited number of available seedlings, *A. schoenoprasum*, *A. pskemense*, and *A. vavilovii* accessions were only tested with isolates UR17-5 and NL131-2.

Seedlings were grown in sterilized pot-soil and transplanted 38 days after sowing to 0.5 l pots containing the same substrate (one plant per pot). The experimental layout consisted of twelve plants per accession-isolate combination, and twelve non-inoculated controls. Plants were randomized within each *Fusarium* treatment, separated from the other treatments to prevent cross-contamination.

Each *Fusarium* inoculum was produced as a suspension of conidia obtained from 10–15 day-old

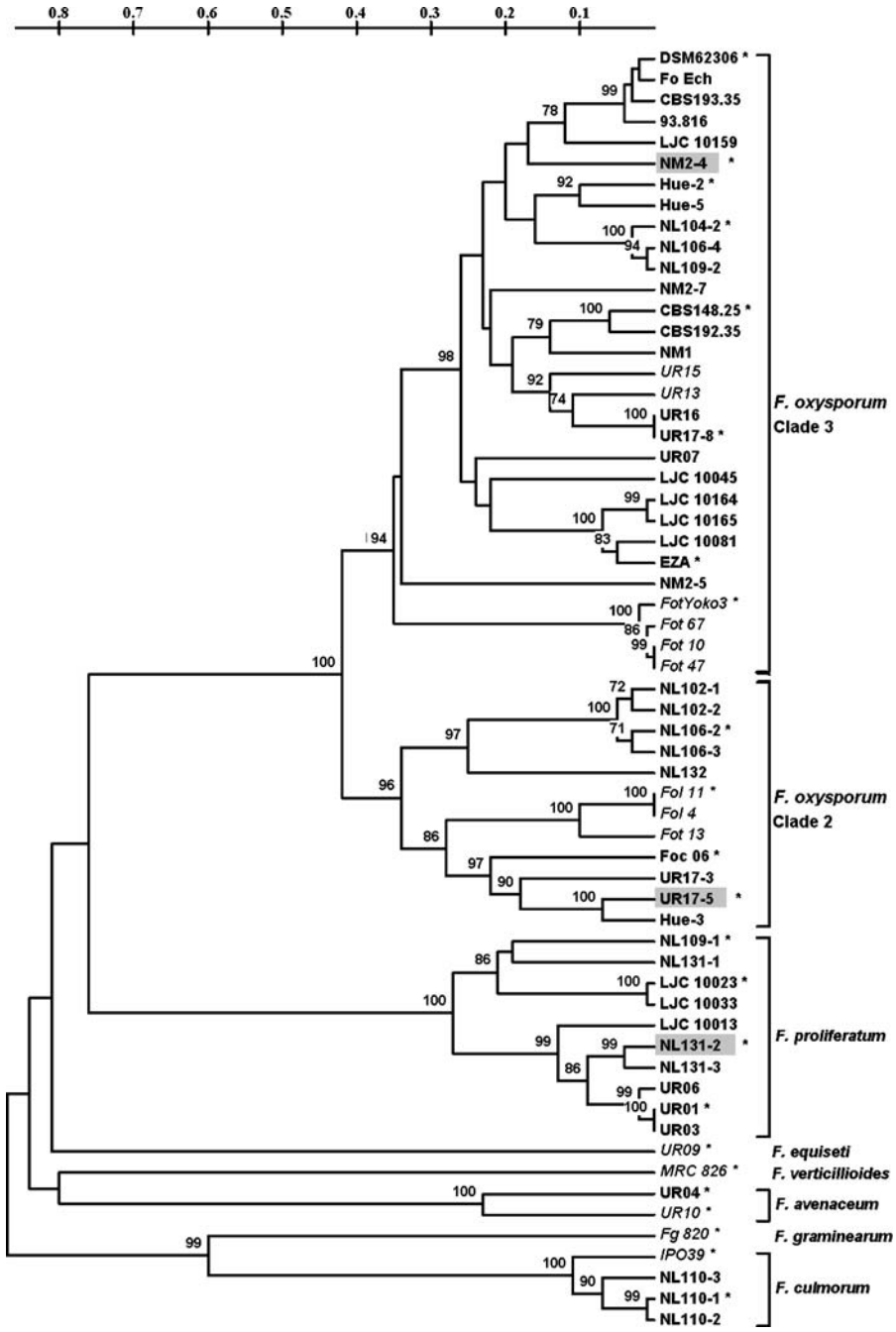

Fig. 1 UPGMA dendrogram of genetic relationships among *Fusarium* isolates, based on AFLP markers from three primer combinations. Bootstrap values >70% (1,000 replicates) are shown above the branches. Nei and Li distances are shown on top. Isolates from onion or *Allium* are presented in *bold* font, and the other ones in *italics*. Isolates used in the screening for resistance are indicated by *grey shaded boxes*, whereas those isolates followed by an *asterisk* were sequenced for the elongation factor 1α gene. *Fusarium oxysporum* Clades were termed according to O'Donnell et al. (1998)

colonies grown on PDA, filtered through cheesecloth, and adjusted to 3×10^5 conidia ml^{-1}. All plants were inoculated twice, 10 and 21 days after transplanting, by pouring each time 40 ml of the suspension into each pot. Average daily temperature during the test ranged from 22 to 27°C.

Plants were harvested by carefully washing the soil from the roots under running tap water. Onion cvs 'Texas E.G. 502' and 'Pantanoso del Sauce CRS' were evaluated 67 days after transplanting, because they showed plant maturity. Other accessions were assessed between 91 and 98 days after transplanting. A disease index (0–3) was established to score the plants based on the necrotic proportion of the basal plate, as follows: 0, no symptoms; 1, slightly infected (<20% of the basal plate infected); 2, moderately

Table 2 *Allium* accessions included in the screening for resistance to *Fusarium* basal rot

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

[a] Onion cultivar types, according to the day length requirement for bulbing. SD: short day (about 10–12 h)
ID Intermediate day (12–13 h); *LD* long day (13–14 h); *LLD* long-long day onion types (>14 h)

infected (20–50%); 3, highly infected (>50%) and rotten bulbs or plants. The presence of *Fusarium* infections in basal plates was confirmed by examination of the developed colonies after incubation in a moist chamber at 27°C during seven days, and observations under the dissection microscope.

