Aspects of resistance of flax and linseed (*Linum usitatissimum*) to *Fusarium oxysporum* f.sp. *lini*

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Aspects of resistance of flax and linseed (*Linum usitatissimum*) to *Fusarium oxysporum* f.sp. *lini*

Aspecten van de resistentie in vezel- en olievlas (*Linum usitatissimum*) tegen *Fusarium oxysporum* f.sp. *lini*

Ineke Kroes

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Bibliographic abstract
In the thesis aspects have been described of the flax and linseed interaction to *Fusarium oxysporum* f.sp. *lini,* the causal agent of flax wilt. Two *in vitro* tests were established to screen for resistance, to investigate race specificity and to study infection and colonization patterns in a resistant and a susceptible cultivar. The fungus appeared to colonize the cortex, causing root rot initially, followed by colonization of the protoxylem vessels. Several aspects of quantitative screening for resistance were investigated by visual disease screening, physiologic parameters, and ergosterol measurements. The results indicated that symptoms are related to resistance rather than tolerance. Race specificity was studied *in vitro,* and in an international field experiment performed at six locations in Europe and three in North America. Although significant genotype * isolate interaction and significant genotype * environment interaction was observed, possible races of the fungus could not be identified. No indication was obtained for race-specific resistance in flax and linseed.

Key words: AMMI, colonization, ergosterol, flax, *Fusarium oxysporum* f.sp. *lini,* infection, interaction, linseed, *Linum usitatissimum* L., race-specificity, races.

Foto omslag: Bloeiend vezelvlas ('Diane') aan de Kanaalkust in Normandië

Spreuken 17: 22

Vrolijkheid geneest je,
Neerslachtigheid verslindt je krachten....

Stellingen

1 Voor waardplant-bodempathogeen interacties bestaat een positieve correlatie tussen de mate waarin het omringende milieu deze interacties beinvloedt en het nut van het ontwikkelen van betrouwbare *in vitro* resistentietoetsen voor dezelfde interactie.

2 Ergosterolbepaling in planten als maat voor resistentie tegen schimmelziekten dient vermeden te worden vanwege de onbetrouwbaarheid van de resultaten en de grote milieubelasting die het met zich meebrengt.

3 Gen-om-gen interacties tussen pathogeen- en waardplantgenotypen veroorzaken altijd fysiospecificiteit, maar niet altijd fysio's.

4 *Fusarium oxysporum* f.sp. *lini* is primair een rotschimmel.

5 Het publiceren in een, voor de meeste wetenschappers, weinig toegankelijke taal, kan tot gevolg hebben dat foutief citeren en plagiaat in de hand gewerkt wordt.

6 De waarde van het huidig onderzoek wordt vooral bepaald door de snelheid waarmee resultaten verkregen worden en door het prijskaartje dat eraan hangt. Daarom heeft het weinig zin zich als toekomstig onderzoeker druk te maken over het al dan niet erudiet zijn.

7 Van de menselijke drang naar het verkrijgen van gelijke rechten zijn diegenen de dupe die hun rechten niet kunnen of niet mogen definiëren.

8 Zolang de prijs van duurzaam geproduceerd tropisch hardhout nog ruim 50% hoger ligt dan die van niet duurzaam geproduceerd tropisch hardhout, hoeft men zich geen enkele illusie te maken over het succesvol introduceren van duurzame landbouwmethoden in de derde wereld.

9 Verzorgende beroepen worden in de huidige maatschappij ernstig ondergewaardeerd.

10 Door de eeuwen heen is door de maatschappij een dusdanig groot *beroep* gedaan op huisvrouwen dat het zijn van huisvrouw de status van **beroep** verdient.

11 Door de mens niet verstoorde natuurontwikkeling is in Nederland onmogelijk en wordt vaak ten onrechte als term gebruikt voor het aanleggen en/of ontwikkelen van natuurlijk uitziende parken.

Stellingen behorende bij het proefschrift, getiteld "Aspects of resistance of flax and linseed (*Linum usitatissimum*) to *Fusarium oxysporum* f.sp. *lini* ", door G. M. L. W. (Ineke) Kroes

Wageningen, 15 december 1997

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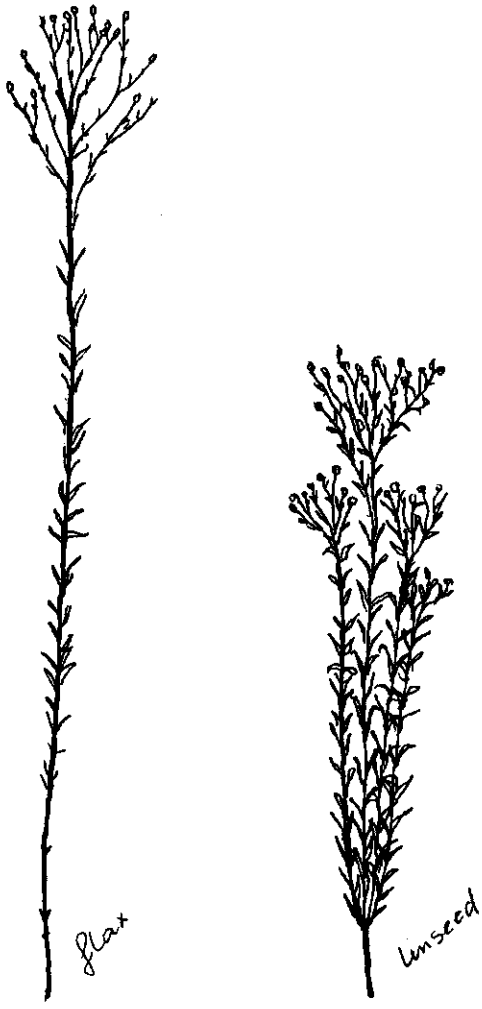
CHAPTER 1

General introduction



FLAX AND LINSEED

Origin and history

Flax (*Linum usitatissimum* L.) is one of the earliest cultivated plant species. The progenitor of small seeded flax is considered to be *L. bienne* L., originating from Kurdistan and Iran, while a distinct type of *L. bienne* with high oil content and high seed weight, is supposed to be the progenitor of flax originating from the Mediterranean region (Murre, 1955, Zeven and De Wet, 1982). The latter is also described as *L. angustifolium.* These species all belong to the family of the *Linaceae.* Flax is the only member of this family with economic importance, except for a few species that are grown as ornamentals. The crop is annual and self-pollinating.

Flax must have been domesticated before 6200 BC in the Mediterranean coastal area and in Turkestan, Afghanistan, India and South Russia (Van Zeist and Bakker-Heeres, 1975; Gill, 1987). About 5000-3000 BC semi-nomads, originating from the Middle East settled in Flanders and introduced flax cultivation. This is considered to be the beginning of flax culture in The Netherlands, Belgium, Northern France and Switzerland. (Dewilde, 1983). Since flax was domesticated, there has been a preference for growing the crop for fiber (linen) in the Western region and for linseed oil in the Eastern region of Eurasia (Gill, 1987). The two types of the crop differ much in agronomic characters. Compared with flax grown for fiber, flax grown for linseed oil has a shorter statue, more branching and a later harvest time. Often the name flax is used for the type grown for fiber, while the other type is called linseed. This nomenclature will also be used in the present thesis.

In the ancient Egyptian (2400-2200 BC), Greek and Roman cultures an advanced linen industry was established. Wearing "linen cloth" was considered to be a sign of aristocracy. The ancient Egyptians used linen for wrapping the royal mummies, additional to embalming the bodies of the deceased pharaohs with linseed oil. In The Netherlands and most likely also in Belgium and Northern France flax has been grown since the ancient times (Dewilde, 1983). In the book Historia Naturalis (29 BC), Plinius praised the

quality and fineness of the linen, originating from these regions (Bostock and Riley, 1856) and nowadays the finest quality linen still originates from Western Europe (Hayward, 1967). Flax fibers are known since 1200 as a source for paper industry (Murre, 1955).

In linseed a distinction has to be made between edible linseed having a high content of linolic acid and a low content of linolenic acid, and non-edible linseed with a high contents of linolenic acid and a low contents of linolic acid. From 700 BC linseed is known to be edible. Historically its flour has been used in cakes (Murre, 1955). Seed extracts were used as a domestic remedy against several illnesses. Even at present the consumption of linseed is considered to improve health.

Products of flax and linseed also are known to be used in the world of art. Since about 1200 (Murre, 1955) the most important base of paints was linseed oil (Jaxtheimer, 1984). For instance, many "old masters paintings" from Rembrandt, Vermeer and later also Van Gogh and Gaugain, were painted with linseed oil based paints, on linen textile. About 150 years ago "linoleum", a floor covering based on non-edible linseed, came on the market. Linseed, containing a high proportion of linolenic acid is the base of environmentally friendly paints nowadays. So, the species name given by Lineaus, *Linum usitatissimum*, which means useful flax is very much to the point.

The market

Flax

Flax has always been an important crop for The Netherlands, with an acreage of about 30.000 ha since the beginning of this century, and an established linen market. The situation of the market for linen changed, when enormous amounts of low price Russian flax were introduced on the West European market in the late 1950. Next, the introduction of synthetic fibers, textiles and textile products, combined with strongly reduced cotton prices, led to a collapse of the linen market. Consequently the flax acreage in The

Netherlands decreased dramatically to about 3.000 ha in 1980. In 1996 the acreage was 3823 ha (Kozlowsky, 1997).

In the last decades the market of agricultural products in the food sector stagnated in the countries belonging to the European Union (Riensema *et al.*, 1990). This led to a re-orientation of the agricultural sector. At the same time, society started to demand a more environmental friendly way of agricultural production in Europe. Thus, the interest grew for so-called "alternative" crops, new or rarely grown crops which can be cultivated by farmers in addition to or instead of the few major crops. Much research has been initiated since to develop new industrial crops, and to develop markets for their products (Riensema *et al.*, 1990; Van Soest, 1990, 1994a, 1994b; Van Dam *et al.*, 1994; Van Kemenade *et al.*, 1996). Among the crops investigated, flax is considered to be one of the most promising crops for Europe because, besides its established market for linen clothing, it has a broad range of utilization possibilities for new markets (see Table 1.1). Furthermore, flax growing cultivation does not need much fertilizers and agrochemicals, and therefore it has an environmental friendly way of production (Vreeke *et al.* 1991). In 1996, 134.000 ha flax was grown in Western Europe. The countries with acreages of more than 10.000 ha in 1996 were France (44.500 ha), Spain (44.000 ha), The United Kingdom (20.500) and Belgium (11.200 ha). In other parts of the world, the countries with acreages of more than 10.000 ha in 1996 were Russia (135.000 ha), Belarussia (78.500 ha), Ukraine (54.500 ha) and Egypt (20.000 ha) (Kozlowsky, 1997).

Linseed

Although the interest in Western Europe in growing linseed always has been much lower than for flax, the production of linseed reached high levels in other parts of the world. In the late 1700s in the USA and Canada, the first crop that was grown on new land was usually flax. After the World War II a large demand for linseed oil developed, with a top of 2.28 million ha

Table 1.1

Plant fibers used or studied in different applications. The numbers represent the amount of different applications used or studied per plant fiber, whereby price of the fiber, technical and chemical properties, performance and environmental aspects were selection criteria (Van Dam, 1994).

Fibers	Matrix composites	Textile	Pulp and paper	Geo-textiles	Non-wovens
Flax	26	14	14	3	5
Hemp	7	5	7	1	3
Kenaf	2	0	7	1	1
Miscantus	9	0	7	0	1
Jute	16	5	2	1	0
Abaca	3	1	8	1	0
Sisal/Agave	10	4	5	0	1
Coir	3	0	2	1	1
Cotton	15	10	10	0	1
Straw (cereals)	8	0	10	0	3
Wood	7	0	0	0	0

grown in the USA and 1.2 million ha in Canada. Since the late 1950s the demand and production of linseed steadily declined (Comstock, personal communication). In 1996 about 600.000 ha linseed was grown in Canada and 120.000 in the USA.

Breeding and cultivation

Except for breeding for the usual goals, such as high yield, lodging resistance, disease resistance, etc. flax and linseed breeding programs focus on various other traits. There are distinct breeding programs for long and fine fibers (textile purpose), for edible linseed (reform-food industry), for non-edible linseed, (paints and linoleum), and breeding for "double-purpose flax", short and low weight fibers for geo-textiles and non-wovens combined with high yields of linseed (Riensema *et al.*, 1990). Most of the flax and linseed

Table 1.2

Diseases of flax and linseed, the causal pathogens and the disease characteristics; economic importance, appearance during the growth season, the way of transmission and the nature of the causal organism.
Abbreviations: ma = main disease, int = disease of intermediate importance, mi = minor disease, e = appearance in seedling stage, m = appearance in flowering stage, l = appearance in adult stage, se = seed-borne disease, so = soil-borne disease, ai = air borne disease, tr = transmission by grasshoppers, (fac) = facultative parasite ; F = fungal disease, V= viral disease.
Sources: Diddens, 1931; Muskett and Colhoun, 1947; Plonka and Anselme, 1956; Verhoeven, 1961; Decognet, 1994; Anonymus, 1996; Malone and Muskett, 1997

Disease	Pathogen	Disease characteristics			
		importance	appearance	transmission	organism
Fusarium wilt	*Fusarium oxysporum* f.sp. *lini* (Bolley) Schnyder & Hansen, Schlecht.	ma	eml	so/se	F
Fusarium stem break	*Fusarium* spp.	mi	e	se	F
Rust	*Melampsora lini* (Ehrenb.) Lév.	ma	eml	ai	F
Scorch	*Pythium megalacanthum* de Bary	ma	ml	so	F
Damping off	*Pythium* spp.	mi	e	so	F
Seedling blight	*Colletotrichum linicola* Pethybridge and Lafferty	int	e	se	F
Stem-break, browning	*Polyspora lini* Lafferty	mi	eml	se	F
Foot rot	*Phoma* spp.	int	e	se	F
Grey mould	*Botrytis cinerea* Persoon ex Fries	int	ml	se/ai (fac)	F
Powdery mildew	*Oidium lini* Škoric	int	ml	ai	F
Sclerotinia	*Sclerotinia sclerotiorum* Massee	mi	eml	so	F
Rhizoctonia	*Rhizoctonia solani* Kühn	mi	e	so	F
Root rot	*Thielaviopsis basicola* Ferraris	mi	em	so	F
Alternaria	*Alternaria linicola* Groves and Skolko	mi	e	se	F
Pasmo	*Sphaerella linorum* Wollenweber	int	eml	se	F
Aster Yellows	micoplasma-like organism	mi	l	tr	
Crinkle	oat blue dwarf virus	mi	l	tr	V

breeding programs follow traditional methodology, i.e. crossing, followed by a selection process up to the sixth selfed generation (F6), followed by three or four multiplication steps. However, methods for genetic transformation and *in vitro* regeneration are available (Mlynárová *et al.*, 1994) and the development of transgenic linseed has been successful (McHughen and Holm, 1995). The possibilities of doubled haploid techniques, to avoid the time consuming selfing process, have been studied (Nichterlein and Friedt, 1993), and this breeding method is available and operating.

Flax and linseed cultivation requires a fine regular soil structure and a good drainage, to be able to sow early. The crop needs a low nitrogen level; sandy clay or loam is the best soil for growing flax. Flax does well after cereals, maize or leguminoses, but after potatoes or sugar beets the soil may be too loose and *Rhizoctonia* may become a problem (Anonymus, 1996, Vreeke *et al.*, 1991).

The main flax pathogens are summarized in Table 1.2. Furthermore, other parasites like thrips (*Thrips angusticeps* Uzel, *Thrips linarius* Uzel), flea-beetles (*Longitarsus parvulus* (Payk.), *Aphthona euphorbiae* (Schrank)), leaf rollers (*Cnephasia communaria* (H.-Sch.)) and nematodes (*Ditylenchus dipsachi* Kuehn), *Meloidogyne hapla* (*Chitwoodi*) may sometimes cause problems in flax growing (Verhoeven, 1961). Therefore a crop rotation is recommended of 6-7 years.

Breeding for disease resistance

World wide, breeding programs for disease resistance in flax and linseed focus on resistance to flax rust (*Melampsora lini*) and *Fusarium* wilt (*Fusarium oxysporum* f.sp. *lini*). Resistance to these diseases is very important to get a new cultivar accepted on the list of recommended cultivars in all parts of the world. In addition, in Western Europe resistance to scorch (*Pythium megalacantum*) is highly desirable for a new cultivar. For the other diseases the control is realized by seed treatment, crop rotation, and/or

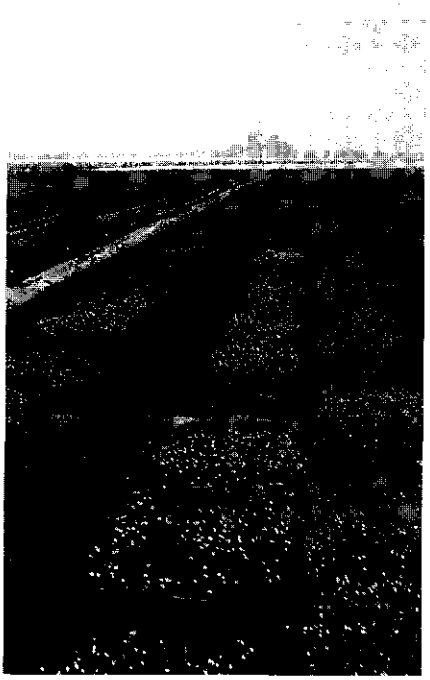


Figure 1.1

Flax wilt nursery in Normandy, France. Photo obtained from the Coopérative Linière de Fontaine Cany, Fontaine le Dun.

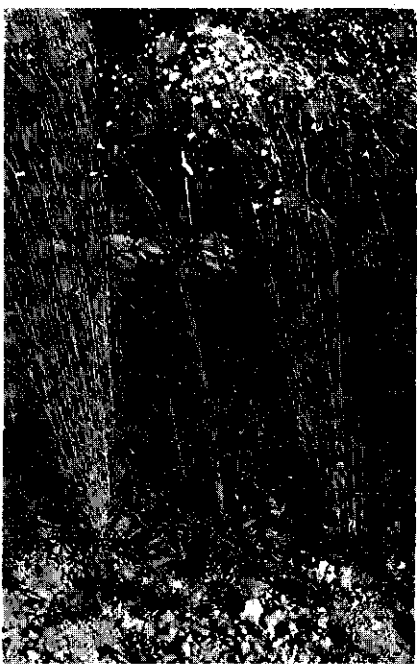


Figure 1.2

Resistant and susceptible flax in the flax wilt nursery in Normandy, France. Photo obtained from the Coopérative Linière de Fontaine Cany, Fontaine le Dun.

sometimes field applications of fungicides (Vreeke *et al.*, 1991, Anonymus, 1996).

Flor (1940) studied both *Fusarium* wilt and flax rust. Based on the studies with flax rust the famous gene-for-gene theory was formulated (Flor, 1955, 1956). The genes involved in the resistance to flax rust are well described now, and cultivars with resistances against all rust races are available.

The genetics and resistance mechanisms of flax wilt were never fully described, although high levels of resistance in flax to *Fusarium oxysporum* f.sp. *lini* have been obtained by recombination and selection (Amy, 1936). Selection for *Fusarium* resistance occurs in a relatively late stage of the

breeding program, in the F5 or F6 generation. For screening, the use of nurseries infested with *Fusarium oxysoprum* f.sp. *lini* is common (Fig. 1.1).

***Fusarium* wilt**

In 29 BC Plinius was the first to note that flax was, "scorching the ground where it is grown and (of) deteriorating the very soil itself" (Bostock and Riley, 1856). Early work on flax in Flanders (Nypels, 1897) indicated that this exhaustion was caused by various diseases, including "vlasbrand" (scorch), "dode harrel" (foot rot), and "kouterplaag" (top death of flax). The symptoms described for "kouterplaag" are very similar to the symptoms of flax wilt (Barker, 1923). The causal agent was assumed to be a fungus called *Fusicladium lini* n.sp. Sorauer (Nypels, 1897). In the first half the 20th century flax wilt was never a serious threat in the flax growing areas of Western Europe, and relatively little West European literature on the subject is available (Friederich, 1962). Around 1970 an outbreak of the disease caused great losses in Normandy, France and at the same time in Groningen, in the north of The Netherlands. Selection for *Fusarium* wilt resistance on diseased fields in North Groningen, The Netherlands, (Trip, personal communication) led to 'Natasja', the first wilt resistant flax cultivar in Europe.

Early work on flax wilt in the USA dates from 1889 by Otto Lugger, in Minnesota, USA. Lugger (1890) discovered that old flax straw could be the cause of wilt in hitherto wilt free fields, and concluded that the straw itself was the cause of flax wilt, instead of an assumed exhaustion of the soil. Snyder (1896) came to the same conclusion. In Japan two reports mentioned a *Fusarium* species, causing flax wilt (Hiratsuka 1897; Tochinai, 1925). In 1901 Bolley demonstrated that flax wilt was caused by a fungus, which he called *Fusarium lini.* This name changed in 1940 in *Fusarium oxysporum* f.sp. *lini* (Snyder and Hansen, 1940). Bolley established the first flax wilt nursery, "Plot 30" in Fargo, North Dakota, USA in 1894, while in 1913 a plot at the University of Minnesota, St. Paul, USA was inoculated with *Fusarium oxysporum* f.sp. *lini.* Selection on these plots led to 'Chippewa' and 'Winota',

the first wilt resistant linseed cultivars (Arny, 1936). The first reports of flax wilt occurrence in Australia date from 1913 (Millikan, 1951), for India from 1923 (Gill, 1987), for Northern Ireland from 1926 (McKay, 1947) and for Great Britain from 1941 (Wilson, 1944). In Eastern Europe the literature is quite difficult to access but in 1973 the disease is mentioned in a Russian publication (Dudin and Sysoenko, 1973). Flax wilt is well known now in all main flax and linseed growing countries and may cause severe losses.

Fusarium oxysporum f.sp. *lini* belongs to the Deuteromycetes (*Fungi Imperfecti*), section *Elegans* (Snyder and Hansen, 1940). The fungus is haploid and can produce three types of asexual spores, uni- (bi-)cellular microconidia, multicellular 4-5 septate macroconidia and chlamidospores. Chlamidospores can survive for a long period in the soil. Houston and Knowles (1949) reported a fifty year survival of *Fusarium oxysporum* f.sp. *lini* in the soil in the absence of flax culture. The host range of the fungus is restricted to flax and linseed (Borlaugh, 1945; Davis, 1967).

The main route of infection is through the roots (Nair, 1956).) Boyle (1934) described root rot and wilt in flax, both caused by *Fusarium oxysporum* f.sp. *lini*. Turlier (1994) described a model of infection and colonization of the pathogen in flax root tissue, slightly deviant from most models for *Fusarium* infection and colonization. Wilt symptoms are classified into early wilt, late wilt, partial wilt and unilateral wilt (Kommedahl *et al.*, 1970). The symptoms can appear through the whole growing season, starting with bending of the top (wilting), followed by yellowing of the leaves, often unilateral, then necrosis of the leaves and finally the death of the plant (Fig. 1.2).

Temperature appears to be the most important environmental factor affecting wilt, and a temperature of 25-28 °C is mentioned to be the optimum for wilt development (Tisdale, 1917). At temperatures of 7-12 °C the fungus germinates slowly (Broadfoot, 1926). The effect of temperature may vary with the cultivars and with the genotypes of the fungus (Kommedahl *et al.*, 1970). Low soil moisture might stimulate the development of wilt but the relationship

is not clear. Soil type does not seem to influence the incidence of wilt, since wilt has been found on most soil types (Bolley, 1901; Kommedahl *et al.*, 1970). Greenhouse experiments in Fargo, North Dakota, using soil from the flax wilt nursery "Plot 30", indicated that the amount of inoculum in the soil need not to be high to cause flax wilt (Kommedahl *et al.*, 1970). However, Nair (1956) illustrated that an increase of inoculum resulted in an increase in the incidence of wilted plants. Fertilizers may have no direct effect on the wilt fungus (Kommedahl *et al.*, 1970). Seed quality seems to be of importance. The use of damaged seed stimulates the development of the fungus in the seedling stage (Nair and Kommedahl, 1957; Anonymus, 1996).

Contradictory results were reported concerning the inheritance of flax wilt resistance and the durability of the flax wilt resistance (Tisdale, 1917; Nelson and Dworak, 1926; Burnham, 1932; Knowles and Houston, 1955; Knowles *et al.*, 1956; Kommedahl *et al.*, 1970; Kamptham *et al.*, 1981; Pavelek, 1983; Goray *et al.*, 1987, Popescu, 1995) but in the majority of the reports the resistance was mentioned to inherit quantitatively. Popescu (1995) concluded that both additive and non-additive effects are involved in the genetics of linseed resistance to *Fusarium* wilt, but that additivity seems to be predominant.

OUTLINE OF THE THESIS

In recent years some Dutch flax cultivars were observed to be less resistant to *Fusarium oxysporum* f.sp. *lini* when grown in France, compared with results obtained six years earlier (Trouvé, unpublished results). This was considered to be an indication that a new race of the fungus might have developed. Therefore investigations concerning the existence of races in the fungus, were started to obtain more detailed knowledge of this host-pathogen relationship.

In the past, screening methods in flax wilt nurseries proved to be too variable to be able to detect race specificity (Kommedahl *et al.*, 1970). For that reason two *in vitro* screening methods were developed (Chapter 2).

Because of the contradictions in published reports concerning histological observations of infection and colonization, as well as the possible existence of root rot caused by the same fungus (Tisdale, 1917; Boyle, 1923; Nair 1951; Turlier *et al.*, 1994), a detailed study of infection and colonization in a resistant and a susceptible flax cultivar was performed. The results are described in Chapter 3. Screening methods for disease resistance are mostly based on secondary characteristics, which can cause difficulties in the precise determination of the nature of the resistance of a genotype. This is also the case in *Fusarium* wilt in flax and linseed. Therefore, the relationship was studied between parameters which show the amount of damage, like disease index, dry weight, and length reduction, and parameters which show the actual presence and quantity of the fungus, the amounts of ergosterol and fusaric acid, produced by *Fusarium oxysporum* (Chapter 4). With the help of one of the *in vitro* screening tests, the evaluation of the possible existence of races in the fungus and race specificity in the host could be studied in more detail (Chapter 5). In Chapter 6 the results are presented of a world-wide field experiment to study host * pathogen * environment interaction.

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CHAPTER 2

Two *in vitro* assays to evaluate resistance in *Linum usitatissimum* to *Fusarium* wilt disease

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Submitted

ABSTRACT

Two types of *in vitro* seedling tests were developed to evaluate resistance in flax (*Linum usitatissimum*) against *Fusarium oxysporum* f.sp. *lini.* In the first test a solid medium was used, seedlings were grown in test tubes containing vermiculite. The second test was based on a liquid medium, seedlings were grown in two-liter preserving jars of which the inner walls were lined with filter paper. Both systems contained a 10% MS-nutrient solution. The seedlings were inoculated with spore suspensions of *Fusarium oxysporum* f.sp. *lini.* Disease severity was assessed after three weeks, by measuring the reduction of sprout length. Both methods proved to be useful for screening for resistance, for evaluating race specificity of resistance and to study pathogenesis. The test tube method proved to be the most accurate for the screening, but the preserving jar method was much less time and labor consuming.

Key words: flax, linseed, *Linum usitatissimum*, *Fusarium oxysporum* f.sp. lini, in *vitro* test, resistance breeding, wilt, disease rating.

INTRODUCTION

In plant breeding efficient selection for disease resistance depends on the availability of representative tests such as infested field tests, greenhouse tests and *in vitro* tests. Much attention has been paid to develop such efficient and reliable screening tests, especially when a soil-borne fungus, such as *Fusarium oxysporum* is involved, a pathogen causing wilt and root rot in many crops (McCoy, 1988; Stephens and Elmer, 1988; Löffler and Mouris, 1989; Islam, 1992; Van Westrhenen *et al.*, 1995).

The *Fusarium* wilt disease results in economical damage in flax and linseed (*Linum usitatissimum* L.) (Beaudoin, 1988) and is caused by *Fusarium oxysporum* f.sp. *lini* (Bolley) Snyd. & Hans.. Within the species *Linum usitatissimum* flax and linseed are two distinguishable groups. Flax is grown for fibers while linseed is grown for oil. The two groups differ much in

agronomic characteristics. Linseed has a reduced height, more branching and a later harvest time. Based on field observations in a flax wilt nursery in Normandy, France, the assumption arose that there might be a difference between the two groups in *Fusarium* resistance.

About the *Fusarium*-flax interaction little is known. Also hardly anything is known about the infection and colonization process and about the resistance mechanisms in flax and linseed, because of the lack of reliable and accurate screening tests, especially *in vitro* ones.

All over the world *Fusarium* resistance is a major objective in breeding programs for flax and linseed (Liu *et al.*, 1993; Ondrej, 1993; Kenaschuk and Rashid, 1993; 1994; Li *et al.*, 1994; Popescu *et al.*, 1994; Gent, 1995). Chlamydospores of the fungus are difficult to destroy by agrochemicals and resistant cultivars are desired to control the disease. Conventional methods for screening of resistance in flax and linseed consist of field trials at infested sites (flax wilt nurseries) with visual assessment of wilt development. Such trials give highly variable results and therefore require adequate replications, both in space and time.

Most soil types contain different *Fusarium* species, and the infestation patterns of *Fusarium oxysporum* in the soil can be variable (Tamietti and Pramotton, 1987). Because of interactions between pathogenic and non-pathogenic *Fusaria* (Davis, 1966) and because the severity of the disease is influenced by the soil type (Alabouvette *et al.*, 1982), it is difficult to predict how different flax and linseed cultivars react at different locations. Also races of *Fusarium oxysporum* f.sp. *lini* have been reported, using field tests, pot tests and greenhouse tests (Broadfoot, 1926; Borlaug, 1945; Kulkarni *et al.*, 1969), but these reports gave rise to discussion. Using a greenhouse test, Fouilloux and Chaboche (1996) found no indication of race specificity. The genetic system of *Fusarium* resistance in flax and linseed is complex (Popescu, 1995) and a simple and reliable screening test would be of great help for detecting the resistance genes involved as well as obtaining insight into the infection and colonization process. Davis (1966) pointed out that

microfloral contaminants competing with fusarial wilt pathogens and cross contamination among *formae speciales* cannot be excluded using greenhouse tests, so *in vitro* screening methods are highly desirable.

In the present study two recently developed *in vitro* tests are evaluated for the use in resistance research and breeding. A comparison is made with data from a flax wilt nursery in Normandy, France, which nursery serves as a guideline for determining *Fusarium* resistance in cultivars mentioned in the French descriptive list for new cultivars (Anonymous, 1992). Furthermore an evaluation is made of the suitability of the two tests in studying fundamental aspects of the *Fusarium*-flax interaction, race specificity, and the infection and colonization patterns.

MATERIALS AND METHODS

Host

Flax seeds from 'Ariane', 'Belinka', 'Laura', 'Marina', 'Regina', 'Saskia' and 'Viking' (CPRO-DLO stock collection) and 'Hermes' (Landbouwbureau Wiersum, Dronten, The Netherlands) and linseed seeds from 'Atalante', 'Barbara' and 'Linda' (CPRO-DLO stock collection) were used. Directly before use the seeds were sterilized for 15 s in 70% ethanol, followed by 15 min in 1% hypochlorite.

Pathogen

Single spore cultures of *Fusarium oxysporum* f.sp. *lini* (Fof), originating from wilted flax straw of 'Regina' grown in a flax wilt nursery at Ingelmunster, Belgium (Fof-B2) and from wilted straw from a flax wilt nursery in Normandy, France (Fof-F1) were provided by Dr. G. Marshall, The West of Scotland College, UK. Stock cultures were kept for long time preservation at -80 °C on Protect Bacterial preservers (Technical Service Consultants Ltd., UK). Before use, the stock cultures were grown on potato dextrose agar in the dark at 24 °C for 14 days. In a pilot experiment, the isolates were determined as aggressive (Fof-B2) and moderately aggressive (Fof-F1).

In vitro screening

Method 1

Test tubes of 15 * 250 mm with glass caps were filled with 0.65 vermiculite and 7.0 ml of a 10% MS-solution (Murashige and Skoog, 1962). The pH was adjusted to 5.8. The tubes were autoclaved for 20 min. Per tube one seed was sown, and 20 tubes per cultivar per isolate treatment were prepared. The tubes were placed in a climate chamber with 16 h light (Philips 84 HF, 1100 lux) per day, at 22 °C. After six days the seedlings had developed just opened cotyledons and of each set of 20 tubes the ten seedlings least variable were selected on the basis of equal length (approx. 4 cm). In this test ten cultivars were exposed to two isolates while in addition ten replicates per cultivar were treated with sterile water as a control, so the test consisted of ten inoculated replicates per cultivar/isolate combination. The selected seedlings were inoculated by adding 1 ml spore suspension of one of the two isolates, containing 10^5 spores per ml, to the vermiculite. For the controls 1 ml sterile water was used. To determine the best parameters for screening, disease symptoms as well as sprout lengths were measured. The following disease symptoms were distinguished: yellowing of leaves, brown spots on leaves, bending of the top, death of the top and death of the stem. Because of cultivar dependent regeneration after damage, which influenced the scores, regeneration as a parameter for disease symptoms was taken into account. The different disease symptoms and the occurrence of regeneration were scored at day seventeen. Length of the sprout was measured at two, four, six, eight, eleven and seventeen days after inoculation. Test tubes filled with vermiculite, and 'Laura' are shown in Fig. 2.1.

Method 2

In two-liter preserving jars a 5 cm high strip of filter paper was placed on the inside of the wall and 100 ml of a 10% MS-solution was poured into the jar. The jars were autoclaved for 20 min. In this test eight cultivars were

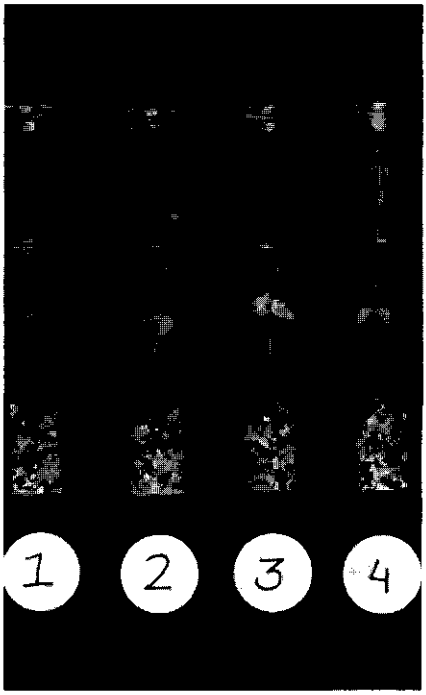

Figure 2.1

Method 1, *in vitro* screening test for *Fusarium* resistance in flax and linseed using test tubes with solid medium and 'Laura'. Tube 1 shows the developmental stage at the day of inoculation, tube 2 shows the healthy control, tube 3 shows 'Laura' at 17 days after inoculation with isolate Fof-B2 and tube 4 shows 'Laura' at 17 days after inoculation with isolate Fof-F1.

Figure 2.2

Method 2, *in vitro* screening test for *Fusarium* resistance in flax and linseed, using a two-liter preserving jar with liquid medium, and 16 randomly placed seeds of eight cultivars, 7 days after inoculation with isolate Fof-B2.

exposed to the two isolates. Per jar two sterilized seeds per cultivar were placed. In total 45 jars were prepared in a randomized block design from which fifteen jars were destinated for inoculation with the isolate Fof-B2, 15 jars with the isolate Fof-F1 and 15 jars were destinated for treatment with sterile water as a control. In this way the test consisted of 30 replicates per cultivar/isolate combination. The seeds were placed on the upper edge of the

paper, so that the young roots could develop between glass and paper. The outsides of the jars were covered with aluminum foil to protect the developing young roots from direct light (Fig. 2.2). The jars were placed in a climate chamber with 16 h light (Philips 84 HF, 1100 lux) per day, at 22 °C. After six days the seedlings were inoculated by adding 1 ml spore suspension of 10^5 spores per ml close to each seedling, between paper and glass. One ml sterile water was used for the controls. Disease symptoms as described for method 1 were observed but not in as much details as for method 1, and therefore are not presented. The sprout lengths of the seedlings were measured 21 days after inoculation.

Field trial

To be able to compare with the results of existing screening methods (field tests) a comparison was made with data, obtained from observations of 1991 in a flax wilt nursery in Normandy, France (Beaudoin, 1991). These data are presented in Fig. 2.3 and Table 2.2.

Statistical procedures

Data of the sprout length measurements from both methods at the final days of the respective tests were subjected to an analysis of variance (ANOVA) and rating of disease severity per cultivar was determined subsequently as % length compared to the average values of the control tests. Average disease severity rating per experiment was calculated as the grand mean over the results with the isolates used. A correlation matrix was calculated from the results of the different data sets, i.e. the best parameter for creating a disease index from method 1, the disease severity per cultivar per isolate from both *in vitro* methods, the disease rating from both *in vitro* methods and the two field data sets. Subsequently, for all of these different data sets the cultivars were ranked from most susceptible to most resistant and from these ranking lists a correlation matrix was calculated as well.

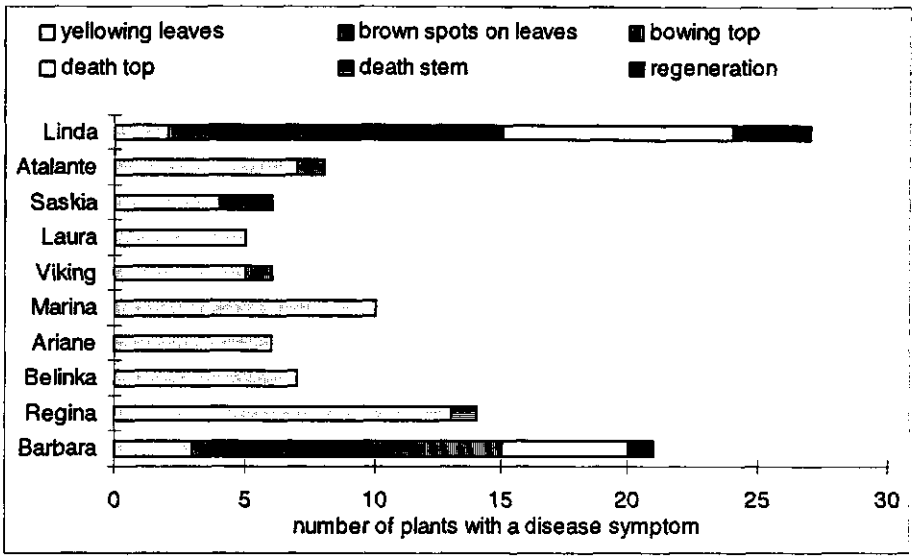

Figure 2.3

Disease symptoms of ten flax and linseed cultivars, inoculated with *Fusarium oxysporum* f.sp. *lini*, isolate Fof-B2 or Fof-F1, scored 17 days after inoculation. At the x-axis the number of plants with a certain disease symptom observed at day 17, using method 1. At the y-axis the cultivars, ordered from most resistant (top) to most susceptible (bottom) according to the field data (Beaudoin, 1991).

RESULTS

Disease symptoms

Disease symptoms were scored in detail in the test tube experiment. The symptoms varied with the different cultivars. In all cultivars the disease was sometimes expressed by a yellowing of the leaves, but not all diseased plants showed this symptom. Several plants of some cultivars showed development of necrotic spots on the leaves ('Barbara', 'Saskia', 'Atalante' and 'Linda'), or reacted with death of the top ('Barbara' and 'Linda'). Cultivars 'Barbara' and 'Viking' started to wilt first and died shortly afterwards, whereas in other cases stem death occurred, followed by death of the seedling or by regeneration from the plant parts located under the dead stem part ('Barbara' and 'Linda'). Because the disease symptoms were different with the cultivars,

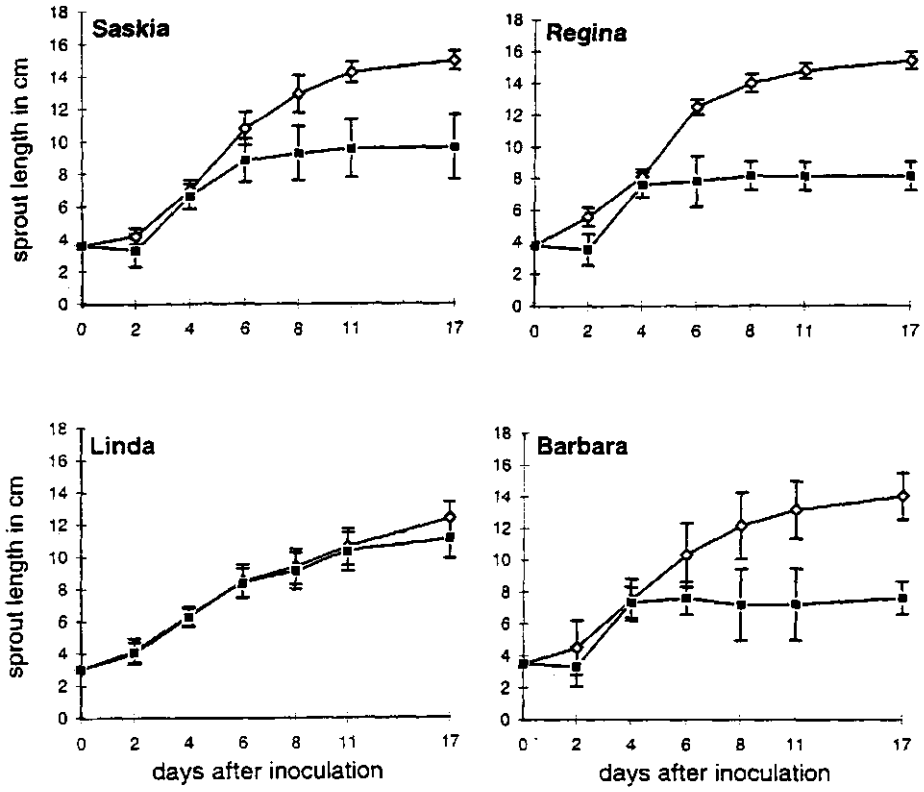

Figure 2.4

Sprout lengths of four cultivars of resistant and susceptible flax ('Saskia' and 'Regina') and resistant and susceptible linseed ('Linda' and 'Barbara'), inoculated with *Fusarium* isolate Fof-B2 (■), and with sterile water (◇), using method 1. Standard deviations at error bars.

no disease scale, based on these symptoms, could be developed. The different symptoms were scored separately and were compared with field data of the flax wilt nursery in Normandy, France (Beaudoin, 1991). As can be seen in Fig. 2.3, no single disease symptom gave a good correlation with these field data and neither did the cumulative disease symptoms. The most common symptom "yellowing of leaves" had the highest correlation with the field data ($r = 0.38$, n.s.) compared to the other disease symptoms.

Table 2.1

Analyses of Variance (ANOVA) of sprout length measurements of flax and linseed seedlings inoculated with *Fusarium oxysporum* f.sp. *lini* isolate Fof-B2, isolate Fof-F1 or with sterile water using method 1 or method 2.

Method	Source of variance	D.f.	S.s.	M.s.	V.r.	F pr.
1	Isolate (or water) (Is)	2	1482.7	741.4	435.1	<.001
	Residual	27	46.0	1.7		
	Cultivar (Cv)	9	438.4	48.7	32.7	<.001
	Cv * Is	18	186.6	10.4	7.0	<.001
	Residual	243	669.5	1.5		
	Total	299	3565.3			
2	Block (Bl)	14	122.8	8.8	1.7	
	Cultivar (Cv)	7	425.6	60.8	11.6	<.001
	Isolate (or water) (Is)	2	287.3	143.6	27.3	<.001
	Cv * Is	14	138.8	9.9	1.9	0.003
	Residual	322	1520.7	5.2	1.7	
	Bl * Cv * Is	360	179.9	3.2		
	Total	719	2624.2			

Plant length measurements

Method 1

A large variation in sprout length was found for all cultivars. The average total sprout length of all seedlings at the end of the experiment was 12.2 cm. The development of the most resistant and the most susceptible cultivars ('Saskia' and 'Regina') and the most resistant and most susceptible linseed cultivars ('Linda' and 'Barbara'), inoculated with the isolate Fof-B2 and compared with the controls is shown in Fig. 2.4. The analysis of variance (Table 2.1) showed that most of the variance was due to the main effects, isolates and cultivars. The isolate * cultivar interaction was significant, but the contribution to the total variance was relatively small. In Table 2.2 the results are presented as the average length as % of the control for ten cultivars and

Table 2.2

Disease severity rating of flax (F) and linseed (L) cultivars, given as the % lengths of the cultivars compared with controls of the same cultivars, calculated from the results of length measurements for two isolates Fof-B2 and Fof-F1 from method 1 or method 2, the overall average disease severity rating per experiment (ADR), and field data from a flax wilt nursery in Normandy, France (Beaudoin, 1991). The overall aggressiveness of the isolates for the two methods is given as the average isolate aggressiveness (AIA).