Analysis of the disease index data concerned the fitting of a Proportional Odds Model using Genstat 9th Ed. (Lawes Agricultural Trust, Rothamsted Exp. St., UK, 2006). The disease infection was scored on an ordinal scale of four classes. Such ordinal data cannot be analyzed under the assumption of normality. These data can be modelled by reference to an underlying latent variable and threshold values associated with the ordinal scores (Proportional Odds Model, McCullagh and Nelder 1989). These parameters (threshold values and means) were estimated by the maximum likelihood method (Cox and Hinkley 1979) and the result is presented as an analysis of deviance (Table 3). The classes need not to represent equidistant measures of infection. In order to improve the balance in the number of observations among the classes, scores 2 and 3 (moderate and severe infections) were merged into one class. In addition, *A. schoenoprasum* was excluded for its biased scores (as no *Fusarium* infection was scored in this accession).

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

Results

Cluster analysis of *Fusarium* isolates

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

Table 3 Accumulated analysis of deviance for the distribution over disease index classes (Proportional Odds Model), testing the effects of *Allium* accessions, *Fusarium* isolates and their interaction

Sequencially added terms to the model	*df*	Deviance	Mean deviance	Deviance ratio	Chi-square
Fusarium isolates	2	7.2	3.60	3.60	0.027
Allium accessions	12	140.6	11.72	11.72	<.001
Isolate × accession	16	27.8	1.74	1.74	0.034
Residual	30	39.0	1.30		
Total	60	214.6	3.58		

and were included initially as controls. This group, with bootstrap values 100, 100, and 99% for the individual primer combinations, was confirmed as *F. oxysporum* by morphologic characteristics, by testing with the species-specific primers, and by sequencing the EF-1α gene.

Within *F. oxysporum*, isolates clustered in two main clusters supported by the topology of all three primer combinations. Isolates obtained in Uruguay were present in both clusters, as well as isolates obtained in The Netherlands (Fig. 1). Some closely related onion isolates (Nei and Li distance <0.10) originated from the same region: e.g. isolates NL102-1, NL106-2, -3 (The Netherlands). However, isolates originating from different countries also showed high genetic similarity, e.g. EZA (Australia) and LJC-10081 (Argentina). In addition, high similarity was found for some isolates obtained from different host plants and locations, e.g. UR17-5 (onion, Uruguay) and Hue 03 (garlic, Spain).

The *F. proliferatum* group (bootstrap 100% in each primer combination) consisted of 10 onion isolates originating from Argentina, Uruguay, and The Netherlands. Species identification was confirmed by morphologic characteristics, species-specific primers, and sequencing the EF-1α gene. The isolates clustered closely together (largest Nei and Li distance, 0.27). *Fusarium proliferatum* isolates were obtained from infected seeds, and from infected basal plates of bulbs collected in the field and after storage. When collecting bulbs it was not possible to discriminate between symptoms caused by *F. proliferatum* or *F. oxysporum*.

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

Resistance in *Allium* species

Three *Fusarium* isolates were used to screen for levels of resistance in onion cultivars and six related *Allium* species. For the *Allium* accessions, large and significant differences were observed in the overall distribution of the number of plants over the disease index (DI) scores (Table 3, Fig. 2). In addition, significant differences between isolates and significant isolate x accession interactions were observed, but not as apparent as the difference between *Allium* accessions (Table 3). The aggressiveness of each isolate was very much dependent on specific isolate-accession combinations, although the onion cv. 'Rijnsburger' was the only one with a distribution of DI scores significantly different between isolates (Table 4).

In general, *A. schoenoprasum* and the four accessions of *A. fistulosum* had consistently the lowest DI scores when evaluated for their resistance to the three *Fusarium* isolates (Table 4, Fig. 2). On the other extreme of the spectrum, four of the five onion cultivars and *A. vavilovii* had consistently the highest DI scores. Among them, onion cv. 'Pantanoso del Sauce' was the most diseased, being different from all *A. fistulosum* accessions for the three isolates. Interestingly, variation in DI scores was found within the onion germplasm tested, as cv. 'Rossa Savonese' contained plants resistant to all three *Fusarium* isolates. Considering the resistance observed in other wild relatives of onion, namely *A. roylei*, *A. pskemense*, and *A. galanthum*, DI scores were intermediate between those of *A. fistulosum*–*A. schoenoprasum*, and those of the four susceptible onion cultivars.

The mean number of roots for each accession in the controls was found to be negatively correlated with the