Cultivar	Type	Method 1			Method 2			Field data
		B2	F1	ADR	B2	F1	ADR	
Linda	L	89.9	93.4	91.7	96.6	108.9	102.7	1.3
Atalante	L	71.8	96.1	84.0	82.7	85.8	84.3	1.5
Laura	F	69.1	86.5	77.8	-	-	-	1.8
Marina	F	67.4	91.3	79.4	81.0	80.4	82.8	1.8
Viking	F	55.8	81.6	68.7	79.7	85.9	82.8	2.3
Hermes	F	-	-	-	98.7	100.9	99.8	2.3
Saskia	F	64.4	92.4	78.4	-	-	-	2.8
Ariane	F	51.5	80.8	66.2	88.3	88.9	88.6	5.0
Barbara	L	54.3	70.9	62.6	73.9	85.0	79.6	7.5
Belinka	F	58.3	91.4	74.9	-	-	-	8.0
Regina	F	53.0	87.7	70.4	74.3	84.8	79.4	9.0
AIA		63.6	87.2		84.4	90.1		

two isolates. As expected, isolate Fof-B2 was the most aggressive while Fof-F1 was less aggressive. 'Barbara' was susceptible, 'Linda' clearly resistant to both isolates. For the other cultivars there was more variation between isolates.

Method 2

Disease symptoms in method 2 showed the same diversification as in method 1, and also a large variation in length was found for all cultivars and for the two isolates used in method 2 (Table 2.2). The average total length at the end of the experiment was 8.3 cm, which was considerably less than the

Table 2.3

Linear correlation coefficients between the disease-parameter "yellowing of leaves" (YL), disease severity rating as the % length reduction of the cultivars for the two isolates, Fof-B2 (B2) and Fof-F1 (F1) using method 1 (M1) and method 2 (M2), the average disease severity rating for the two methods (ADR), furthermore for the results from the field data obtained from a flax wilt nursery in Normandy, France (FR).

	M1 YL	M1 B2	M1 F1	M1 ADR	M2 B2	M2 F1	M2 ADR	FR
M1YL	1.00**							
M1B2	-0.69*	1.00**						
M1F1	-0.42	0.62	1.00**					
M1ADR	-0.65*	0.94**	0.85**	1.00**				
M2B2	-0.63	0.72*	0.46	0.67*	1.00**			
M2F1	-0.33	0.74*	0.29	0.61	0.84**	1.00**		
M2ADR	-0.49	0.76**	0.39	0.66*	0.95**	0.96**	1.00**	
FR	0.38	-0.69*	-0.42	-0.65*	-0.63	-0.33	-0.49	1.00**

* Significant correlations at 5% level
** Significant correlations at 1% level

average lengths from method 1, 12.2 cm. The ANOVA again showed that the variance for cultivars and for isolates were the major contributors to the total variance (Table 2.1), while the cultivar * isolate interaction variance, though significant, was small again. Also in this experiment the isolate Fof-B2 was more aggressive, and was 'Linda' clearly resistant. The susceptibility of 'Barbara' was less clear.

Correlations between screening methods

In general most significant correlations were found using method 1 and isolate Fof-B2, the most aggressive isolate. The parameter which was most useful as a disease index, namely "yellowing of leaves", was correlated only at a significant level (P = 0.05) with length reduction as caused by isolate Fof-B2 using method 1, and the average disease severity rating (ADR) determined by method 1 (Table 2.3). Parameters of method 1 (isolate Fof-B2

Table 2.4

Rank correlations between the disease parameter "yellowing of leaves" (YL), disease severity rating as the % length reduction of the cultivars for the two isolates, Fof-B2 (B2) and Fof-F1 (F1) using method 1 (M1) and method 2 (M2), the average disease rating for the two methods (ADR), furthermore for the results from the field data obtained from a flax wilt nursery in Normandy, France (FR).

	M1 YL	M1 B2	M1 F1	M1 ADR	M2 B2	M2 F1	M2 ADR	FR
M1YL	1.00**							
M1B2	0.32	1.00**						
M1F1	-0.21	0.79**	1.00**					
M1ADR	-0.07	0.82**	0.96**	1.00**				
M2B2	0.35	0.43	0.54	0.61	1.00**			
M2F1	0.71	0.18	0.04	0.14	0.68*	1.00**		
M2ADR	0.31	0.41	0.50	0.58	0.99**	0.76*	1.00**	
FR	0.36	0.89**	0.75*	0.79**	0.75*	0.43	0.74*	1.00**

* Significant correlations at 5% level
** Significant correlations at 1% level

and ADR) correlated well with the French field data, but parameters of method 2 did not correlate significantly with the field data (Table 2.3).

When using rank correlations no significant correlations were found for the parameter "yellowing of leaves" (Table 2.4). However, with ranked data many significant correlations were found between the *in vitro* methods and the French flax wilt nursery, but not between the two *in vitro* methods.

DISCUSSION

Disease symptoms

Because of the diversity of disease symptoms between and within cultivars (Fig. 2.3) and the lack of correlation with the resistance scores in the fields (Table 2.3 and 2.4) these symptoms are not suitable for a representative disease rating. Jouan and Sailly (1991) developed an *in vitro* seedling test for *Fusarium*-flax, using test tubes with vermiculite, and rating based on yellowing of leaves, wilting and death of plants. *Fusarium* spores

were added immediately at sowing. For getting a reliable disease screening the incubation time was 70 days, while three different levels of spore concentration were needed. However, the authors stated that this *in vitro* test was not an effective way to screen a large number of accessions because the test is too laborious. In the *in vitro* test for *Fusarium* disease resistance in flax developed by Van Westrhenen *et al.* (1995) rating also was based on disease symptoms. It was not clear what kind of disease symptoms were used. The results from method 1 in the present study showed that screening for disease symptoms is unreliable.

Length measurements

Bos and Parlevliet (1995) stated that reduction of growth can be a significant disease symptom. In *Fusarium* assessments of length reduction measurements have been used successfully as a parameter for disease rating (Löffler *et al.*, 1997). However, a disadvantage of length measurements in flax and linseed is that growth differs between cultivars, also because of differences in selection criteria for flax and linseed. On average linseed is shorter than flax. Linseed is grown for seed production and therefore selected for branching while flax is selected for fiber production and therefore selected for a long main stem. For that reason a disease rating based on length measurements was used by comparing the average length of the diseased cultivar with that of the healthy control of the same cultivar, the latter being set at 100%. Compared with the French field data (Beaudoin, 1991) this disease rating gave good correlations for both methods of the present study (Table 2.3 and 2.4). Thus, using disease severity rating based on sprout length of diseased plants compared with that of controls of the same cultivar, proved to be successful for the screening of flax as well as linseed with the *in vitro* methods.

The isolate Fof-F1 was moderately aggressive and gave moderate discrimination between cultivars. Better results were obtained using the more aggressive isolate Fof-B2. For both *in vitro* methods this isolate was capable

to distinguish between resistant, moderately resistant and susceptible cultivars. The correlation with the field data was better for the aggressive isolate Fof-B2 than for the less aggressive isolate Fof-F1. In the field it is most likely that the most aggressive *Fusarium* strains are expressed strongest, explaining the better correlation between the *in vitro* test results of the more aggressive isolate and the field data. This suggests that in the French field more aggressive *Fusarium* strains dominate. The French field data corresponded well with the Dutch and French descriptive lists of cultivars (Anonymous, 1986; 1988; 1990; 1992; 1994; Ebskamp and Bonthuis, 1993, 1997) Therefore, the use of an aggressive isolate is an important factor for reliable and representative results in *in vitro* screening tests.

Application of *in vitro* methods

Method 1 gave the best correlation with the field data, and gave a better discrimination between the cultivars, compared with method 2. This can be explained by the fact that in the procedure of method 1 a selection for equal sprout length took place at the day of inoculation, removing a source of environmental variation. This procedure is not feasible for method 2 because of the lack of separate units. However, method 2 consisted of easier manageable units to work with and for that reason this method was much less time and labor consuming. With method 2, one person could test about three times more accessions in the same time compared with method 1.

Fusarium resistance in flax and linseed is a very important selection criterion and it is desirable to select for this trait in an earlier stage than is the case at present. In the two *in vitro* methods 20 or 30 seeds per cultivar-isolate combination were required, while in field trials about 1000 seeds are used in general. The multiplication rate of flax and linseed is low. This means that screening for *Fusarium* resistance in field trials can be performed only after several multiplication steps, i.e. later in the breeding program. Using one of the *in vitro* methods, a test for *Fusarium* resistance can already be carried out in an early stage of the breeding program. While field trials have to be

repeated for at least two years, the *in* vitro tests can be performed within the time span of one month.

Cultivar * isolate interaction was observed but the analysis of variance showed that this interaction was of little importance. Although, for both *in vitro* methods it seems possible to trace interaction patterns and the interaction seems to be better quantifiable using method 1 (Table 2.1).

Method 2 was based on a liquid medium, which included that the roots stayed intact during the development of the disease. This was in contrast with method 1, where the roots almost completely damped off in a very short time. So method 2 gives the additional possibility to perform more fundamental histological studies for infection and colonization processes of the pathogen.

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CHAPTER 3

Infection and colonization of flax seedling roots (*Linum usitatissimum*) by *Fusarium oxysporum* f.sp. *lini*

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Submitted

ABSTRACT

Infection and colonization of flax seedling roots by *Fusarium oxysporum* f.sp. *lini* was studied using semi-thin sections of plastic-embedded roots of a susceptible and a resistant flax cultivar. Within two days, the fungus colonized the calyptra cell layers by intercellular, and subsequently also intracellular, growth. Attempted intercellular penetration of calyptra cell layers and eventually the epidermis induced the formation of distinct appositions next to penetration hyphae. Other cells next to penetration hyphae collapsed, which was accompanied by swelling of the neighbouring cells. Invasion of calyptra cell layers and growth towards the epidermis was counteracted by enhanced production of new layers of calyptra cells by the meristematic epidermis (protodermis), and their successive detachment and sloughing off. The epidermis and cortex were reached and penetrated in four days, which was followed by rapid and massive colonization of the entire root tip. From eight to sixteen days after inoculation, the lower parts of the roots were colonized throughout and the cortical region was degraded. Colonized tissues were severely plasmolyzed. Heavily colonized roots were hollowed out, the only remaining tissues being the epidermis and exodermis outside, and the colonized vascular tissue inside. Eventually the vascular region was also degraded. Upward spread of root rot was restricted in the period studied to the first 10 mm from the root tip, the upper parts of the root and the hypocotyl being unaffected except for invasion through lateral roots infected at their respective tips. Mature roots with a well-developed epidermis and exodermis were not invaded from outside. Disease development was similar in resistant 'Hermes' and susceptible 'Regina', although occlusion of intercellular spaces with gum-like components and cell wall enforcement with phenolics seemed to be more prominent in the resistant cultivar.

Key words: colonization, flax, *Fusarium oxysporum* f.sp. *lini,* infection, *Linum usitatissimum,* root tip, wilt.

INTRODUCTION

Fusarium oxysporum f.sp. *lini* (Bolley) Snyder & Hansen, causal agent of wilt disease in flax (*Linum usitatissimum* L.), is one of the most important diseases in this crop all over the world. The fungus is soil-borne and infection takes place mainly through the roots, although the hypocotyl may also be infected (Nair, 1956). Several early studies on this disease concerned the mode of infection and colonization of flax by *F. oxysporum* f.sp. *lini* (Tisdale, 1917; Boyle, 1934; Millikan, 1951; Nair, 1956; Nair and Kommedahl, 1957). More recently, Turlier *et al.* (1994) described the infection process in hydroponically grown flax seedlings using a GUS-transformed strain of the pathogen. Based on studies using thick (7 µm) sections of paraffin-embedded samples, they concluded that the fungus penetrates into undifferentiated protodermis cells and root cap cells, and then reaches the subapical meristem where it remains endophytically as a permanent internal site of infection for the differentiating xylem. In other host species, however infection is supposed to take place through transversal, mainly intercellular, growth of hyphae from the epidermis towards the stele (Bishop and Cooper, 1983). The present study on semi-thin (2 µm) sectioned samples was carried out to obtain further details on the infection process, using both a resistant and a susceptible flax cultivar.

MATERIALS AND METHODS

Host and pathogen

Flax seeds from 'Hermes' (Landbouwbureau Wiersum, Dronten, The Netherlands) and 'Regina', (CPRO-DLO stock collection) were used. Directly before use the seeds were sterilized for 15 seconds in 70% ethanol, followed by 15 min in 1% hypochlorite. Single-spore cultures of *Fusarium oxysporum* f.sp. *lini* (Fof), isolate Fof-F60 were provided by Dr. G. Fouilloux, INRA Versailles, France. Stock cultures were stored at -80 °C on PROTECT bacterial preservers (Technical Service Consultants Ltd, UK). Before use,

cultures were grown on potato dextrose agar in the dark at 24 °C for 14 days.

Inoculation of seedlings

An *in vitro* test was performed on liquid medium (see Chapter 2) using 'Hermes' and 'Regina', and the isolate Fof-F60. During the test 16 preserving jars were used. Three seeds of each cultivar were randomly placed in each jar. After six days of growth the seedlings from 12 jars were inoculated by pouring 1 ml spore suspension of 10^5 spores per ml onto each root, while four jars were treated with sterile water as a control. After two, four, eight, and sixteen days, nine seedlings of three inoculated jars and three seedlings of a control jar were harvested for histological examination. Length of the seedlings was determined at harvesting.

Light microscopy

Immediately after harvesting, segments of 3 mm length of root tips, branched root segments, root segments from the part of the root 1 cm below the junction of stem and root, and hypocotyl segments, were fixed in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8). Fixed segments were dehydrated in a graded ethanol series and embedded in Technovit 7100 (Heraeus Kulzer GmbH, Friedrichsdorf, Germany). From all harvests, four to ten samples of root tips of both cultivars were examined, and two to six samples per cultivar of root branching sites, of parts 1 cm below the junction of stem and root, and of the hypocotyl. Using a Jung 2040 rotary microtome and Ralph glass knives, sections of 1.5 - 2.0 µm thickness were made at various levels of the root tips, from 0.1 mm up to 0.5 cm above the root tips, and from the other plant parts described. The sections were stained with toluidine blue O (Jensen, 1962) and viewed with a Zeiss Axioplan microscope (Zeiss-Nederland B.V., Weesp, The Netherlands) using bright-field light microscopy and differential interference contrast. Sections were photographed using Zeiss MC-100 photographic equipment and recorded on Ilford Pan F50, Kodak Technical Pan and Kodak Ektachrome 320 T film.

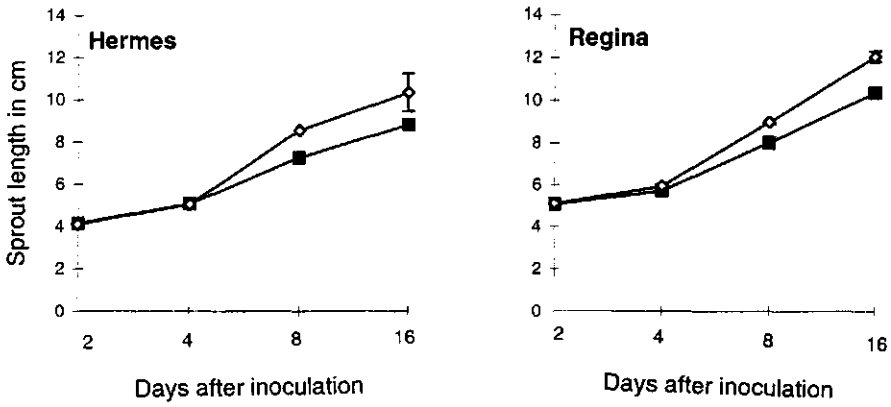


Figure 3.1

Sprout lengths of 'Hermes' and 'Regina', inoculated with *Fusarium oxysporum* f.sp. *lini* isolate Fof-F60 (■) or with sterile water (◇), using method 2 described in Chapter 2. Standard deviations (error bars) mostly were very small and consequently have disappeared under some of the markers.

RESULTS

Macroscopic symptoms

The fungus showed a distinct preference for the root tip throughout the experiment. Two days after inoculation, the fungus was ubiquitously present around the root tips but much less so in the zone of elongation or at lateral root branches. Three to five days after inoculation, most root tips turned purple over the first 1.5 mm, most likely due to the production of fungal pigments. After about eight days the root tips decayed and turned brown. After twelve days, the brown zone extended to 3 - 5 mm from the tip. Decay spread right across the root, eventually resulting in hollow roots consisting of merely the epidermis as outer coat and, internally, remainders of the stele. After sixteen days, decay had spread to maximally 10 mm from the tip, and both cultivars had developed disease symptoms such as leaf yellowing and necrosis of the shoot apex. Disease development was similar in both cultivars, as was shoot length reduction (Fig. 3.1). Only at the end of the experiment some distinction was observed between both cultivars, all plants

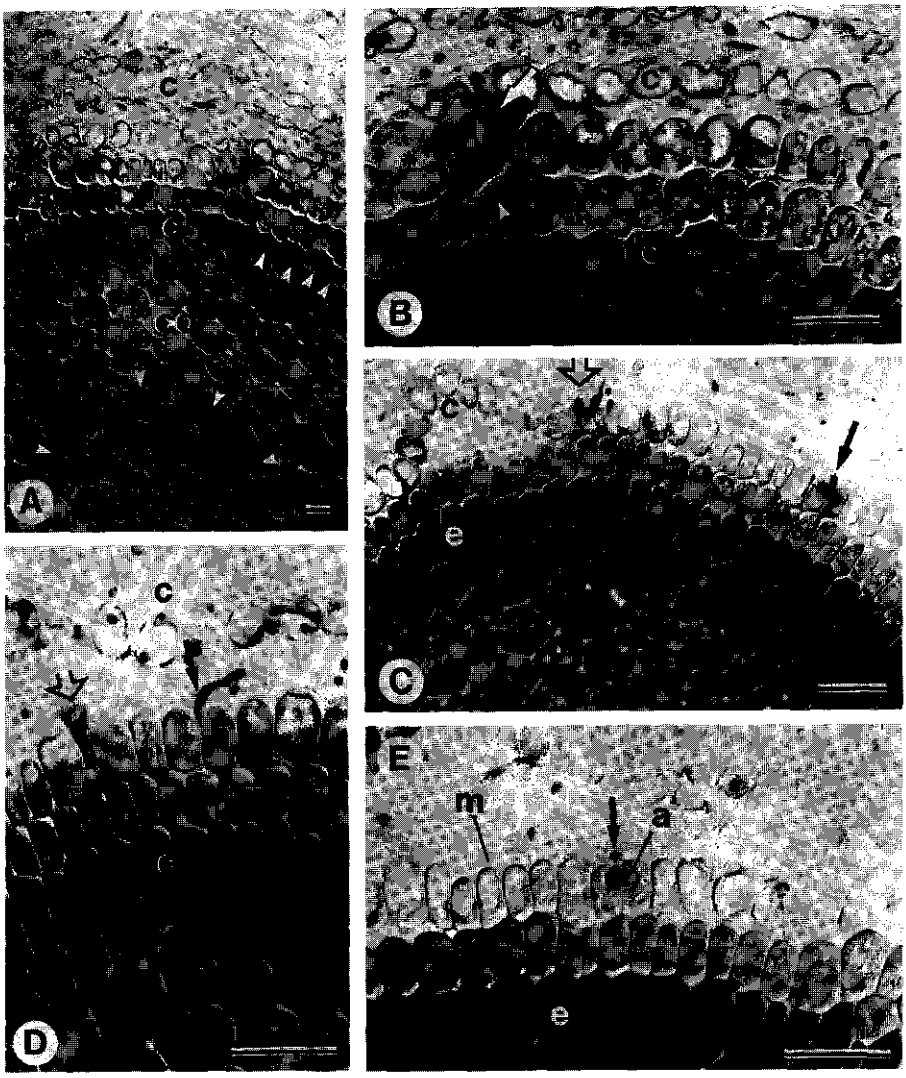
of 'Regina' being reduced in length while several plants of 'Hermes' were not.

Infection of the root tip

Two days after inoculation, fungal hyphae proliferated in both cultivars in between the cell layers of the root cap as well as inside the cells of the outer, senescent and already detached layers of the calyptra (Fig. 3.2A to E). From the colonized outer layers, the fungus attempted to invade the inner, healthy layers of the root cap and the epidermis (*sensu* Esau, 1977) or protodermis (*sensu* Weier *et al.*, 1974) itself. In flax, a clear distinction between epidermis and root cap cell layers does not exist, because the successive cell layers of the root cap are formed by iterative longitudinal division of the epidermis. The newly formed inner cells develop into cortex cells, and the outer ones into successive layers of root cap cells (this is the so-called body-cap concept; Esau, 1977). Fungal hyphae grew through the middle lamella, inside the outer wall of calyptra cells or in the mucilage that covered them (Fig. 3.2E). Fungal growth in new root cap layers initially was intercellularly only, although rapidly followed by intracellular growth in detaching cell layers.

Figure 3.2 A - E

Micrographs of roots of 'Regina' (A and B) and 'Hermes' (C to E) seedlings, two days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of the meristematic zone, 0.25 - 0.30 mm from the tip. Bar = 20 µm.
A. General view. Fungal hyphae are present abundantly in between the cell layers of the root cap. The meristematic epidermis (e), or protodermis, is covered by several cell layers that together form the calyptra (c). The cortex (co) consists of isodiametric cells. Note meristematic activity in the epidermis and the pericycle (arrow heads).
B. Detail of A. Cell collapse at the site of invasion (arrow) of a fungal hypha into the middle lamella in between anticlinal calyptra cell walls. Cell contents are intensely stained with toluidine blue O. Neighbouring calyptra cells are swollen (*), and calyptra cells underneath have formed an apposition-like structure (arrow head).
C. Overview. Note the cell collapse (open arrow) and appositions (black arrow) in calyptra cells upon fungal penetration. The calyptra cell layer involved is sloughing off.
D. Detail of an area as shown in C. Note swollen cells (*) next to a collapsed cell (open arrow), and the appositions produced in other calyptra cells next to a penetration hypha (black arrow).
E. Detail of C. The penetration hypha (arrow) that has incited apposition (a) formation initially grew through the mucilage-like distended outer cell wall (m).

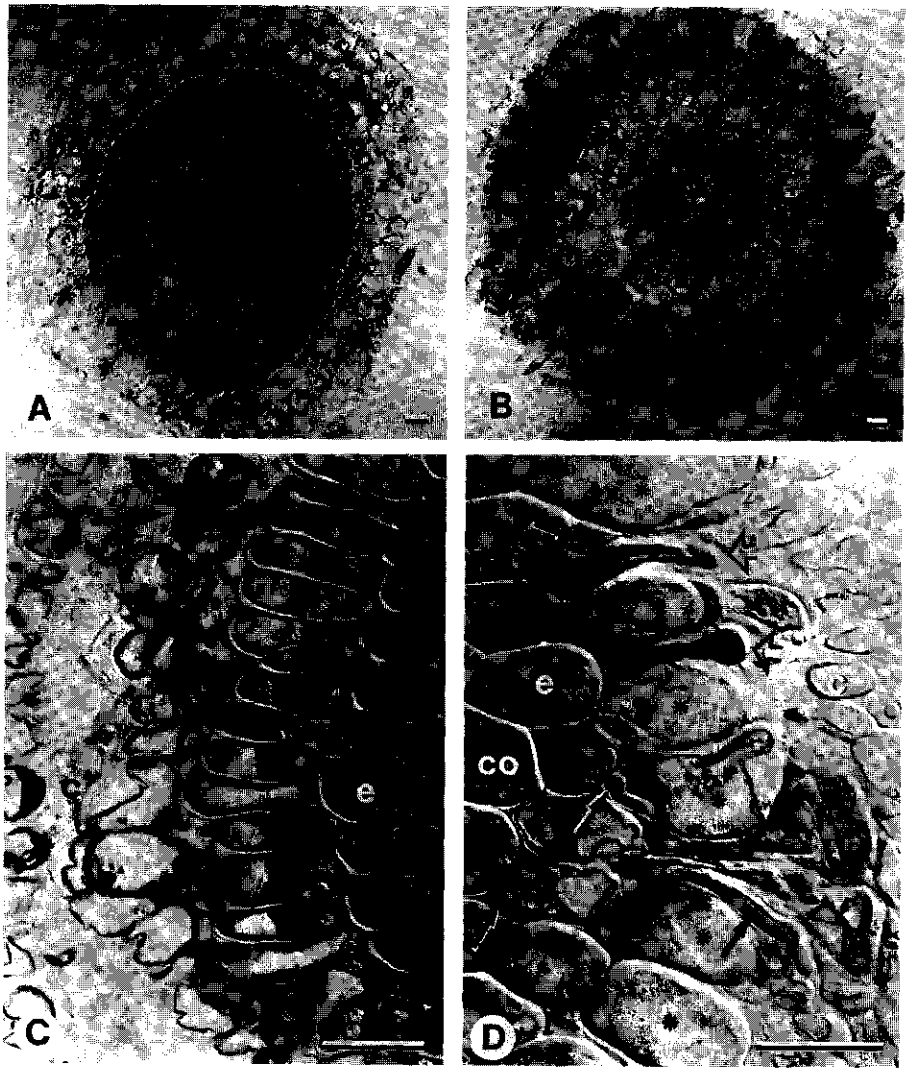

A
c
B
c
C
c
e
D
c
E
m
a
e

Penetration of hyphae into the middle lamellae between the anticlinal walls of calyptra and epidermis cells induced the production of disc-shaped to globose appositions in these cells (Fig. 3.2C to E), as well as the collapse of cells (Fig. 3.2A to D). Collapsing cells were often bordered by swollen cells, and had darkly staining contents. Appositions were particularly formed in resistant 'Hermes', and stained blue-green with toluidine blue O, indicative of the deposition of phenolics (O'Brien and McCully, 1981). Appositions were also observed in senescent, plasmolyzed root cap cell layers in the process of detachment (Fig. 3.2C and E) and at later stages of pathogenesis often also in cells of fully detached root cap layers that had been sloughed off (not shown). Fungal attempts to penetrate the root were thus counteracted by production and detachment and sloughing off of new layers of root cap cells, generated from the epidermis.

Four days after inoculation (Figs. 3.3, 3.4 and 3.5), the calyptra had been massively invaded in both cultivars up to 1.5 mm from the root tip. Most calyptra cells, including the ones having produced appositions at an earlier stage (Fig. 3.3C), were heavily plasmolyzed and seemed dead. The fungus had passed the calyptra and reached the epidermis at several places both in 'Regina' (not shown) and in 'Hermes' (Fig. 3.3D). Collapsing, darkly staining cells neighboured by inflated and swollen ones were at this stage commonly observed in or close to the epidermis rather, than in the calyptra (Fig. 3.3B and D).

Figure 3.3 A - D

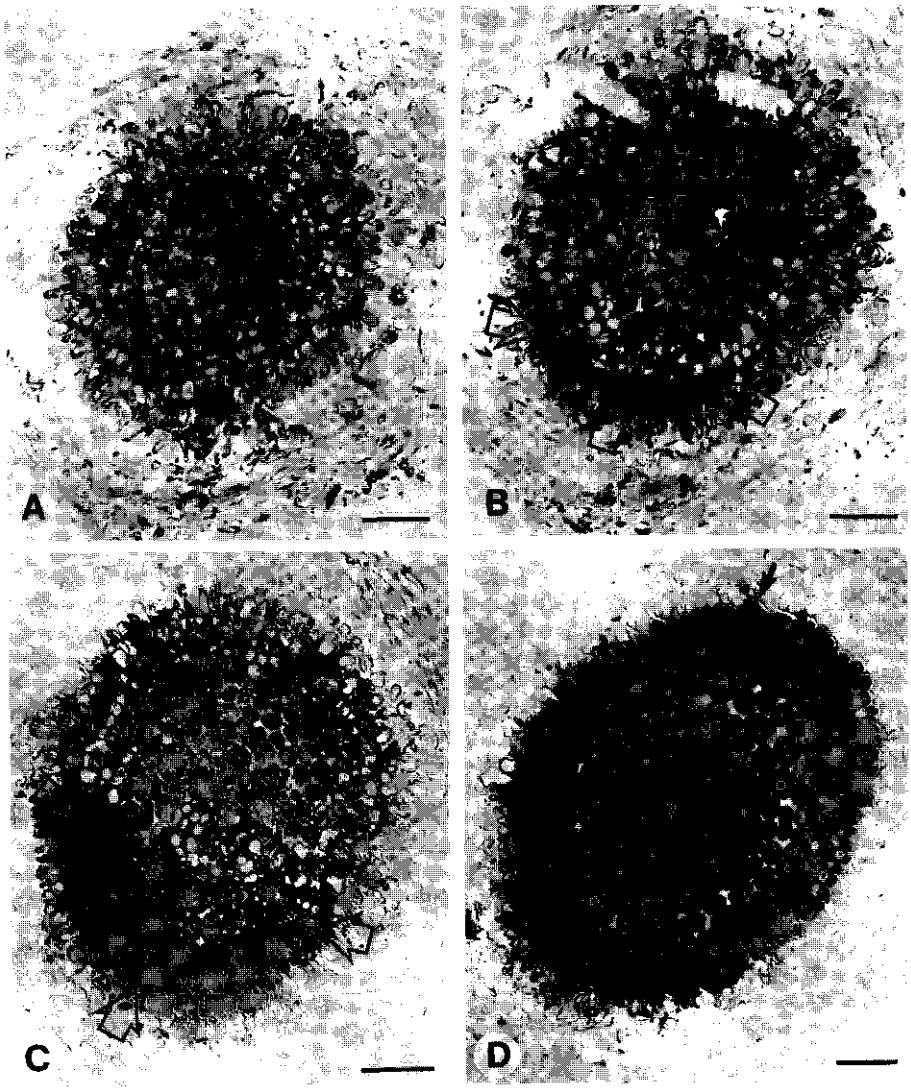
Micrographs of roots of 'Regina' (A and C) and 'Hermes' (B and D) seedlings, four days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of the meristematic zone, 0.25 - 0.30 mm from the tip. Bar = 20 µm.
A. General view of a superficially infected root.
B. General view of a more deeply infected root.
C. Detail of A. Invasion of penetration hyphae through middle lamellae in between anticlinal calyptra cell walls. Note the appositions in calyptra cells in the process of detachment (arrow). Abbreviations: c, calyptra; e, epidermis.
D. Detail of B. Many cells have collapsed (open arrows), and their contents are intensely stained with toluidine blue O. Neighbouring cells are heavily swollen (*). The penetration hyphae (black arrows) have reached the epidermis and the cortex (co).


A
B
e
C
e
co
D

Once the cortex was reached by the fungus (Fig. 3.3D), the entire root tip was colonized rapidly and massively, as demonstrated in Fig. 3.4 and Fig. 3.5 for successive sections of the same root tip of 'Hermes' at four days after inoculation. From the root tip to 0.20 mm off (Fig. 3.4A), the fungus had colonized the root throughout, including the cortical and stelar tissues. Cellular disorganization and incipient plasmolysis were observed throughout the root. At 0.23 mm from the tip (Fig. 3.4B), the same condition was observed except for one side of the root where a single row of living, more or less unaffected epidermal cells was present. At 0.26 mm from the tip (Fig. 3.4C, overview and Fig. 3.5A and B, detail), about two-thirds of the root was diseased whereas one third part was unaffected. A sharp boundary existed between affected and unaffected cells. No hyphae were present in the unaffected region, that apparently connected with the single row of unaffected epidermal cells at 0.23 mm from the tip. At 0.30 mm from the tip (Fig. 3.4D), the entire root (including the stele) seemed unaffected except for the calyptra. Typical appositions were not produced in the cortex (Fig. 3.3D). The cortex was colonized intercellularly, although the fungus was also observed inside the cell walls and eventually also inside the cell lumina (Fig. 3.5A and B). Colonized parts of the cortex underwent plasmolysis and cell contents were disorganized (Fig. 3.3D; Fig. 3.5A and B).

Figure 3.4 A - D

Micrographs of successive sections from the same root of a 'Hermes' seedling, four days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of the meristematic zone, 0.20 - 0.30 mm from the tip. Bar = 50 µm.
A. Section taken at 0.20 mm from the root tip. Hyphae are present abundantly in the calyptra layers surrounding the root tip. All parts of the root have been invaded at this level.
B. Section taken at 0.23 mm from the root tip. Same condition as in A, except that locally the epidermal cells are unaffected (open arrows).
C. Section taken at 0.26 mm from the root tip. Two-third of the root is infected as in A and B, while a third part (connecting to the healthy epidermal cells shown in B) is unaffected. A sharp boundary exists between invaded and unaffected areas (open arrows).
D. Section taken at 0.30 mm from the root tip. The root is unaffected throughout, except for the presence of fungal hyphae in the calyptra (arrow).

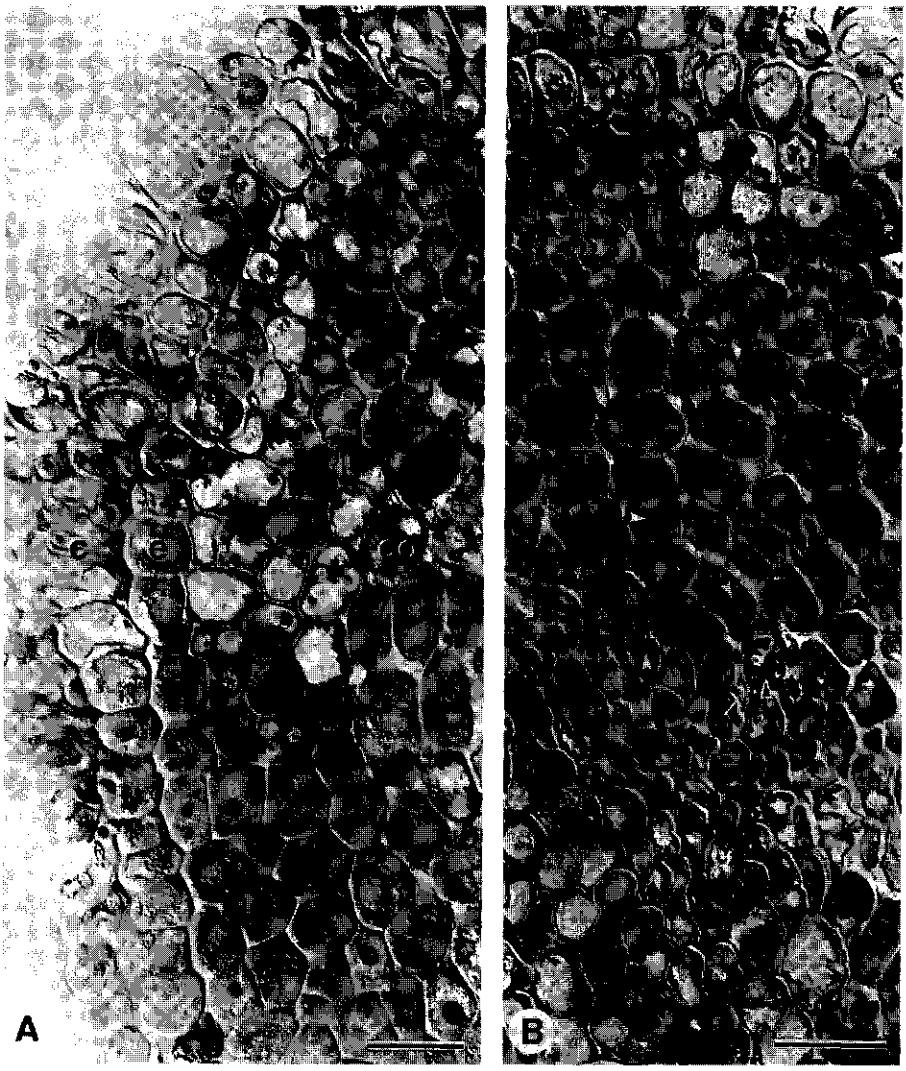

A
B
C
D

Eight days after inoculation (Fig. 3.6A to D), root tips of both cultivars were colonized throughout and the contents of colonized cells stained little or not compared with four days earlier. Cell disorganization and plasmolysis was more severe than at four days after inoculation. Empty-looking cortical cells were colonized both intercellularly and intracellularly, although in the latter case upon closer scrutiny fungal hyphae often seemed to grow inside the distended cell wall, rather than inside the cell lumen (not shown). Intracellular colonization seemed to be preceded by a transient intercellular phase, as evidenced by root areas that still were colonized intercellularly only (Fig. 3.6B). Fungal hyphae were often poorly stained, suggesting a withdrawal of fungal cytoplasm from abundantly colonized areas to the colonization front. Gum-like compounds were incidentally observed intercellularly in 'Hermes' (Fig. 3.6C and D) but not in 'Regina'.

Sixteen days after inoculation, tissue death was complete up to 10 mm from the root tip and, judged from their staining ability, fungal hyphae had withdrawn most of their cytoplasm from this region. Dissolution of host cell walls was extensive, although remainders of darkly stained cell contents were occasionally observed (Fig. 3.7A). A single observation was made of a wedge-shaped lateral invasion site in a root, containing severely plasmolyzed cells and bordered by cells with blue-green stained walls suggestive of phenolics (Fig. 3.7B).

Figure 3.5 A - B

Details of Figure 3.4 C. Micrographs of a section of a root of a 'Hermes' seedling, four days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of the meristematic zone at 0.26 mm from the tip. Bar = 20 µm.
A. Transition zone from unaffected root tissues (lower part) to invaded ones (upper part of micrograph). Fungal hyphae are present abundantly in between cortical cells and often also inside these. Affected cells have darkly stained contents and show incipient plasmolysis; several of them appear to be largely empty.
B. Invaded cortical and stelar tissues. Hyphae (arrows) are present abundantly in between the cortical and stelar cells, and also inside several cortex cells. Do not confuse with cell nuclei (arrow heads). Incipient plasmolysis is seen in many cortical cells of which the cytoplasm otherwise seems relatively unaffected (compare with lower part of A).


A
B

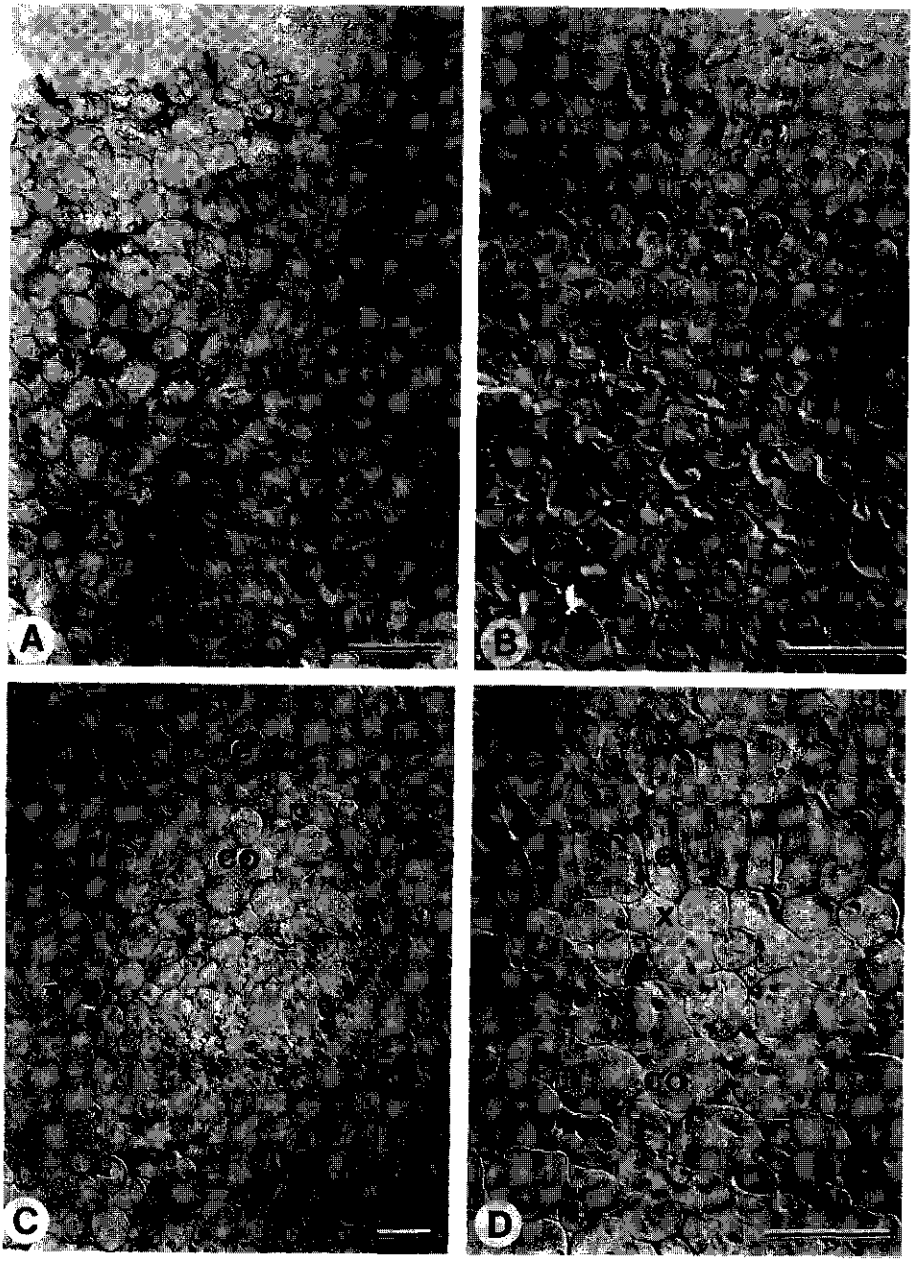
Disease development was similar in 'Hermes' and 'Regina', although occlusion of intercellular spaces with gum-like components and cell wall enforcement with phenolics seemed to be more prominent in 'Hermes'.

Infection of mature parts of the root

Behind the zone of elongation (> 2 mm from the root tip), infection via the epidermis was not observed, even though fungal hyphae did now and then occur on the surface of mature parts of the root (Fig. 3.8A). However, roots were often colonized from within, apparently from lower situated colonized parts (Fig. 3.8B and C, Fig. 3.9). Intercellular growth of fungal hyphae induced severe plasmolysis of neighbouring cortical cells (Fig. 3.9C) and preceded intracellular colonization, dissolution of cell walls and decay of the colonized tissues (Fig. 3.8B and D). Typically, the cortex of colonized roots was hollowed out, the diseased roots being covered by the intact suberized exodermis and the epidermis (Fig. 3.8B). Internally, the stele remained relatively unaffected, although the protoxylem vessels were clearly colonized from out of the cortex (Fig. 3.8B, overview and Fig. 3.9, detail). At

Figure 3.6 A - D

Micrographs of roots of 'Regina' (A) and 'Hermes' (B to D) seedlings, eight days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of the meristematic zone, 0.25 - 0.30 mm from the tip. Bar = 30 µm.
A. Root tip in similar condition as in Fig. 3.3 (four days after inoculation) except that cell disorganization and plasmolysis are more severe. Few or no cortical cells have normal appearing cytoplasm; rather, cell contents have condensed against the wall. Degradation of cell walls is not yet apparent. Fungal hyphae in the calyptra (arrows) are poorly stained compared with those in the cortex.
B. Root tip showing restricted intercellular growth of hyphae in between cortex cells (arrow heads). Intracellular growth occurs in the calyptra but not in the cortex. Fungal hyphae in the calyptra are poorly stained compared with those in the cortex.
C. The cortex and stele have been colonized intensely. Note severe plasmolysis in otherwise empty-looking cortical cells. Degradation of cell walls is not yet apparent. Gum-like compounds are present in some of the intercellular spaces (arrow heads). Abbreviations: e, epidermis; co, cortex.
D. Detail of C. Cortical cells have been heavily colonized and are probably dead. Fungal hyphae in the calyptra are poorly stained compared with those in the cortex. Note the hypha in between two epidermal cells (arrow heads) touching upon towards the exodermis (x).


A
B
C
CC
C
D
X
CC

the end of the experiment, the vascular area had been completely degraded as well (Fig. 3.10A to C). While this was invariably the case in susceptible 'Regina', degradation was less extensive in 'Hermes', which at this stage had often produced gum-like compounds in the intercellular spaces and had greenish stained remainders of cortical cell walls (Fig. 3.10D).

In a number of cases, fungal hyphae were encountered inside the cortex and xylem of roots at branching sites (not shown). Such observations were only made at 16 days after inoculation. Infection in all cases seemed to have taken place at the tip of the lateral root.

Upper root parts and the hypocotyl region

Throughout the experiment, fungal hyphae remained confined to the lower parts of the roots. In the upper parts of the root (10 mm from the hypocotyl) and in the hypocotyl region itself no fungal material or defense responses to colonization were observed at all. Vessel occlusions were not encountered either (not shown).