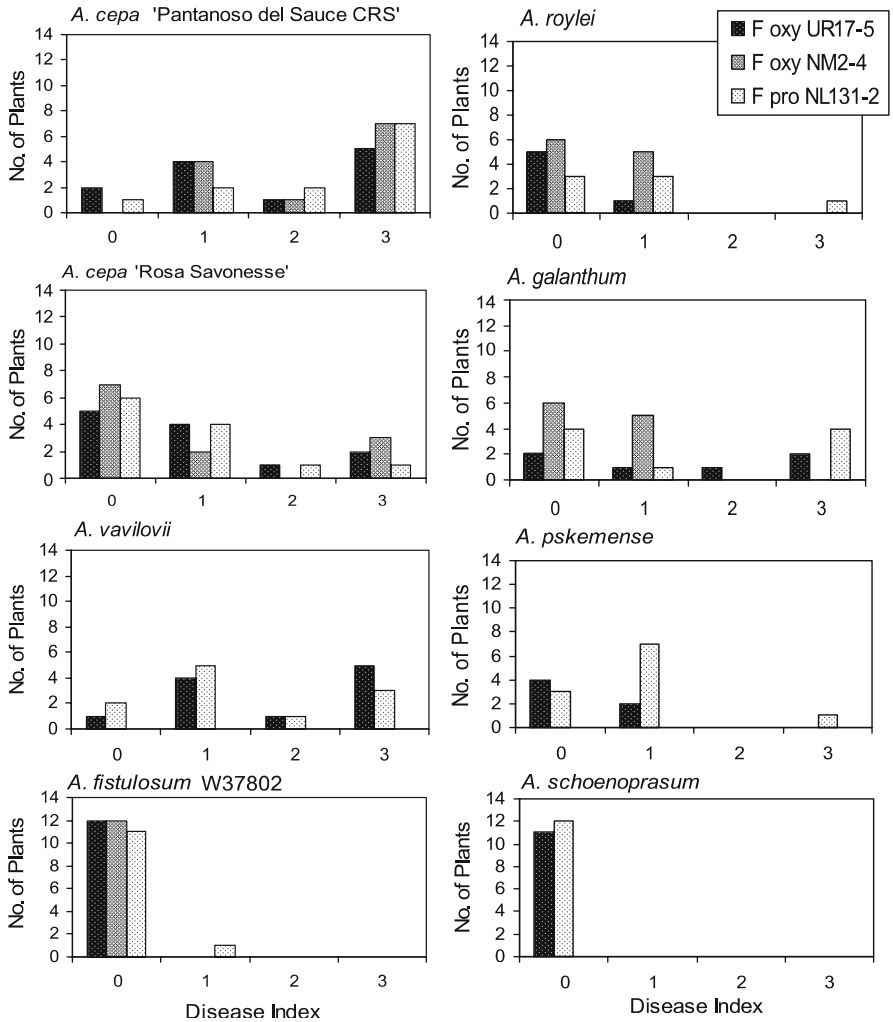

Fig. 2 Division of numbers of plants per *Allium* accession scored in each disease index (*DI*) class after inoculation of individual plants by *Fusarium oxysporum* isolates UR17-5 and NM2-4, and *F. proliferatum* NL-131-2. DI classes are based on the estimation of the affected proportion of the basal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

disease indices (linear regression, data not shown; NL131-2, $P<0.001$; NM2-4, $P<0.065$; UR17-5, $P<0.016$). For example, large numbers of roots were found in *A. fistulosum* accessions (29 roots per plant on average) and *A. cepa* cv. 'Rossa Savonese' (28 roots per plant), whereas 'Pantanoso del Sauce', 'Texas E.G. 502', 'Rijnsburger', and 'Jumbo' had 8, 8, 10, and 14 roots per plant respectively.

Discussion

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

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

Allium accessions	Number of plants	Groups[a]								
NM 2-4 (*Fusarium oxysporum* Clade 3)[b]										
A. fistulosum W37802	12	a								
A. fistulosum W00501	12	a								
A. fistulosum PRI97166	12	a	b							
A. fistulosum UR2003-1	12	a	b	c						
A. galanthum	11	a	b	c						
A. roylei	7	a	b	c						
A. cepa cv. 'Rossa Savonese'	12		b	c						
A. cepa cv. 'Texas E.G. 502'	12			c	d	e	f	g		
A. cepa cv. 'Rijnsburger'	11				d	e	f	g	h	i
A. cepa cv. 'Pantanoso del Sauce'	12							g	h	i
A. cepa cv. 'Jumbo'	12								h	i
UR17-5 (*Fusarium oxysporum* Clade 2)										
Allium schoenoprasum[c]	11	–								
A. fistulosum W37802	12	a								
A. fistulosum UR2003-1	12	a								
A. fistulosum W00501	12	a								
A. roylei	6	a	b							
A. pskemense	6	a	b	c						
A. fistulosum PRI97166	12	a	b	c						
A. cepa cv. 'Rossa Savonese'	12		b	c	d					
A. cepa cv. 'Jumbo'	12			c	d	e	f	g	h	
A. galanthum	6			c	d	e	f	g	h	i
A. cepa cv. 'Pantanoso del Sauce'	10				d	e	f	g	h	i
A. vavilovii	10					e	f	g	h	i
A. cepa cv. 'Texas E.G. 502'	12							g	h	i
A. cepa cv. 'Rijnsburger'	11									i
NL131-2 (*Fusarium proliferatum)*										
Allium schoenoprasum[c]	12	–								
A. fistulosum W00501	12	a								
A. fistulosum UR2003-1	12	a								
A. fistulosum W37802	12	a								
A. fistulosum PRI97166	12	a	b	c						
A. cepa cv. 'Rossa Savonese'	12		b	c						
A. roylei	7		b	c	d					
A. pskemense	11		b	c	d	e				
A. cepa cv. 'Rijnsburger'	12			c	d	e	f			
A. galanthum	9			c	d	e	f			
A. vavilovii	11			c	d	e	f	g	h	
A. cepa cv. 'Texas E.G. 502'	12						f	g	h	i
A. cepa cv. 'Pantanoso del Sauce'	12								h	i
A. cepa cv. 'Jumbo'	12								h	i

[a] Combinations in a group with the same letter do not differ mutually. Comparisons between combinations with no letter in common differ significantly at the level of 5%, ranking from most resistant (level a) to most susceptible (level i) reactions.