DISCUSSION

Flax seedling roots were infected at the root tip, by invasion of the root cap and the epidermal layer that generates the successive cell layers of the calyptra. Mature parts of roots, with a fully differentiated epidermis and a suberized exodermis, were not subject to infection. Mature roots were colonized either from the invaded root tip, or from diseased lateral roots that had been invaded at their own tips. These observations support those of Turlier et al. (1994), who reported that hyphae of a GUS-marked transgenic strain of *Fusarium oxysporum* f.sp. *lini* were particularly active at the root tip and the tips of lateral roots, while mycelium was not active on the mature non-exudating root surface. Resistance of the mature root surface, comprising both the epidermis and a suberized exodermis, to infection has also been observed in lilies infected by *F. oxysporum* f.sp. *lilii* (Baayen and Rijkenberg, unpublished results).

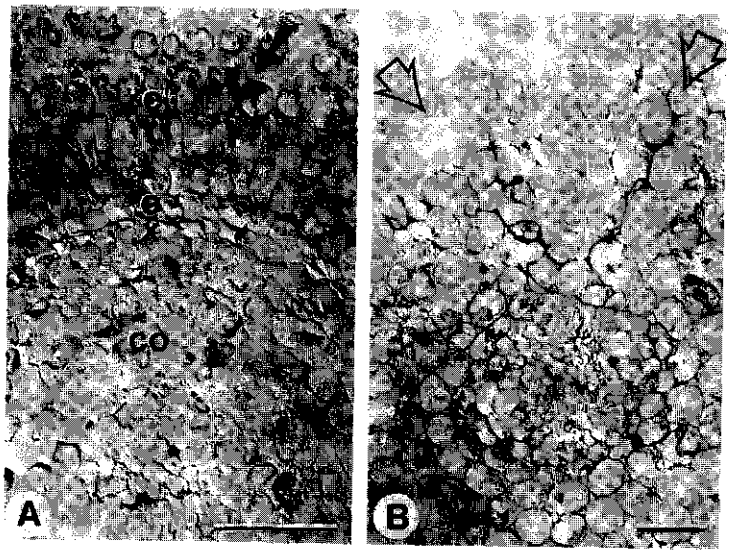


Figure 3.7 A - B

Micrographs of roots of 'Regina' (A) and 'Hermes' (B) seedlings, sixteen days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of the meristematic zone, 0.25 - 0.30 mm from the tip. Bar = 50 µm.
A. Cell wall degradation of the colonized cortex has resulted in incipient hollowing out of the root. Fugal hyphae are largely unstained. Note remaining apposition in calyptra (arrow). Abbreviations: c, calyptra; e, epidermis; x, exodermis; co, cortex.
B. Wedge-shaped lateral invasion of the root (open arrows), associated with severe plasmolysis of the cells involved and with degradation of their walls. Cells next to the invasion front have darkly stained walls. Eventually the entire root has been colonized, and plasmolysis has spread throughout the root.

Figure 3.8 A - D

Micrographs of roots of 'Regina' (A and C) and 'Hermes' (B and D) seedlings, four (A) and eight days (B, C and D) after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of young parts of roots at 1.3 - 1.4 mm from the tip. Bar = 30 µm.
A. The root is unaffected. Hyphae are incidentally found on the root surface (arrows).
B. Heavily colonized and partially hollow root. Plasmolysis is visible throughout the cortex. Whereas many cell walls are in the process of degradation, others are darkly stained with toluidine blue O and are relatively unaffected (left hand part of root). Note gum-like materials in intercellular spaces at bottom part of root. A well-developed exodermis (x) is present.
C. Intercellular growth of hyphae, although transient, precedes intracellular colonization of the cortex. Note severe plasmolysis of cells in the invaded area.
D. Detail of B, showing colonization of the stele and degradation of the cortex (*).

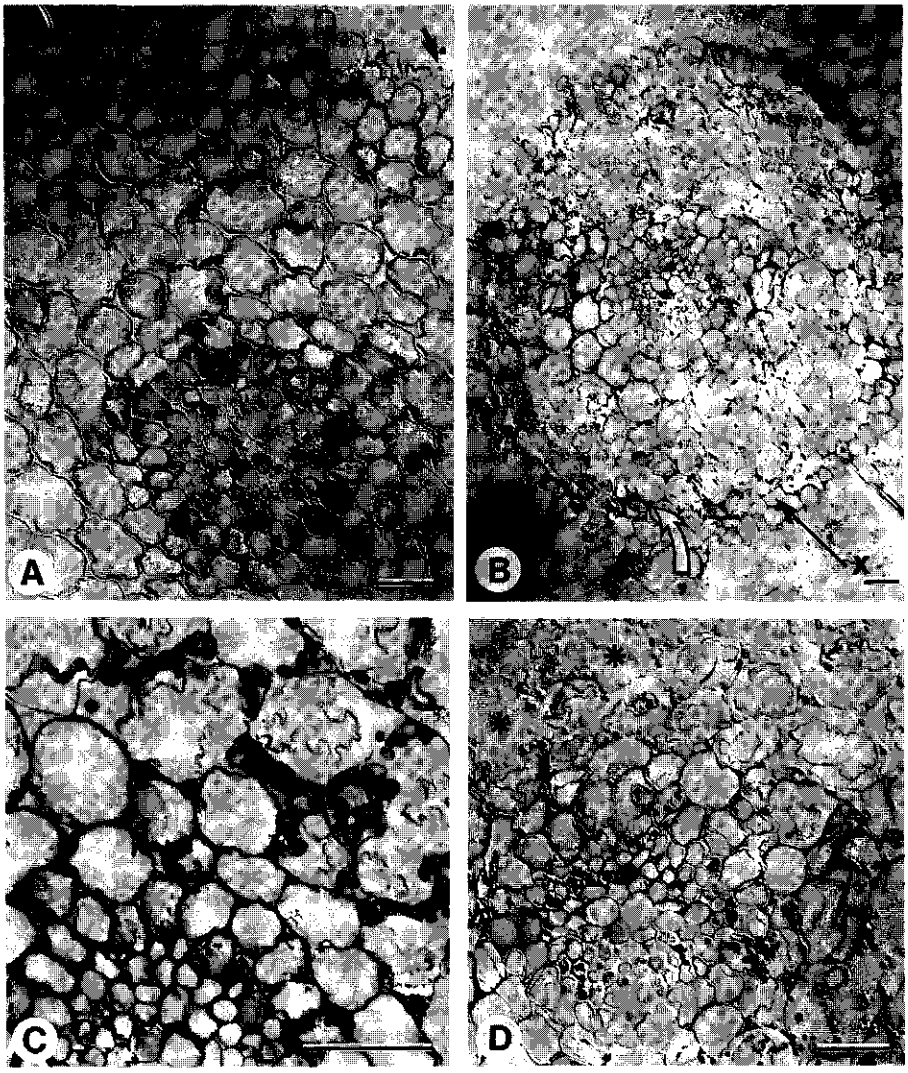
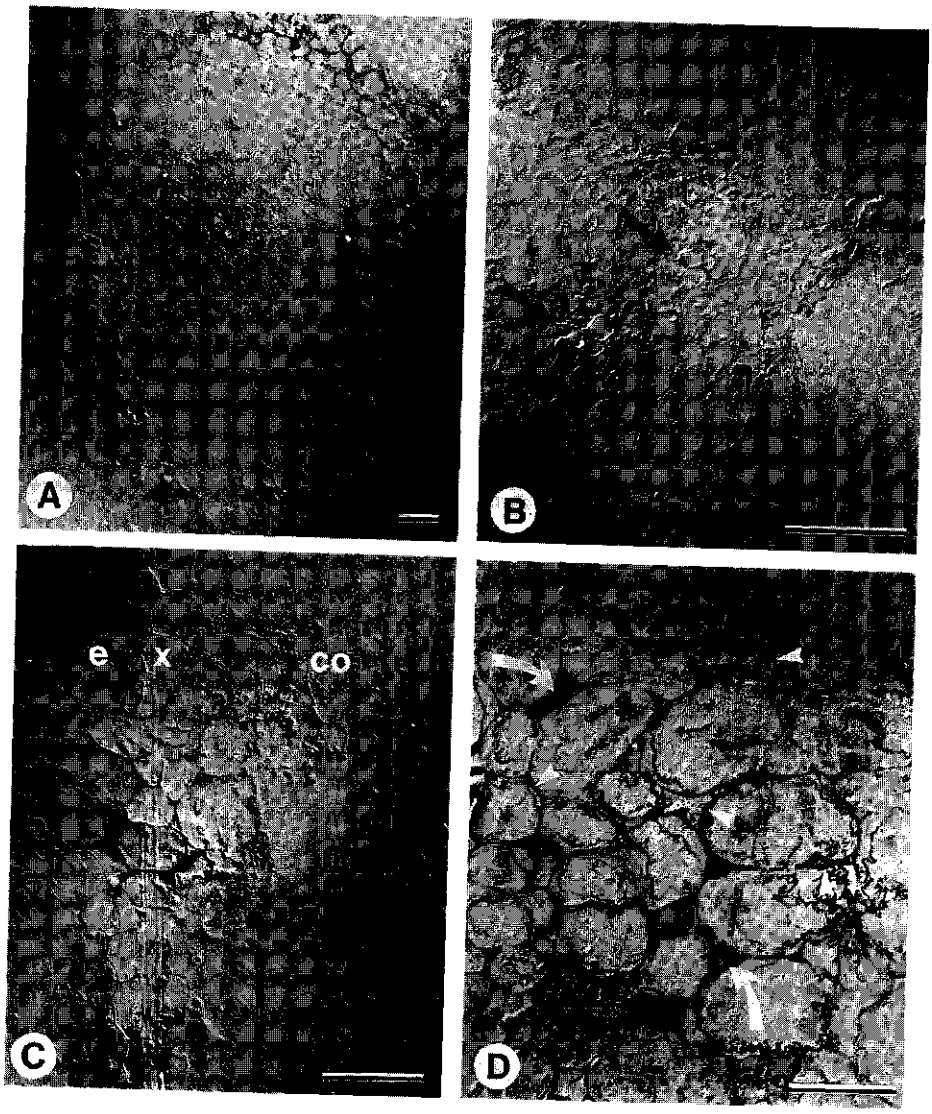

A
B
C
D

Figure 3.9

Detail of Fig. 3.8 B. Fungal hyphae have colonized the cortical and stelar parenchyma, and have reached the protoxylem vessels (px; arrow heads). Hyphae initially colonize parenchyma cells walls and, from these, invade the cell lumina (arrows). Bar = 10 µm.

Figure 3.10 A - D (page 58)

Micrographs of roots of 'Regina' (A, B *and* C) and 'Hermes' (D) seedlings, sixteen days after inoculation with *Fusarium oxysporum* f.sp. *lini*. Sections of young root parts at 3.0 mm from the tip. Bar = 50 µm.
A. General view of a hollow root. The epidermis and exodermis have remained.
B. Detail of A showing remainders of protoxylem vessels (arrows).
C. Detail of A showing epidermis (e) and exodermis (x). Except for a few darkly stained walls, little remains of the cortex (co).
D. A *similar area in 'Hermes'* as shown in C for 'Regina'. Thickened, darkly stained walls (arrow heads) and gum-like compounds in the intercellular spaces (arrows) are slightly more abundant.


A
B
e
x
co
C
D

Invasion of the cell layers of the root cap was counteracted by enhanced meristematic activity of the epidermis, and by successive detachment and sloughing off of the invaded root cap layers. Although distinct appositions were also produced next to penetration hyphae in the root cap cells and the epidermal cells, the present observations suggest that enhanced production, detachment and sloughing off of root cap layers may be equally effective for prevention or retardation of infection. Such a mechanism may be unique to plant species in which the root cap is similarly derived from meristematic epidermal (protodermal) cells, rather than from an apical root cap meristem. In flax, the homology of root cap cells and epidermal cells proper is supported by the present observation that appositions were produced in both cell types, but never in the cortical cells underneath the epidermis. To the knowledge of the authors, such a defense mechanism has not been described previously. However, some similarity exists with the sloughing off of occluded xylem tissue from roots and incidentally also from the stems of carnations infected with *F. oxysporum* f.sp. *dianthi* (Baayen *et al.*, 1989, 1996).

Once fungal hyphae had reached the epidermis, colonization of the cortex and stele was rapid and massive. The fungus rapidly degraded the cortex, hollowing out the root, and eventually also the xylem. While protoxylem vessel elements were invaded by the fungus, their colonization was no more rapid than that of cortical cells. Colonization and disease in flax seedling roots thus resemble root rot such as has been described for lily root tips infected by *F. oxysporum* f.sp. *lilii*, including plasmolysis of affected cells and degradation of their walls (Baayen, 1992, 1996; Baayen and Rijkenberg, unpublished results). Although *F. oxysporum* f.sp. *lini* induces wilt symptoms in flax typical root rot symptoms are a well-known part of the disease syndrome. Boyle (1934) even considered root rot and wilt of flax to represent two distinct types of the disease, each with independently inherited resistance factors. Hollowed-out roots as presently described have been observed commonly and consistently in field trials of the first author (Kroes, unpublished results). During later stages of pathogenesis, the fungus

supposedly moves from the rotten parts of the root through the xylem into the stem, thus switching to the vascular phase of its life cycle, mentioned by previous authors (Millikan, 1951; Turlier *et al.*, 1994).

The observations of this study lead to a model of infection and colonization that differs from that proposed by Turlier *et al.* (1994). These authors suggested that the fungus colonizes the undifferentiated cells in the meristematic zone and remains viable inside these cells in the form of endomycelium. Endophytic fungal growth would follow cell division, the hyphae being later eliminated from the differentiated cortex and phloem cells while remaining alive in the epidermis, and in and between the vessels and the stelar parenchyma. In the present study, however, colonized epidermal and cortical cells underwent severe plasmolysis and disorganization of cell contents and often suffered cell wall degradation. Shortly, colonized cells appeared moribund or dead rather than supportive of endophytic hyphae; fungal growth inside cortical cells without apparent damage was at best a short transient phase in disease development. Also, the protoxylem was reached by a massive front of hyphae, rather than by differentiation of endophytically colonized undifferentiated cells into protoxylem vessels. No indications at all were obtained that the fungus spreads endophytically into the epidermis and cortex, later on to be eliminated from the cortex but not from the epidermis. The presence of hyphae in stele and epidermis but not in the cortex mentioned by Turlier *et al.* (1994) may have been due to progressive colonization of the stele after onset of the vascular phase of the disease, coinciding with restricted invasion of the upper outskirts of the root cap. In contrast to the model proposed by Turlier *et al.* (1994), the observations presented here fit closely with earlier models of root infection by *F. oxysporum* (Bishop and Cooper, 1983; Benhamou *et al.*, 1994), in which the root tip xylem is reached by centripetal growth of hyphae through the cortex and paratracheal parenchyma.

Appressoria were not observed in the present study. Turlier *et al.* (1994) interpreted short hyphal branches as representing appressorium-like

structures, although the mycelium never seemed to penetrate under these structures. In the present study fungal hyphae rather invaded the middle lamella region of the anticlinal cell walls of calyptra and epidermis cells in the same manner as described for lily, pea and tomato infected by *F. oxysporum* f.sp. *lilii*, f.sp. *pisi* and f.sp. *lycopersici*, respectively (Baayen and Rijkenberg, unpublished results; Bishop and Cooper, 1983). Infection of the inner cell layers of the root cap and the epidermis induced the formation of appositions in some cells, while other cells collapsed. The reason for these different responses is not clear. Swelling of cells adjacent to collapsing ones may be a matter of osmotic pressure.

Disease development in resistant 'Hermes' did not differ appreciably from that in susceptible 'Regina', although after 16 days reduction in sprout length was more common in 'Regina' than in 'Hermes'. Microscopically, differences were minimal as well although some plant reactions, like the formation of appositions and intercellular gom-like and phenolic compounds were observed earlier in 'Hermes', in general. Differences in pathogenesis between susceptible and resistant cultivars have been reported previously, the fungus being restricted to the cortex in 20-day-old seedlings of resistant 'Redwood', whereas the phloem and xylem of susceptible 'Punjab' had been invaded by that time (Nair, 1956; Nair and Kommedahl, 1957). A possible explanation for the minimal differences found between 'Regina' and 'Hermes' may be the development of rot rather than wilt symptoms in the present study. Furthermore, it might be plausible that the slight differences in early reactions may have larger consequences in later growth stages.

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CHAPTER 4

The use of ergosterol and fusaric acid as a determinant of the resistance of flax (*Linum usitatissimum)* to *Fusarium oxysporum* f.sp. *lini*

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Submitted

ABSTRACT

Measurements of ergosterol and fusaric acid were carried out, using plants of flax ('Hermes' and 'Laura') and linseed ('Culbert' and 'Linda'), all cultivars with field resistance to *Fusarium oxysporum* f.sp. *lini,* and of a susceptible flax cultivar ('Regina'). Plants were grown in a flax wilt nursery and in an *in vitro* test. Very low concentrations of ergosterol were detected in the healthy looking material of the resistant cultivars originating from the field test as well as the *in vitro* test, whereas the concentrations in the diseased cultivar were very variable. Fusaric acid was detected in none of the experiments *in planta.* Ergosterol levels appeared too variable to be useful in estimating levels of resistance or tolerance in flax. Fusaric acid was not detectable at all in these cultivars. As the fungus was present in the apparently resistant cultivars in very low levels and in the susceptible cultivar in variable, but clearly higher amounts, resistance seems the case for these cultivars, rather than tolerance.

Key words: ergosterol, flax, fusaric acid, *Fusarium oxysporum* f.sp. *lini,* linseed, *Linum usitatissimum,* resistance, tolerance.

INTRODUCTION

Flax wilt is one of the most frequent diseases in flax (*Linum usitatissimum* L.), and is caused by the fungus *Fusarium oxysporum* f.sp. *lini* (Bolley) Snyder and Hansen. While flax defense mechanisms have been studied extensively (Tisdale, 1917; Nelson and Dworak, 1926; Burnham, 1932; Knowles and Houston, 1955; Knowles *et al.*, 1956; Pavelek, 1983, see also Chapter 3), it is still open to discussion whether the plant defense mechanism is based on resistance or tolerance, and whether toxins play a role in fungal development. According to Agrios (1988) resistance is the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or damaging factor, while tolerance is the ability of a plant to sustain the effects of a disease without dying or suffering serious

injury or crop loss. Also the amount of toxic residue allowable in or on edible plant parts is under the law.

The fungal sterol ergosterol has been used to quantify the amount of fungal material *in planta* (Seitz *et al.*, 1979; Gretenkort and Ingram, 1993; Liljeroth *et al.*, 1996) and in cases where *Fusarium* was the causal agent of the disease, the results indicated good correlations with fungal development (Snijders and Krechting, 1992; Gretenkort and Helsper, 1993; Remotti, 1996a). Remotti (1996a) found that even very small amounts of ergosterol were detectable, indicating that quantification of the fungal material by measuring the amounts of ergosterol *in planta* may be used to determine the presence of fungal material even in healthy looking plants.

Snijders and Krechting (1992) found a reasonable correlation between the fungal biomass of *Fusarium culmorum* (W. G. Smith) Saccardo in wheat as determined by ergosterol measurements, and the toxin, produced by this fungus, deoxynevalenol (DON) *in planta*. Remotti (1996b) could not find a relationship between the amount of ergosterol in gladiolus, inoculated with *Fusarium oxysporum* f.sp. *gladioli* (Mass.) Snyder & Hansen, and the toxin produced by this fungus, fusaric acid. *Fusarium oxysporum* f.sp. *lini* produced large amounts of fusaric acid *in vitro*, compared with other *forma speciales* of *Fusarium oxysporum* that cause wilt (Remotti, 1996b). Fusaric acid has been found in various crops (Gapilout *et al.*, 1996; Remotti and Löffler, 1996). Davis (1969) was able to detect reasonable amounts of fusaric acid in flax *in planta*. Apparently fusaric acid plays a role in the pathogenicity of the fungus. With help of ergosterol measurements to quantify the amount of the fungus, and by fusaric acid measurements to assess the possible level of pathogenicity and disease assessments, it might be possible to see whether resistance or tolerance to *Fusarium oxysporum* f.sp. *lini* is operating and if there is a relationship between the amount of ergosterol and the amount of fusaric acid *in planta*.

Field tests at two flax wilt nurseries, and an *in vitro* test, using five flax and linseed cultivars and an isolate of the fungus, were carried out to

determine the nature of the plant reaction. Ergosterol as well as fusaric acid contents were measured repeatedly and compared with disease ratings in the field experiment, and with sprout length measurements in the *in vitro* experiment.

MATERIALS AND METHODS

Field experiment

In a pilot experiment straw of 'Regina' was used, grown at a flax wilt nursery at Ingelmunster, Belgium. After harvest, flax straw with severe wilt symptoms was collected randomly. The straw was stored in a shed for two years. Afterwards the fungus was isolated from the straw, purified and characterized by exposing 'Regina' flax seedlings to the fungus in an *in vitro* test, according to the rules of Koch (Agrios, 1988). The seedlings showed the wilting symptoms as observed to be caused by *Fusarium oxysporum* f.sp. *lini* (see Chapter 2). Three random samples of the straw were used for ergosterol extraction.

In a field experiment seeds of 'Culbert' and 'Hermes' (Coopérative linière de Fontaine Cany, Fontaine le Dun, France), 'Laura', 'Linda' and 'Regina' (CPRO-DLO stock collection) were used. The cultivars were grown in a flax wilt nursery in Normandy, France (see Chapter 1, Fig. 1.1) and in a non-infested field near this nursery. A split plot design, in three replicates, with the factors harvest and cultivar, was used for both fields. The plot size was two rows of 2.5 m, 30 cm apart. The density of sowing was 1.0 g m^{-1}. Between every harvest-block of five cultivars two rows with a resistant cultivar ('Hermes') was sown, to prevent possible side effects caused by the occurrence of empty spaces from the early harvests. From six weeks after sowing, cultivars were harvested every fortnight, after disease scoring according to Rashid and Kenaschuk (1993), six harvests in total. The plants were counted and freeze-dried. Per sample total dry weight was determined. The plants were stored at -20 °C until extraction. The fungus was isolated from randomly taken soil and from flax samples and characterized according

to the rules of Koch, as in the pilot experiment.

***In vitro* experiments**

Seedlings of the same five cultivars were grown in liquid medium, in preserving jars (see Chapter 2) and exposed to the *Fusarium* isolate Fof-F60 (see Chapter 3). Per jar, 16 seeds of one cultivar were sown. For both ergosterol and fusaric acid measurements, 120 jars were inoculated with the isolate Fof-F60 and 120 jars with sterile water as a control. Sprout lengths were measured before the harvests at one, two and three weeks after inoculation. The plants were freeze-dried, dry weight per sample was determined and the samples were stored at -20 °C until extraction. The total experiment consisted of a randomized block design with five cultivars, two treatments (isolate and control), three harvest times, two types of measurements (ergosterol and fusaric acid), and two replicates in space and two replicates in time.

Extraction and measurement of ergosterol and fusaric acid

The assay procedure for ergosterol was a modified version of that of Gretenkort and Helsper (1993). Three samples of 0.5 g of the two year old 'Regina' straw, samples of 0.5 g (in duplo) of harvests one to three of the field experiment, and samples of 1.0 g (in duplo) of harvest four to six of the field experiment, were cut into pieces and ground in a mortar. The same procedure was followed for the total amounts of all dried plants of the *in vitro* experiment. The ground material was transferred to teflon-lined screw-cap test tubes and saponified in 5 ml 20% methanolic KOH (w/v), 30 min, 70 °C, under constant agitation. After saponification 1.8 ml demi-water and 3.8 ml hexane was added to the test tubes and firmly shaken for 5 min. The upper hexane-phase was collected in 4 ml vials and dried stepwise at 35 °C, under a flow of nitrogen. This step was repeated three times. The residue was resuspended in 1 ml methanol. Aliquots of 20 to 100 µl were analyzed by reverse-phase HPLC on a C_{18} column (Spherisorb ODS 2, 125 * 4 mm,

particle size 3 µm) heated to 25 °C, with methanol (0.6 ml/min) as the mobile phase.

Where GC analysis was employed to confirm the presence of compounds detected by HPLC analysis, aliquots of 1.0 µl were injected (injection temperature 300 °C) onto a Chrompack CP Sil5 column (length 50 m, coating thickness 0.12 µm, oven temperature 250 °C, carrier H_2 gas). The compounds were detected using a flame ionization detector (temperature 300 °C) and analyzed using a Shimadzu CR3A integrator.

For fusaric acid extraction, samples of 0.5 g (in duplo) of harvests one to three of the field experiment, and samples of 1.0 g (in duplo) of harvest four to six of the field experiment, were cut into pieces and ground in a mortar. The same procedure was followed for the total amounts of all dried plants of the *in vitro* experiment. The ground material was transferred to teflon-lined screw-cap test tubes and suspended in 5 ml methanol, adjusted to pH 10 with 5% NH_4OH. After 30 min extraction under constant agitation, samples were centrifuged at 1500 rpm for 5 min at 4 °C. The supernatants were stepwise collected in 4 ml vials and dried under nitrogen. The cake was resuspended in 5 ml methanol, re-extracted and supernatants were collected in the respective vials and again dried under nitrogen. The residue was methylated with 0.2 ml of diazomethane (Furniss *et al.*, 1989). After 5 min, the excess of diazomethane was evaporated. The residue was extracted three times with 5 ml hexane-ethyl acetate (9:1) and supernatant was concentrated to 1 ml, under nitrogen. Aliquots of 1 µl were analyzed by GC on a Chrompack CP Sil8 column (lenght 25 m, coating thickness 0.12 µm, injection temperature 200 °C, oven temperature 150 °C for 5 min, then a gradient of 10 °C m^{-1}, and final temperature 200 °C, held for 7 min, carrier H_2 gas). The compounds were detected using a flame ionization detector (temperature 250 °C) and analyzed using a Shimadzu CR3A integrator.

Data processing

The numbers of plants recorded in the field experiment were used to calculate the average number of plants per cultivar, per harvest time and per treatment (infested or not). The averages of the infested fields were divided by the corresponding averages of the non-infested fields to obtain the relative plant number per cultivar, per harvest time. The same procedure was followed for the *in vitro* experiment. Also the calculation of relative dry weights of the field and *in vitro* experiments, and the relative plant lengths of the *in vitro* experiment were carried out similarly.

RESULTS

Field experiment

In the two years old, heavily infested flax straw of 'Regina', ergosterol was found in large amounts with little variation in quantity (0.42 ± 0.05 mg g^{-1} straw).

In the new field experiment in Normandy, 'Culbert', 'Hermes', 'Laura' and 'Linda' showed hardly any symptoms according to the disease scoring, 'Regina' however, showed the disease from the fourth harvest date onward (*Table* 4.1). 'Culbert', 'Hermes and 'Linda' had a reduced germination in the infested field compared to the uninfected field. 'Laura' germinated nearly the same in both fields and 'Regina' germinated slightly better in the infested field. Once germinated, the plants grew and developed well until the second harvest date in both fields. In the infested field, from the second harvest date in 'Regina' and from the fourth harvest date in the other cultivars, individual plants started to wilt, showing symptoms like top bowing and necrosis while in the non-infested field no signs of wilt were observed until the last harvest date. The plants finally seemed to dry out but remained standing. The average dry weight of all cultivars in the first harvest was higher for the infested field, compared with the non-infested field. After the first harvest the relative dry weight decreased for all cultivars, and most drastically for 'Regina'.

Table 4.1

Disease scores of five flax and linseed cultivars, taken at six harvest times during the growing season, according to Rashid and Kenaschuk (1993) in a field experiment at a flax wilt nursery in Normandy, France. The relative number of plants was determined by comparing the amounts of plants in the infested field with a parallel experiment in the same year at a non-infested field nearby. The relative dry weight of the plants grown at the infested field are compared with the dry weights of the plants from the parallel experiment at the non infested field. Ergosterol was assessed in the plants originating from the infested field.

Cultivar	Harvest	Disease scoring	Relative number of plants	Relative dry weight	Ergosterol mg g^{-1}
Culbert	1	1.0	0.61	1.59	0.000 a[1]
	2	1.0	0.66	0.70	0.001 a
	3	1.0	0.68	0.87	0.001 a
	4	1.0	0.56	0.33	0.001 a
	5	1.0	0.56	0.35	0.000 a
	6	1.5	0.64	0.46	0.001 a
Hermes	1	1.0	0.77	2.23	0.001 a
	2	1.0	0.86	0.81	0.000 a
	3	1.0	0.86	0.36	0.000 a
	4	1.0	0.84	0.40	0.001 a
	5	1.0	0.65	0.28	0.001 a
	6	1.0	0.65	0.49	0.001 a
Laura	1	1.0	0.83	2.15	0.000 a
	2	1.0	0.98	1.01	0.000 a
	3	1.0	0.84	0.31	0.000 a
	4	1.0	1.01	0.91	0.000 a
	5	1.0	0.98	0.57	0.000 a
	6	1.5	0.92	0.59	0.001 a
Linda	1	1.0	0.62	1.68	0.001 a
	2	1.0	0.59	0.73	0.001 a
	3	1.0	0.66	0.34	0.000 a
	4	1.0	0.59	0.47	0.000 a
	5	1.0	0.86	0.70	0.000 a
	6	1.0	0.65	0.59	0.000 a
Regina	1	1.0	0.92	2.86	0.000 a
	2	1.0	0.98	0.87	0.001 a
	3	1.0	1.10	0.54	0.001 a
	4	3.0	1.07	0.29	0.020 a
	5	4.0	1.02	0.21	0.105 b
	6	7.0	1.03	0.16	0.182 c

[1] Values in column followed by unlike letters are significantly different at $P < 0.05$ according to LSD test; $LSD_{0.05} = 0.76$.

Table 4.2

Relative lengths as a measure of the disease (see Chapter 2), of five flax and linseed cultivars in an *in vitro* experiment. The relative number of plants is determined by dividing the average amounts of the germinated plants per cultivar in the inoculated jars by the average amounts of germinated plants per cultivar of the control jars. The relative dry weight is determined by dividing the average dry weights per cultivar in the inoculated jars by the average dry weights per cultivar in the control jars. Ergosterol was detected in the plants originating from the inoculated jars.

Cultivar	Harvest	Relative length	Relative number of plants	Relative dry weight	Ergosterol mg g^{-1}
Culbert	1	109	1.05	0.89	0.001 a[1]
	2	112	1.05	0.88	0.001 a
	3	88	1.12	1.00	0.002 a
Hermes	1	91	1.05	1.09	0.001 a
	2	95	1.04	1.11	0.001 a
	3	83	1.07	0.96	0.002 a
Laura	1	94	1.11	1.12	0.001 a
	2	97	1.03	1.09	0.002 a
	3	87	0.90	1.04	0.002 a
Linda	1	97	0.96	0.93	0.001 a
	2	101	1.21	0.98	0.002 a
	3	81	1.12	0.99	0.002 a
Regina	1	92	0.98	0.98	0.001 a
	2	89	1.02	1.07	0.002 a
	3	76	1.05	0.94	0.003 b

[1] Values in column followed by unlike letters are significantly different at $P < 0.05$ according to LSD test; $LSD_{0.05} = 0.002$.

Ergosterol was not detected in any cultivar in the non-infested field until and including the last harvest, and also the amounts of ergosterol in 'Culbert',

'Hermes', 'Laura' and 'Linda', grown in the infested field, were very low or negligible (Table 4.1). Ergosterol was detected in clearly higher amounts in 'Regina', from the fourth harvest of the infested field, but these amounts were very variable (respectively 0.020 ± 0.029; 0.105 ± 0.178 and 0.182 ± 0.149).

Fusaric acid was not detected in any cultivar neither from the non-infested field, nor from the infested field.

In vitro experiment

The parameters, dry weight and number of germinated plants, from the *in vitro* experiment showed less pronounced differences, compared with the field test. Germination was equal for all cultivars for non-inoculated and inoculated plants. Dry weight did not distinguish cultivars, only length measurements gave a difference between 'Regina' and the other cultivars in the last stage.

Ergosterol contents were rather low in this experiment too, but relative to the dry weights the amounts of ergosterol were higher than in the field test, except for the pilot experiment (Table 4.2). Fusaric acid was not detected in any of the samples from the *in vitro* test.

DISCUSSION

The measurements of resistance is dependent on the possibilities of measuring the amount of the fungus *in planta*. To assess the amount of fungal material, pathogens can be distinguished in partially visible pathogens and pathogens which cause direct effects on the one hand, and pathogens with indirect effects, such as for instance wilting and withering, on the other hand (Parlevliet, 1993). In the first group of pathogens, assessment of the amount of tissue affected, gives a good estimate of the amount of pathogen present, so the resistance can easily be determined. For the second group of pathogens, with true disease symptoms, no reliable way is available to estimate the amount of pathogen present, and in this group it is difficult to estimate resistance. The *Fusarium*-flax interaction appears to be an

outstanding example of the second group, an interaction whereby indirect effects are measured, such as disease symptoms, dry weight, sprout length measurements, or numbers of dead plants (Table 4.1, Rashid and Kenaschuk, 1991). To assess the amount of fungal material by ergosterol measurements proved to be an acceptable method to determine the amounts of fungal material. Ergosterol was not found in the controls, and was present in all inoculated cultivars in the *in vitro* experiment. Furthermore the ergosterol contents of the flax straw, originating from the pilot experiment, showed little variation.

It proved to be difficult to find a relation between the indirect effects and the true amount of fungus or its toxin. In the field experiment 'Regina' was most affected while the other cultivars were little or not affected at all, according to the disease scoring. The relation between the disease scoring and the ergosterol measurements was weak, while the relation between disease scoring and the other measured indirect effects, relative number of plants and relative dry weight, was poor. The relation between the relative number of plants and relative dry weight was poor, as was the relation between the relative number of plants and the results of the ergosterol measurements. However, ergosterol measurements correlated reasonable well with sprout length measurements in the *in vitro* experiment.

There was measurable damage in the field. The relative dry weights decreased in the infested field for all cultivars, up to 40 - 50% for 'Culbert', 'Hermes', 'Laura' and 'Linda' and up to 84% for 'Regina'. The infested field and the control plots had a similar soil composition and were about 300 m apart, excluding weather as a differential factor. It is therefore reasonable to assume that the decrease of dry weight in the infested field in the healthy looking cultivars was caused by *Fusarium oxysporum* f.sp. *lini*. Very low levels of ergosterol were found in the resistant cultivars from the field experiment. The low level might have been caused by the fact that, while rinsing the soil from the roots, rotten root material got lost. In Chapter 3 *Fusarium oxysporum* f.sp. *lini* was described as a vessel parasite of flax, which also causes root rot.

The present results fit well in this model and the loss of fungal material by rinsing the rotten roots might be an explanation of the low levels of ergosterol.

Only a weak relation between the true disease symptoms and the amount of pathogen, as measured by ergosterol, was observed. Sprout length reduction in *in vitro* conditions and dry weight reduction in field conditions appeared to be the best way ways to measure resistance. In both experiments the amounts of fungus in the susceptible cultivar was higher than in the resistant cultivars, indicating resistance in the cultivars with reduced disease symptoms. In case of tolerance one would have expected similar levels of ergosterol in all cultivars, irrespective of the level of disease symptoms, and this was not the case in both experiments.

Although all *in vitro* inoculated cultivars contained ergosterol, the amounts were too variable to be able to put forward an hypothesis about resistance mechanisms based on the ergosterol contents. Variation in ergosterol contents also was found in some other ergosterol studies (Nout *et al.*, 1987; Lumsden, 1990; Gunnarsson *et al.*, 1996), so it seems that the fungus is present in variable amounts in diseased cultivars. The observation that completely healthy plants are regularly present in susceptible cultivars, (Kroes, unpublished results, see also Chapter 1, Fig. 1.2) indicates, that escapes occur even in plots of susceptible plants and this might be a cause of the variable amounts of fungal material in 'Regina'.

The fact that in all *in vitro* grown cultivars ergosterol was found while inoculation took place by adding the fungus close to the roots, indicates that the fungus is able to enter the roots of resistant cultivars.

Although *Fusarium oxysporum* f.sp. *lini* produces high amounts of fusaric acid in *in vitro* cultures (Remotti, 1996b), it was not possible to detect any fusaric acid *in planta,* not even in any harvest of the ergosterol containing cultivars, so a relationship between amounts of fungal material and of toxin could not be detected. While the amounts of ergosterol, representing the amount of fungal material, were very low, it is likely that the amounts of the toxin were at least very low too. It is reported that fusaric acid can readily be

broken down *in planta* (Kuo and Scheffer, 1964; Davis, 1969), but the present results do not indicate that resistance is related to a possible accelerated breakdown of fusaric acid.

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CHAPTER 5

Do races in *Fusarium oxysporum* f.sp. *lini* exist? A study for race specific interactions in flax wilt

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ABSTRACT

Prospects of resistance in flax and linseed to *Fusarium* wilt disease have been evaluated by testing the aggressiveness and virulence of 25 isolates of *Fusarium oxysporum* f.sp. *lini* towards a number of different sources of resistance in flax and linseed (*Linum usitatissimum*). Isolates differed strongly in aggressiveness as did the host in resistance. Significant interaction variance was observed between host genotypes and pathogen isolates, but its magnitude was small compared to that of the main effects, the resistance of the cultivars and the aggressiveness of the isolates. The interactions, probably due to race specific effects, were mainly caused by a group of isolates with low aggressiveness originating from the American continent. The race specific interactions might be are based on minor gene differences among isolates.

Keywords: flax, linseed, *Linum usitatissimum*, *Fusarium oxysporum* f.sp. *lini*, races, wilt, race specificity, virulence, interaction, *in vitro*.

INTRODUCTION

Flax and linseed (*Linum usitatissimum* L.) are grown for fibers or oil, respectively. Western Europe, North Africa and Asia have a long tradition in growing flax for textile manufacturing, whereas North and South America and Australia are continents where linseed is grown. An important problem in growing flax as well as linseed is the *Fusarium* wilt disease caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *lini* (Bolley) Snyder & Hanssen. In flax as well as linseed the disease can cause up to 90% losses in yield. The predominant symptoms are wilting and withering of the plant in the seedling or in the adult plant stage (Kommedahl *et al.*, 1970). The chlamydospores may survive in the soil for decades. Houston and Knowles (1949) described an outbreak of flax wilt in a field on which flax had not been grown for fifty years. The disease can be prevented by seed treatment with fungicides, combined with a crop rotation scheme in which flax is grown only once in six years (Vreeke *et al.*, 1991) on not infected soils. Control of the disease by cultivation

of *Fusarium* resistant cultivars will increase the possibilities of growing flax and linseed.

High levels of resistance were found in some Dutch and French flax cultivars (Anonymous, 1986; 1988; 1990; 1992; 1994; Ebskamp and Bonthuis, 1993; 1997) and in some American and Argentinean linseed cultivars (Popescu *et al.*, 1994). In flax this resistance is regarded to inherit quantitatively (Pavelek, 1983). If durable, these sources of resistance can be used to develop new resistant flax and linseed cultivars. However, very little of the inheritance of flax wilt resistance and the durability of resistances is yet understood, despite extensive research (Tisdale, 1917; Nelson and Dworak, 1926; Burnham, 1932; Knowles and Houston, 1955; Knowles *et al.*, 1956; Pavelek, 1983). Field trials and greenhouse tests indicated that *Fusarium oxysporum* f.sp. *lini* may comprise an indefinite number of races (Armstrong and Armstrong, 1968; Kommedahl *et al.*, 1970; Islam, 1991), but results may also be interpreted to indicate a single common race with minor gene differences among isolates. According to Parlevliet (1985) races in parasitic fungi can only be defined and designated on the basis of their virulence/avirulence pattern on a set of differential host cultivars if the race-specific effects are sufficiently large. Recent field trials and greenhouse tests could not solve the question whether race specificity in flax and linseed and thus identifiable races in *Fusarium oxysporum* f.sp. *lini* exist (Fouilloux *et al.*, 1991; Ondrej, 1993).

It was observed that some resistant cultivars, like the flax cultivar 'Natasja' in Western Europe (Sneep *et al.*, 1972; Bonthuis and Ebskamp, 1992) and the linseed cultivar 'Bison' in the USA (Kommedahl *et al.*, 1970) kept their resistance for at least 20 year. The observation that some highly resistant flax cultivars like 'Laura' and 'Marina', descendants from 'Natasja', lost their resistance in Normandy, France in about six years (Trouvé, personal communication) may be an indication that a new race had developed there. In the present study a recently developed *in vitro* seedling tube test for *Fusarium*-flax interactions (see Chapter 2) was used to test whether race specificity and

identifiable races exist in a group of *Fusarium oxysporum* f.sp. *lini* isolates originating from a wide geographical range. It also was studied whether a new race in France could be detected by comparing isolates originating from the French field where the above mentioned observations were made with an isolate from a Dutch *Fusarium*-flax nursery.

MATERIALS AND METHODS

Host and pathogen

Seeds from resistant, moderately resistant and susceptible flax and linseed cultivars originating from Europe and North and South America were used. The origin of the cultivars is summarized in Table 5.1.

From the flax wilt nursery "Lelystad" the Flevopolder, the Netherlands, two soil samples were taken (soil sample 1 and 9, Table 5.2) and from the flax wilt nursery "La Gaillarde", Normandy, France, seven soil samples were randomly taken (samples 2 - 8, Table 5.2). Spores of *Fusarium oxysporum* were isolated using a slightly modified Komada medium (Komada, 1975), according to the method described by Löffler and Mouris (1989), and were characterized as *Fusarium oxysporum* f.sp. *lini* by microscope studies and by bio-assay, using an *in vitro* seedling tube test (see Chapter 2). From the soil samples 1, 6, 7, 8 and 9 one single spore was isolated and from the soil samples 2, 3 and 4 two single spores. From sample 5 one single spore was isolated, multiplied and two duplicates were taken from this isolate. The cultures of these single spores were stored on PROTECT bacterial preservers (Technical Service Consultants Ltd, UK), at -80 °C.

Isolates of *Fusarium oxysporum* f.sp. *lini*, originating from plant material, were obtained from European and North and South American institutes. From these isolates single spores were cultivated and stored in the same bacterial preservers at -80 °C.

Isolates selected for virulence tests were revitalized on OXOID potato dextrose agar plates (PDA) for 14 days in a growth chamber at 23 °C in dark conditions. The spores were rinsed from the PDA plates with sterile water,

Table 5.1

Flax type, origin and resistance levels of the 16 cultivars used in the present study. Data ITL are field observations from 1991 of the flax *Fusarium* wilt nursery "La Gaillarde", Normandy, France. Resistance of the cultivars was visually determined using a disease severity scale from 1 - 9, whereby 1 = very resistant and 9 = very susceptible (Beaudoin, 1991).

Cultivar	Abbr.	Type	Origin	Data ITL	Resistance*
Ariane	ARI	flax	France	5.0	MR
Atalante	ATA	linseed	France	1.5	R
Barbara	BAR	linseed	Hungary	7.5	S
Bison	BIS	linseed	USA	-	R
Culbert	CUL	linseed	USA	-	R
Evelin	EVE	flax	The Netherlands	1.8	R
Hera	HE1	flax	The Netherlands	-	S
Hermes	HE2	flax	France	2.3	R
Laura	LAU	flax	The Netherlands	1.8	MR
Linda	LIN	linseed	France	1.3	R
Marina	MAR	flax	The Netherlands	1.8	R
Natasja	NAT	flax	The Netherlands	3.5	R
Ocean	OCE	linseed	France	9.0	S
Regina	REG	flax	The Netherlands	9.0	S
Tape Parana	TAP	linseed	Argentina	-	R
Viking	VIK	flax	France	2.3	R

*Resistance visually determined by Kroes, unpublished data. R = resistant, MR = moderately resistant, S = susceptible.

filtered over glass-wool and the spore suspension was adjusted to 10^5 spores per ml, using a Buerker Turk haemocytometer.

In Table 5.2 the code names of the *Fusarium* isolates used are given, as well as the place of origin, the isolation source and the aggressiveness, determined in an earlier *in vitro* pilot experiment. Fof stands for *Fusarium oxysporum* f.sp. *lini*.

Table 5.2

Source, place of origin, source of isolation and aggressiveness of the 25 isolates used in the present study. Aggressiveness was determined in an earlier pilot experiment (Kroes, unpublished results), ++ very aggressive; + aggressive, ± moderately aggressive; -- unknown.