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

[c] Because of the requirements of the statistical model, *A. schoenoprasum* was not included for its biased scores (no *Fusarium* infected plants were observed in this accession).

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

Fusarium oxysporum is assumed to be the causal agent of onion basal rot, and was indeed predominantly present in the collection. Our results showed a significant diversity among *F. oxysporum* isolates from onion, grouped in two main clades, namely Clade 2 and Clade 3 (O'Donnell et al. 1998), as supported by the position of isolates Fol4 in Clade 2, and Fol10 in Clade 3 determined earlier by Baayen et al. (2000a) (Fig. 1). Some *F. oxysporum* isolates collected in one region were found to cluster closely together, suggesting a clonal origin (e.g., NL109-2, NL106-4). In contrast, diversity was also observed at the regional level. For example, isolates from a sampled region fell in different *F. oxysporum* clades (e.g., NL109-2, NL106-3), leading to genetic variation in a single location or a single field. The finding of multiple vegetative compatibility groups (VCG) within f. sp. *cepae* (Swift et al. 2002), likewise, indicated genetic variation in this forma specialis, because VCG can be considered as distinct clonal lineages in a *F. oxysporum* population (Kistler 1997).

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

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

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

Fusarium culmorum, found on a farm in The Netherlands, has been reported before as a pathogenic species of onion in Mexico (Montes et al. 2003). In leek (*Allium porrum* L.), *F. culmorum* is known to cause leek rot disease (Blancard et al. 2003; Koike et al. 2003). *Fusarium avenaceum* is known to cause rot of pumpkin fruits (*Cucurbita* sp.), which are cultivated in rotations with onion and garlic (P.H. González, Uruguay, unpublished data). It is not known to what extent *F. culmorum* and *F. avenaceum* could be relevant species in the basal rot disease of onion, or merely if they have onion as an alternative host (Dhingra and Coelho 2001).

In order to test the resistance of onion cultivars and *Allium* species, we selected for a first and general screening one strain from each *F. oxysporum* subgroup, and one from *F. proliferatum*. All tested onion cultivars appeared susceptible to *Fusarium* basal rot, with moderate to high levels of infections on average. An exception was 'Rossa Savonese', confirming results obtained by Özer et al. (2004). The high number of roots per plant of this accession might partially explain this response. Every onion accession had also plants without any infection. As onion is an outcrossing crop, each accession is genetically heterogeneous, which may explain the finding of resistant plants within the set of cultivars tested. In that case, recurrent selection of non-infected plants may gradually result in populations with larger proportions of plants resistant to *Fusarium.* This approach has resulted in the successful development of selections with higher levels of resistance (Gutierrez and Cramer 2005; Cramer 2006). Alternatively, non-infected plants may be the result of escapes from infection during the screening assay.

Allium fistulosum accessions were affected at a very low incidence. These results are in agreement with Holz and Knox-Davies (1974), who found very low levels of infection in *A. fistulosum* caused by a *F. oxysporum* f. sp. *cepae* isolate, compared to a set of onion cultivars. One of the differences between *A. cepa* and *A. fistulosum* concerns the rooting system,

which is much denser and larger for the latter (De Melo 2003). *Allium fistulosum* also differs from *A. cepa* in the lack of bulbing and dormancy. These morpho-physiological differences might influence the response to *Fusarium* basal rot, leading to mechanisms of escape. *Allium fistulosum* keeps actively growing, developing new roots, and as a result may overcome *Fusarium* root infections. In contrast, *A. cepa* stops growing after bulb formation, and root and basal plate tissues become senescent. However, the complete absence of *Fusarium* symptoms in a large number of *A. fistulosum* plants may suggest the presence of true resistance against *Fusarium* isolates from onion.

High level of resistance was also found in *A. schoenoprasum*, where only root infections were observed. Intermediate levels of resistance were found in *A. roylei*, *A. galanthum*, and *A. pskemense*. *Allium vavilovii*, which is closely related to the cultivated *A. cepa* (Van Raamsdonk et al. 2003), showed a larger proportion of susceptible plants. This accession has a denser root system than onion, showing that plants with a larger and denser rooting system may also be susceptible.

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

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