Code name	Place of origin	Isolation source	Aggressiveness
Fof-N4	Lelystad, The Netherlands	soil sample 1	--
Fof-F7	La Gaillarde, France	soil sample 2	--
Fof-F7a	La Gaillarde, France	soil sample 2	--
Fof-F8	La Gaillarde, France	soil sample 3	--
Fof-F8a	La Gaillarde, France	soil sample 3	--
Fof-F9	La Gaillarde, France	soil sample 4	--
Fof-F9a	La Gaillarde, France	soil sample 4	--
Fof-F10	La Gaillarde, France	soil sample 5	--
Fof-F10a	La Gaillarde, France	soil sample 5, (duplo of Fof-10)	--
Fof-F11	La Gaillarde, France	soil sample 6	--
Fof-F12	La Gaillarde, France	soil sample 7	--
Fof-A1	Castellar, Argentina	linseed	+
Fof-A2	Castellar, Argentina	linseed	±
Fof-B1	Ingelmunster, Belgium	flax	++
Fof-B2	Ingelmunster, Belgium	flax	++
Fof-C2	Morden, Canada	linseed	+
Fof-C3	Morden, Canada	linseed	±
Fof-F1	Rennes, France	flax	+
Fof-F26	La Gaillarde, France	soil sample 8	++
Fof-F60	Versailles, France	flax	++
Fof-N1	Wageningen, The Netherlands	flax	+
Fof-N3	Metslawier, The Netherlands	flax	++
Fof-N10	Lelystad, The Netherlands	soil sample 9	++
Fof-U1	Torzhok, Russia	flax	++
Fof-U2	Torzhok, Russia	flax	+

Virulence screening

Cultivars were screened in two experiments using the *in vitro* seedling tube test (see Chapter 2). Resistance of the cultivars as well as aggressiveness of the isolates were expressed as the length of the sprouts of the tested plants relative in % to the length of the sprouts of healthy plants of the same cultivars. This means that the more susceptible the cultivar or the more aggressive the isolate the lower the relative length.

In the first experiment six plants from eight flax cultivars, all except 'Hermes', and six linseed cultivars, all except 'Linda', (Table 5.1) were tested. Isolates originating from the French and Dutch soil were used in this experiment, (Fof-N4, Fof-F7 - 12, including the a-isolates, Table 5.2) and the isolate Fof-F60, a highly aggressive isolate used as a standard in French greenhouse tests (INRA, Versailles) to determine *Fusarium* resistance in new flax cultivars. The experiment consisted of six randomized blocks, each containing a singular sample of all cultivar-isolate combinations, plus a cultivar-water combination (healthy control).

In the second experiment all nine flax cultivars and seven linseed cultivars (Table 5.1) were inoculated with 14 isolates from a wide geographical range, (Fof-A1, Fof-A2, Fof-B1, Fof-B2, Fof-C2, Fof-C3, Fof-F1, Fof-F26, Fof-F60, Fof-N1, Fof-N3, Fof-N10, Fof-U1, Fof-U2, Table 5.2), thus including the French standard isolate Fof-F60. The experiment was replicated three times, and each replication included three randomized blocks, each containing a singular sample of all cultivar-isolate combinations, and in addition a cultivar-water combination (healthy control).

Statistical analysis

The data were analyzed by an Analysis of Variance, using the GENSTAT program and by agglomerative clustering (Corsten and Denis, 1990). Strongly interactive cultivar * isolate combinations were indicated, using an adapted tetrad module for tracing combinations, significantly deviating from additivity (Bradu and Hawkins, 1982; Van Eeuwijk, CPRO-DLO, unpublished results).

RESULTS AND DISCUSSION

Resistance

In both experiments the average resistance of the different flax and linseed cultivars ranked from very resistant to very susceptible. The flax cultivars 'Natasja' and 'Hermes' proved to be very resistant while 'Culbert' was the most resistant linseed cultivar in this study. The flax cultivar 'Regina' and the linseed cultivar 'Ocean' were most susceptible. The measured resistances correlated reasonably well with field data from the flax wilt nursery "La Gaillarde" obtained in 1991 (Table 5.1); the correlation coefficient, r, between these field data and the data from experiment 1, the experiment testing isolates originating from this field, was significant at P = 0.05 (r = -0.90*). The correlation between experiment 2 and the field data was lower and not significant (r = -0.57). The correlation coefficient between the two experiments was significant at P = 0.05 too (Fig. 5.1, r = 0.69*), although the good correlation was mainly due to two of the 16 cultivars, the extremes: susceptible 'Ocean' and resistant 'Culbert' (Table 5.1). In flax wilt nurseries small differences in levels of attack are registered from year to year, caused by differences in temperature (Kikuchi, 1934; 1940) and moisture (Kommedahl *et al.*, 1970), or by the influence of various biotic factors (Tursunkhodzhaev, 1965). Using the *in vitro* tube test these influences are reduced but not excluded. Temperature and moisture are standardized as much as possible, but although the seeds are externally sterilized, the incidence of seed-borne parasites like *Alternaria linicola* Groves & Skolko cannot always be excluded. This might be a cause of variation. Another cause of variation is that, although flax is a self pollinator, the flax genome is not completely stable. Changes in the genome can be environmentally induced (Durant, 1972). In this light the observed correlations between field data and *in vitro* data are quite acceptable.

Aggressiveness

A large variation in aggressiveness of the isolates was found in both

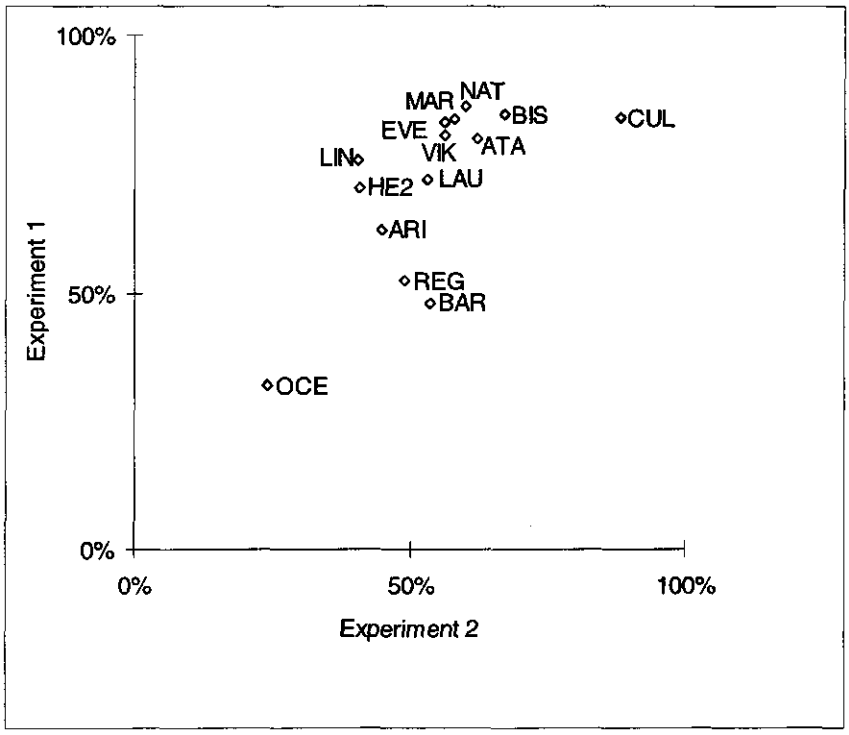

Figure 5.1
Correlation between experiment 1 and experiment 2. Relative sprout length per cultivar was plotted on the x-axis for experiment 2 and on the y-axis for experiment 1. r = 0.69*, P = 0.05.

experiments (Tables 5.3 and 5.4). In the first experiment, where different isolates from one field were tested, the variation in average aggressiveness was less than in the second experiment, where isolates were tested from diverse continents. The mean level of attack in experiment 1 was lower than in experiment 2.

In both experiments the French standard isolate, Fof-F60 caused great sprout length reductions. In the first test, plants inoculated with this isolate reached on average 58% of the length of the control plants, while in the second test the relative sprout length of the inoculated plants was only 38%. All isolates from the French soil were less aggressive than the isolate Fof-F60. In the second experiment two other isolates were found to be more aggressive

Table 5.3

Disease score values, expressed as relative sprout lengths, compared with control plants of the same cultivar, of 14 flax and linseed genotypes inoculated with 12 isolates of *Fusarium oxysporum* f.sp. *lini*, sorted towards decreasing aggressiveness of the isolates (horizontally) and towards increasing resistance of the genotypes (vertically). Data from experiment 1. AR1 = average resistance per cultivar for experiment 1. AA1 = average aggressiveness per isolate for experiment 1.

	Fof F60	Fof N4	Fof F7	Fof F9	Fof F10	Fof F8a	Fof F7a	Fof F10a	Fof F12	Fof F11	Fof F8	Fof F9a	AR1
OCE	19	32	26	25	27	33	34	34	33	28	40	51	32
BAR	35	55	22	45	55	47	59	52	49	40	42	76	48
REG	49	37	57	34	37	40	52	40	71	64	55	94	53
ARI	40	49	41	49	63	51	65	63	65	79	75	104	62
HE1	38	19	86	70	73	94	98	74	62	34	101	96	70
LAU	45	65	56	74	67	82	72	65	88	67	87	96	72
LIN	70	49	79	75	75	88	81	81	50	82	82	97	76
ATA	76	86	65	79	81	56	68	80	85	95	88	97	80
VIK	57	71	61	62	75	90	96	90	82	94	79	109	81
MAR	81	81	42	83	81	82	72	89	77	96	100	112	83
EVE	66	50	83	87	79	86	79	87	91	87	102	105	84
BIS	65	73	79	90	70	82	69	85	78	92	116	114	84
CUL	63	86	70	90	84	74	64	76	90	117	86	104	84
NAT	101	78	72	79	82	66	69	79	105	105	91	105	86
AA1	58	59	60	67	68	69	70	71	73	77	82	97	71

than Fof-F60, namely the Russian Fof-U1 and the Dutch Fof-N3. Comparing the first and the second experiment the ranking order of the cultivars after screening with Fof-F60 was not always the same. In experiment 1 'Natasja' was not affected at all and in experiment 2 this cultivar showed a more intermediate reaction. 'Marina' and 'Atalante' were relatively little affected in experiment 1 and rather strongly in experiment 2.

Isolates originating from one soil sample varied considerably in their ability to reduce seedling growth, except the two identical isolates, Fof-10 and

Table 5.4

Disease score values, expressed as relative sprout lengths, compared with control plants of the same cultivar, of 16 flax and linseed genotypes inoculated with 14 isolates of *Fusarium oxysporum* f.sp. *lini*, sorted towards decreasing aggressiveness of the isolates (horizontally) and towards increasing resistance of the genotypes (vertically). Significant multiple outliers, traced by a tetrad model, are underlined. Data from experiment 2. (AR2 = average resistance per cultiuvar for experiment 2, AA2 = average aggressiveness per isolate for experiment 2.

	Fof U1	Fof N3	Fof F60	Fof F26	Fof N10	Fof B1	Fof B2	Fof U2	Fof F1	Fof N1	Fof C2	Fof C3	Fof A2	Fof A1	AR2
OCE	21	13	16	12	22	23	21	16	31	10	12	25	59	56	24
HE1	21	25	18	36	15	23	23	33	38	56	34	70	108	***72***	41
LIN	27	23	22	21	27	42	62	26	37	39	57	95	56	34	41
ARI	21	35	13	37	26	19	22	36	51	50	68	91	84	75	45
REG	20	35	22	40	44	23	41	43	56	65	53	47	97	99	49
LAU	35	25	38	33	28	53	43	55	64	62	61	78	88	81	53
BAR	28	27	37	36	31	29	29	38	34	***120***	52	53	71	***169***	54
VIK	38	37	36	43	39	42	35	49	78	74	66	74	85	92	56
MAR	36	37	43	41	29	47	56	35	49	76	68	91	91	87	56
EVE	32	34	32	40	55	47	55	55	63	73	74	84	82	87	58
NAT	45	41	35	42	41	58	49	70	67	64	80	82	98	71	60
ATA	30	51	43	39	51	56	51	63	50	71	***94***	91	94	85	62
TAP	36	45	52	40	64	51	51	***90***	58	50	75	86	90	99	63
BIS	44	53	57	45	48	52	59	72	62	68	93	72	111	106	67
HE2	52	54	78	65	56	80	85	90	77	86	93	108	98	106	81
CUL	51	73	73	74	93	87	97	109	88	96	108	101	94	91	88
AA2	34	38	38	40	42	46	49	55	57	66	68	78	88	88	56

Fof-10a. These identical isolates of Fof-10 had an average difference in aggressiveness of 3% while the other isolates originating from one soil sample had mutual differences of 10 up to 30% (Table 5.3). This indicates clearly that the isolates in this soil were not genetically identical. It can be concluded that in the flax wilt nursery "La Gaillarde" different strains exist closely together in

the soil. This phenomenon was described too for the flax wilt nursery at St. Paul, Minnesota, where much flax research was performed between 1900 and 1970 (Kommedahl *et al.*, 1970).

From the second experiment it can be seen that all isolates originating from the European continent caused a higher level of length reduction than the isolates from Canada and Argentina (Table 5.4). The Argentinean isolates hardly attacked the seedlings, compared with other isolates from experiment 2, whereas the Canadian isolates caused intermediate reductions, compared with other isolates from experiment 2. The levels of aggressiveness of the Canadian isolates were comparable with many isolates of the first experiment. Within the group of European isolates, large differences existed between the least and the most aggressive isolate originating from one region. Only the two Belgian isolates did not differ much in average aggressiveness. Fof-N10 and Fof-F26, two isolates originating from soil samples, did not deviate from isolates from plant material in their ability to retard seedling growth.

Interaction; the existence of races

The main objective of this research was to get information about the possible existence of identifiable races of *Fusarium oxysporum* f.sp. *lini*. The statistical analyses were therefore focused on interaction effects. In separate Analyses of Variance (ANOVA) the cultivar effect, the isolate effect and the effect of cultivar * isolate interaction was calculated for each of the experiments. The ANOVA showed in both cases that the variance was mainly due to cultivar effects and isolate effects, corresponding with resistance of the *Linum* cultivars and aggressiveness of the *Fusarium* isolates, respectively (Table 5.5). The magnitude of the cultivar * isolate interaction variance was small but significant in both experiments, indicating the possible existence of race-specific effects.

To detect a pattern in the interaction effects an agglomerative clustering (Corsten and Denis, 1990) was performed for both experiments. By this clustering process cultivar * isolate interaction patterns were clustered. For

Table 5.5

Analyses of Variance of sprout length measurements of flax and linseed seedlings inoculated with *Fusarium oxysporum* f.sp. *lini* isolates (Table 5.2) or with sterile water from experiment 1 and 2 (Table 5.3 and 5.4).

Exp.	Source of variation	D.f.	S.s.	M.s.	V.r.	F pr.
1	Block	5	27.4	5.5	2.4	
	Isolate (or water) (Is)	12	700.4	58.4	25.4	<.001
	Cultivar (Cv)	13	1270.2	97.7	42.4	<.001
	Cv * Is	156	549.9	3.5	1.5	<.001
	Residual	905	1992.0	2.3		
	Total	1091	4456.4			
2	Replicates (Rep)	2	279.5	139.7	16.5	0.004
	Residual	6	50.9	8.5	3.6	
	Isolate (or water) (Is)	14	3677.6	262.7	109.8	<.001
	Cultivar (Cv)	15	4276.6	285.1	119.2	<.001
	Rep * Cv	30	478.1	15.9	6.7	<.001
	Rep * Is	28	171.9	6.1	2.6	<.001
	Cv * Is	210	1304.9	6.2	2.6	<.001
	Rep * Cv * Is	420	1239.7	3.0	1.2	0.003
	Residual	1434	3030.6	2.4		
	Total	2159	13379.0			

experiment 1 clusters were formed for the isolates as well as the cultivars (Fig. 5.2), but there was very little difference between any cluster. The P-value of all steps in the clustering procedure was 1.0, so division into any group had no significance at all. This means that the interaction patterns found for the isolates originating from the flax wilt nursery "La Gaillarde" when clustered with the Corsten method had little importance, although the ANOVA showed a small but significant interaction variance.

For experiment 2, the P-values varied inbetween 1.0 and 0.0 indicating

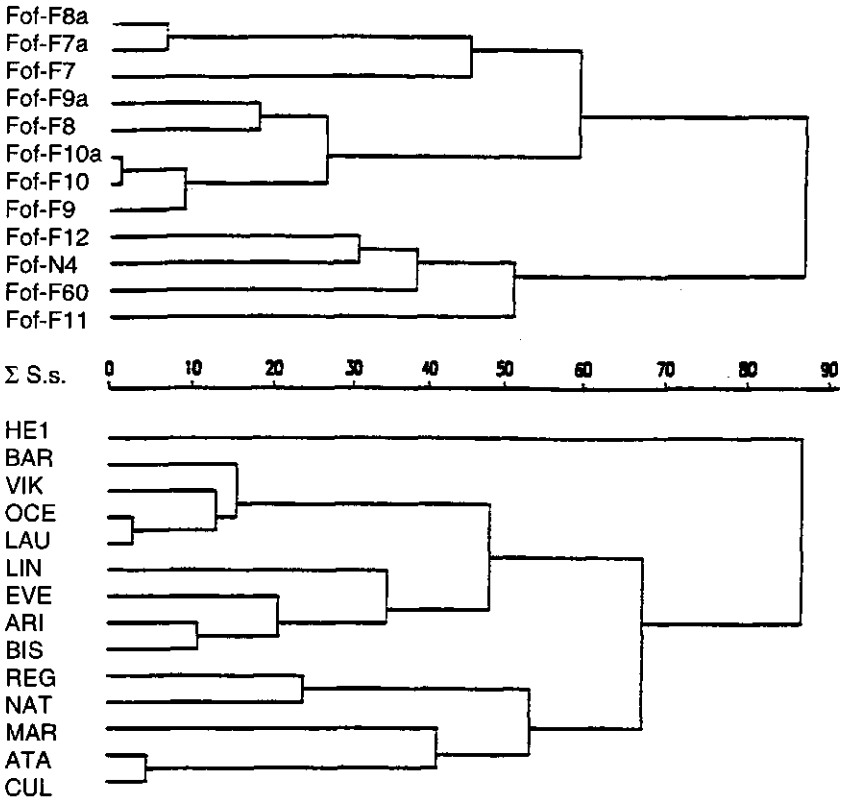

Figure 5.2
Dendrograms produced by the cluster analysis of fungal isolates and host cultivars according to Corsten and Denis (1990). The analysis was performed on the sprout length measurements of experiment 1. Isolates and cultivars are clustered successively according to similarity, in terms of minimal contribution for interaction. See Table 5.1 and 5.2 for abbreviations of cultivars and codes for *Fusarium* isolates respectively.

more difference between clusters, compared with experiment 1. At the level of P = 0.001, the cultivars formed five clusters (Fig. 5.3) and the isolates formed four clusters at this level (Fig. 5.3). Although the cultivars clustered, no recognizable pattern was found. Clustering of the cultivars into fiber flax and linseed was not observed, neither of European versus American or resistant versus susceptible cultivars (Table 5.6). At the level of P = 0.05, the

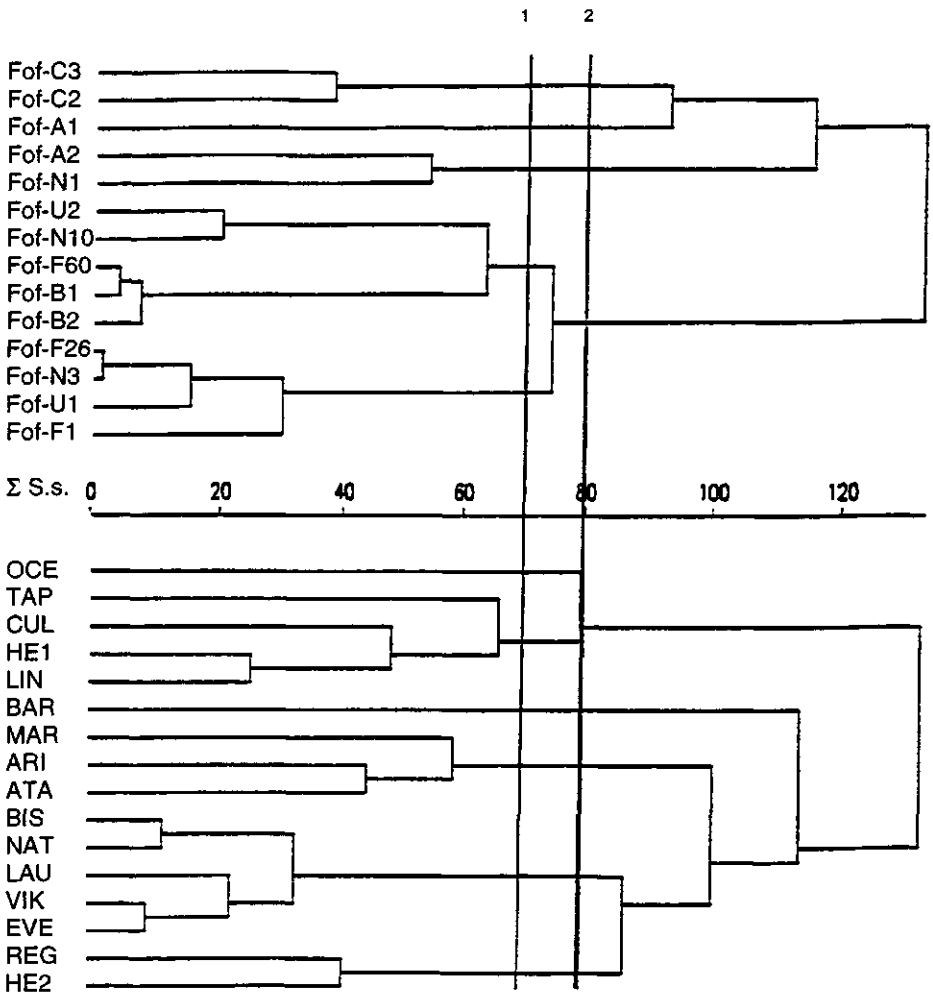

Figure 5.3
Dendrograms produced by the cluster analysis of fungal isolates and host cultivars according to Corsten and Denis (1990). The analysis was performed on the sprout length measurements of experiment 2. Isolates and cultivars are clustered successively according to similarity, in terms of minimal contribution for interaction. See Table 5.1 and 5.2 for abbreviations of cultivars and codes for *Fusarium* isolates respectively. ($^1P = 0.05$, $^2P = 0.001$)

susceptible linseed cultivars 'Ocean' and 'Barbara' were separated as single members of a cluster. The linseed cultivar 'Linda', determined as susceptible in experiment 2 (Table 5.4) but resistant in the French flax wilt nursery

Table 5.6

Number of flax and linseed cultivars and of isolates, used in experiment 2 according to the method of Corsten and Denis (1990), in each cluster (P = 0.001).

Cultivars	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Linseed	4	1	1	1	
Flax	1		2	4	2
Europe	3	1	3	4	2
America	2			1	
Resistant	3		3	5	
Susceptible	2	1			2

Fusarium isolates	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Europe			1	9
America	2	1	1	
Aggressive				9
Less aggressive	2	1	2	

according to Beaudoin (1991) and Kroes (Table 5.1), was placed in a main group.

All European isolates except the Dutch isolate Fof-N1 were placed in one group, the Canadian isolates were placed in one group, the Argentinean Fof-A2 and the Dutch Fof-N1 were placed in the same group and the Argentinean Fof-A1 was placed apart (Fig. 5.3). The first clustering was between the relatively highly aggressive isolates originating from the European continent, except Fof-N1, and the lesser aggressive isolates of the American continent. The Atlantic Ocean as a barrier would possibly fit well in a situation of adaptation. However, some interaction variance may also be caused just by the level of the aggressiveness of the isolates. The fact that Fof-N1, which has the lowest aggressiveness among the European isolates, was clustered with

Table 5.7

Examples of deviations from the additive pattern, as observed in experiment 2, cultivar * isolate interactions in flax and linseed cultivars inoculated with isolates of *Fusarium oxysporum* f.sp. *lini* . See Table 5.1 and 5.2 for abbreviations of cultivars and codes for *Fusarium* isolates respectively.

	Fof F60	Fof C3		Fof B1	Fof A2		Fof B2	Fof A1
ARI	13	91	LIN	42	56	LIN	62	34
BAR	37	53	ARI	19	84	ARI	22	75
	Fof B2	**Fof F1**		**Fof U2**	**Fof C2**		**Fof F26**	**Fof C2**
VIK	35	78	ATA	63	94	HE1	36	34
MAR	56	49	TAP	90	75	LIN	21	57

the American isolates, might be considered as a confirmation of the latter conclusion.

The data set originating from experiment 2 was analyzed in more detail. To detect potential races the data set was analyzed in a tetrad module, whereby unexpected levels of length reduction were detected. In Table 5.4 and 5.7 a number of deviations from the additive pattern (absence of cultivar * isolate interactions) can be seen. All these possible interactions are fairly small, too small to identify reliably possible races, but together they can account for part of the observed, small but significant interaction variance. It should be realized though, that some of the interaction variance comes from incidental and unaccountable deviating values, such as those of 'Barbara' with Fof-N1 (120%) and Fof-A1 (169%), values that are considerably higher than those of the healthy controls, although 'Barbara' belongs to the more susceptible cultivars. 'Barbara' had extremely high sprout lengths, in combination with isolates Fof-A1 and Fof-N1, much longer than the controls. These high values were not caused by only one extreme value. The data for 'Barbara' for the three replicates were 45%, 156% and 159% for the

combination with Fof-N1 and 69%, 271% and 167% for the combination with Fof-A1. The other isolates in combination with 'Barbara' did not give these high deviations from the controls. If these extreme data were not taken into account 'Barbara' would have been ranked as second most susceptible, after 'Ocean', the same situation as in experiment 1. 'Barbara' was observed as susceptible in the field too (Table 5.1). It is not clear why 'Barbara' in combination with the isolates Fof-A1 and Fof-N1 grew this tall in the second and the third replicate of experiment 2.

The French isolates as a group were not distinguishable from the other European isolates. None of the isolates originating from the French soil showed an increased capability to attack the cultivar 'Natasja', nor the cultivars 'Laura' or 'Marina', the cultivars which had shown an increased susceptibility in the flax *Fusarium* wilt nursery "La Gaillarde". So no evidence was found for a new race in Normandy. The observed increased susceptibility might have been caused by other factors than the development of a new race. Furthermore, although interaction effects were significant, no major cultivar * isolate interaction effect was found in the experiments. Kommedahl *et al.* (1970) stated that *Fusarium oxysporum* f.sp. *lini* may comprise an indefinite number of races, but their results may also be interpreted to indicate a single common race with minor gene differences among isolates. The results of the present experiments indicated that the test with isolates originating from different continents, showed greater differences between isolates than the test whereby isolates from one field were tested. *Fusarium oxysporum* is known as a fungus with an abundance of forms (Armstrong and Armstrong, 1968; Aloi and Baayen, 1993), and is described as a primitive and versatile organism. Besides, within the flax genome, induction of genetic variation by environmental factors has been described for various characters (Durant, 1972), which indicates that a relatively fast co-evolution between fungus and flax might be possible.

If the interaction would be caused by a gene-for-gene relationship, in which major resistance genes and major avirulence genes are involved, clear

and large cultivar * isolate interactions would have been found. As argued by Parlevliet and Zadoks (1977) a gene-for-gene relationship may also exist at minor gene level, so that adaptation of the fungus to one or more minor resistance genes would result in isolates differing slightly from one to another in their virulence. The rather small interactions, found in both experiments may point in this direction.

It has been suggested that *Fusarium* resistance in flax is a quantitatively inherited character (Pavelek, 1983; Popescu and Schuster, 1985). A gene-for-gene relationship at a minor gene level, where genetic adaptations on plant and fungal level occur often, is a quantitative system where the individual minor genes are difficult to recognize. The results presented fit well in this relationship.

If races are defined as a group of similar isolates, causing major interaction effects, as Van der Plank (1968) did, no races *in Fusarium oxysporum* could be detected in the present sets of isolates and interaction would be caused by differences in aggressiveness only. However, if races are defined as a group of isolates, causing race-specific interaction effects not only on major but also on minor gene level (Parlevliet and Zadoks, 1977), races in *Fusarium oxysporum* f.sp. *lini* may very well exist but cannot reliably be identified.

In this hypothesis it is plausible that races, based on minor gene effects exist and that the most important minor differences might be found between *Fusarium oxysporum* f.sp. *lini* originating from the European continent and *Fusarium oxysporum* f.sp. *lini* originating from the American continent.

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CHAPTER 6

Assessment of resistance to *Fusarium oxysporum* f.sp. *lini* in flax and linseed across locations and years

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ABSTRACT

Thirty flax and linseed cultivars were evaluated for resistance to *Fusarium oxysporum* f.sp. *lini* at various flax wilt infested test fields in Europe and North America in two consecutive years. Resistance levels of cultivars were measured using a standardized protocol, determining disease severity on a scale of 1-9, at four plant stages during the growing season. In analyses of variance significant main effects and significant cultivar * environment interaction effects were found at all plant stages ($P < 0.001$). Interactions at the seedling and the harvest stage were further investigated by three statistical models. Agglomerative clustering was related to cultivar susceptibility. A tetrad analyze showed that in the French flax wilt nursery interaction specific reactions occurred most frequently. In an Additive Main effects and Multiplicative Interaction effects (AMMI) model, interaction was written as the product of a cultivar and a environment score. Cultivar * environments interaction scores could be described in two terms, which indicated a relatively simple interaction pattern. The interaction effects were mainly caused by differences in environmental factors and were not linked to differences between the wilt nurseries. However, the group of susceptible cultivars also caused interaction effects.

Key words: Additive Main effects and Multiplicative Interaction effects (AMMI) model, agglomerative cluster analysis, cultivar by location interaction, flax, *Fusarium oxysporum* f.sp. *lini, Linum usitatissimum*, vascular wilt

INTRODUCTION

Flax wilt, caused by the host specific fungus *Fusarium oxysporum* f.sp. *lini* (Bolley) Snyder & Hanssen, is a major problem in flax and linseed (*Linum usitatissimum* L.). Breeding for resistance to this fungus is one of the most important activities of flax breeders. High levels of quantitative resistance in flax and linseed cultivars have been found (Ebskamp and Bonthuis, 1993; Beaudoin, 1991; Popescu *et al.*, 1994), but complete resistance was never

observed. Based on differences in wilt from one location to another Kommedahl *et al.* (1970) stated that the fungus comprises an indefinite number of races in the field. However, their results might also be interpreted to indicate a single common race with minor gene differences among isolates. In an *in vitro* study no evidence was found for race-specificity based on major interaction effects, and although races in *Fusarium oxysporum* f.sp. *lini* may exist, they could not be identified (see Chapter 5). So it is not known whether races of this fungus exist and whether the resistances are race-specific. The influence of environmental factors is of unknown importance in the *Fusarium*-flax interaction. Several factors, which might cause interaction have been studied individually. Kommedahl *et al.* (1970) described different kinds of wilting in linseed, early wilt or seedling blight, late wilt and partial wilt. These different types of wilting might affect interaction patterns. Seed quality seems to have an influence on wilt severity (Nair, 1956; Nair and Kommedahl, 1957). Wilt in flax and linseed is influenced by to various environmental factors like temperature (Milikan, 1945; Tisdale, 1917; Tochinai and Takee, 1950), moisture (Tursunkhodzhaev, 1965), soil type (Kommedahl *et al.*, 1970) and inoculum density in the soil (Nair, 1956). A positive direct effect of fertilizers is uncertain. Although temperature and moisture seem to be the predominant factors in determining wilt development in general, it is not known whether variation in these factors can induce interaction patterns. In recent years the linseed cultivar 'McGregor' showed a different response in the St. Paul's flax wilt nursery in Minnesota, compared with the response in the flax wilt nursery in Fargo, North Dakota ("Plot 30") (Hammond, personal communication), but the cause is uncertain. In India races of *Fusarium oxysporum* f.sp. *lini* were reported (Kulkarni *et al.*, 1969), but these trials were not executed simultaneously under similar conditions, so the possible influence of the differential effects of environmental factors were not excluded. In a pilot experiment carried out in 1994 at flax wilt nurseries in Normandy, France, in Flevoland, The Netherlands, and in Manitoba, Canada, very different interaction patterns were found for flax and linseed cultivars, especially

between Canadian results versus French and Dutch results (Rashid, personal communication), but also in this case the tests were not executed simultaneously under comparable conditions, so the possible influence of the environmental differences were not excluded, and an unambiguous interpretation of the results is not possible.

To obtain an better understanding of the nature and the magnitude of the cultivar * environment interactions, a wide ranging international field test was carried out in 1995 and 1996 with the use of a standardized protocol and seeds from common sources per cultivar. Nine locations in Europe and Northern America were involved, and 30 cultivars of fiber flax as well as linseed were used. The protocol was designed in such a way that the test could be synchronized for the different locations. Three statistical models were used to interpret the interaction patterns.

MATERIALS AND METHODS

Field trials

In two consecutive years, cultivar trials for resistance to *Fusarium oxysporum* f.sp. *lini* were carried out at nine flax wilt nurseries in Europe and North America (Table 6.1). Because the development of the wilt disease per location is never identical for different years, each year * location test was considered to be one environment. This means that the cultivars were exposed to 18 environments.

Seeds of 30 flax and linseed cultivars, 1.5 kg per cultivar, varying from very susceptible to very resistant, and from very distinct origin (see Table 6.2), were obtained from different sources in Europe and North America. From this seed stock 60 g per cultivar was distributed over the nine nurseries, 30 g for each year. A completely randomized block design with three replicates, was used for all locations and years. The plot size was two rows of 5.0 m, 30 cm apart. The density of sowing was 1 g m^{-1}.

Table 6.1

Location (city and country), abbreviation, soil type, climate and year of introduction of the flax wilt nurseries used in the nine international trials on flax wilt in flax and linseed in 1995 and 1996.

City	Country	Abbr.	Soil type	Climate	In use since
Lelystad, Flevopolder	The Netherlands	NL	heavy clay	maritime	1992
Ingelmunster	Belgium	BE	loose sandy loam	maritime	1972
La Gaillarde, Normandy	France	FR	sandy loam	maritime	1990
Asendorf	Germany	GE	sandy loam	maritime	1991
Šumperk	The Czech Republic	CZ	sandy clay	continental	1974
Torzhok	Russia	RU	sandy loam	continental	1945
Fargo ND (Plot 30)	USA	US	silt loam	continental	1894
Morden, Manitoba	Canada	CM	black chernozemic	continental	1916
Indian Head, Saskatchewan	Canada	CS	black chernozemic	continental	1934

Protocol

To make the experiments comparable a protocol was developed, based on the disease scoring according to Rashid and Kenaschuk (1993). In order to be able to take in account the difference in development between the cultivars, the cultivar 'Viking' was chosen as a reference, because, compared with all other cultivars used, 'Viking' can be considered 'intermediate' for many important and/or useful characteristics, medium earliness, medium flowering time, medium maturity and moderate resistance to wilt and other diseases. Emergence was scored for all cultivars when the seedlings of 'Viking' were 5 - 10 cm above the ground, to detect possible difficulties in adaptation caused by differences in climate, and furthermore to test the

Table 6.2

Name, type, wilt resistance, and origin of the cultivars used in the international trials on flax wilt in flax and linseed in 1995 and 1996.

Cultivar	type	Resistance[1]	Source[2]	Origin[3]
Alexim	flax	MR	VNILL	Russia
Ariane	flax	S	CPRO-DLO	France
Atalante	linseed	HR	CPRO-DLO	France
Barbara	linseed	VS	CEBECO	Hungary
Bison	linseed	HR	USDA-NDSU	USA
Culbert	linseed	HR	USDA-NDSU	USA
AC McDuff	linseed	MR	AAFC-MRC	Canada
Elise	flax	MR	Procotex	The Netherlands
AC Emerson	linseed	MR	AAFC-MRC	Canada
Escalina	flax	MR	CEBECO	The Netherlands
Evelin	flax	HR	Wiersum	The Netherlands
McGregor	linseed	MR	AAFC-MRC	Canada
Hera	flax	S	Wiersum	The Netherlands
Hermes	flax	R	Wiersum	France
K-5327	flax	MS	VNILL	Russia
Laura	flax	R	CPRO-DLO	The Netherlands
Linda	linseed	HR	CPRO-DLO	France
AC Linora	linseed	MR	AAFC-MRC	Canada
Liflora	linseed	MS	DSV	Germany
Marina	flax	R	CPRO-DLO	The Netherlands
Natasja	flax	MS	CPRO-DLO	The Netherlands
Nike	flax	R	IKWN	Poland
NorLin	linseed	MR	AAFC-MRC	Canada
Ocean	linseed	VS	Lin 2000	France
Raisa	flax	MR	Van de Bilt	The Netherlands
Regina	flax	VS	CEBECO	The Netherlands
Slavny 82	flax	MR	VNILL	Russia
Texa	flax	HR	AGRITEC	The Czech Republic
Verne	linseed	HR	USDA-NDSU	USA
Viking	flax	R	CPRO-DLO	France

Foot note see p. 111 bottom.

quality of the seed material used. A scale was used of 1 - 5, whereby 1 = good emergence and 5 = poor emergence. Four times during the growing season the cultivars were scored for wilt, using a scale of 1 - 9, whereby 1 = healthy, no signs of wilt, and 9 = all plants severely wilted or dead. The dates of scoring were related to the moments that the standard cultivar 'Viking' reached the following stages: 1 = plants are 5 - 10 cm high, 2 = plants are 30 cm high, 3 = just before flowering and 4 = green boll stage, the stage of development of the seeds. The protocol has been used by all collaborators in both years.

Statistical analyses

Data regarding emergence and the four wilt scores were subjected separately to an analysis of variance (ANOVA), using GENSTAT (1992). To study the interactions for early and late wilt and to structure the interactions in a more manageable form, the first and the fourth wilt score were analyzed using several methods.

An agglomerative clustering procedure was carried out (Corsten and Denis, 1990), structuring rows and columns from a two way table and identifying groups in the rows and columns.

A tetrad module was used for tracing combinations deviating from

[1] Resistance is given according to the information obtained by the source of origin or by the descriptive List of Varieties from the country concerned. HR = highly resistant, R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, VS = very susceptible.
[2] VNILL = Flax Research Institute, Torzhok, Russia; CPRO-DLO = Centre for Plant Breeding and Reproduction Research, Wageningen, the Netherlands; CEBECO = CEBECO Seeds, Lelystad, The Netherlands; USDA-NDSU = United States Department of Agriculture - North Dakota State University, Fargo, USA; AAFC-MRC = Agriculture and Agri-Food Canada, Morden Research Centre, Canada; Procotex = Procotex Breeding, St.Jansteen, The Netherlands; Wiersum = Landbouwbureau Wiersum, Dronten, The Netherlands; DSV = Deutsche Saatveredelung, Zuchtstation Hof Steimke, Asendorf, Germany; IKWN = Institute for Natural Fibres, Poznan, Poland; Lin 2000 = Lin 2000, Grandvilliers, France; Van de Bilt = Van de Bilt Zaden, Sluiskil, The Netherlands; AGRITEC = AGRITEC Research, Breeding and Services Ltd, Šumperk, The Czech Republic.
[3] Country where the cultivar originally was selected.

additivity (Van Eeuwijk, unpublished results). This tetrad module is a statistical tool for identifying multiple deviant values in row-column combinations in a two-way (average) table (Bradu and Hawkins, 1982). The multiple deviant values are detected by calculating all possible interactions per cell (tetrads) in a robust way.

Furthermore, the data from the first and the fourth stage were analyzed with a two-way Additive Main effects and Multiplicative Interaction (AMMI) model The model is written as

$$Y_{ijk} = \mu + \rho_i + \gamma_j + \sum_{l=1}^{L} u_{li} v_{lj} + \in_{ijk}$$

whereby the multiplicative terms, or scores for the cultivars are given by u_{li}, those for the environments by v_{lj}, while L is the number of multiplicative terms needed for an adequate description of the interaction. AMMI-analyses were performed using the average tables of wilt scores for cultivars and environments.

RESULTS

Emergence

The average emergence of most of the cultivars (data not shown) was intermediate. Over all locations, it varied between 1.7 and 3.4. There were no cultivars with extremely poor or exceptionally good emergence, except for the cultivars 'Hera' and 'Ocean', which emerged relatively poor in all trials in the first year. Germination tests proved that the poor germination of 'Hera' was caused by poor seed quality. For that reason the seeds of 'Hera' were replaced in the second year, resulting in better emergence. The poor emergence of 'Ocean' in both years was not caused by poor seed quality. Most likely it was caused by the susceptibility of this cultivar for *Fusarium*. Emergence per location (Table 6.3) differed more than emergence per cultivar. In 1996 the emergence in the Belgian nursery was very low. Because of a period of drought directly after sowing, the seeds germinated in two waves. This caused difficulties in the judgment of the disease in the first and

Table 6.3

Emergence of 30 flax and linseed cultivars at nine flax wilt nurseries located in Europe and Northern America, in 1995 and 1996, using a scale from 1 - 5 according to Rashid and Kenaschuk (1993).

	NL[1]	BE	FR	US	CZ	GE	RU	CM	CS	Mean
1995	2.8	1.9	--	--	2.8	1.2	--	2.7	1.8	2.2
1996	1.3	4.7	2.5	--	2.7	2.4	--	2.5	1.4	2.5
Mean	2.1	3.3	2.5	--	2.8	1.8	--	2.6	1.6	2.4

[1] for abbreviations see Table 6.1
-- = data not obtained.

second scoring. However, the difference in plant development within the plots was straightened out after the second scoring.

Disease screening

Development of the disease in the cultivars

Results of disease scoring in stage 1 and 4 are summarized in Table 6.4 and 6.5. Interaction effects between the years were not significant. The cultivars which showed high levels of wilting in the first stage viz. 'Ocean', 'Barbara', 'Hera' and 'Regina', proved to show high levels of wilting in the later stages too. The same holds true for the cultivars 'Liflora', 'Slavny 82', 'K-5327', 'McGregor' and 'Natasja', which were scored moderately affected in all stages. Most of the other, more resistant cultivars (Table 6.2) were not or barely affected in the first stage, so an estimation of a relationship with wilt occurrence in the fourth stage was impossible. The cultivars 'AC Emerson', 'Atalante' and 'Evelin' were moderately affected in the first stage but proved to be hardly affected at the fourth scoring, while 'Bison', 'Viking' and 'Ariane' were healthy in the first stage and moderately affected in the fourth stage. 'AC Linora' and 'Hermes' were healthy at the first scoring and stayed healthy, whereas 'Escalina' and 'Laura' were moderately affected in all stages (Tables 6.4 and 6.5).

Table 6.4

Score 1, average level of attack of 30 flax and linseed cultivars, determined at the first stage when plants were 10 - 15 cm high, at nine flax wilt nurseries in Europe and North America, averaged across 1995 and 1996. A scale was used from 1 - 9 according to Rashid and Kenaschuk (1993). Cultivars are ordered towards increasing level of average resistance in the fourth stage, locations are ordered towards decreasing level of average attack of *Fusarium oxysporum* f.sp. *lini* in the different nurseries during the fourth stage. Because of the unbalanced data set per location no mean was given for the cultivars.

	US[1]	FR	CM	CS	CZ	GE	RU	BE	NL
Ocean	--	1.5	6.3	7.8	3.8	2.5	--	2.2	2.2
Barbara	--	1.0	6.3	4.8	4.0	1.7	1.0	2.5	1.3
Regina	--	1.0	5.3	3.2	3.7	1.5	2.7	2.2	1.7
Hera	--	1.7	5.8	3.7	3.5	2.7	2.7	1.7	1.7
Liflora	--	1.5	6.7	4.8	3.2	1.7	1.0	1.8	1.5
Slavny 82	--	1.0	5.8	2.8	2.7	1.5	1.7	1.7	1.5
K-5327	--	1.0	6.1	3.2	3.0	2.4	1.0	1.8	1.2
McGregor	--	1.3	6.0	2.2	3.2	1.7	1.5	1.3	1.2
Natasja	--	1.0	5.5	3.3	2.7	1.2	2.2	1.2	1.0
Elise	--	1.0	5.5	2.3	2.3	1.3	1.0	1.0	1.0
Ariane	--	1.0	5.3	2.0	2.0	1.2	1.0	1.0	1.0
Escalina	--	1.0	5.5	2.5	2.2	1.2	1.5	1.0	1.0
Raisa	--	1.0	5.8	3.0	3.0	1.2	1.0	1.0	1.0
Viking	--	1.0	4.7	1.7	1.2	1.0	2.0	1.0	1.2
Laura	--	1.0	5.5	2.0	1.8	1.5	2.0	1.0	1.0
Alexim	--	1.0	5.2	3.0	2.0	1.3	1.5	1.3	1.0
Texa	--	1.3	5.3	2.0	1.0	1.5	1.0	1.0	1.0
Linda	--	1.2	5.5	2.2	1.7	1.0	1.0	1.0	1.0
NorLin	--	1.0	4.7	1.8	2.0	1.5	1.5	1.0	1.0
Bison	--	1.0	3.8	1.8	2.0	1.0	1.0	1.0	1.0
Culbert	--	1.2	5.2	2.2	1.3	1.2	1.0	1.0	1.2
Marina	--	1.0	5.7	2.3	2.2	1.3	1.0	1.0	1.0
Nike	--	1.0	5.5	1.7	1.0	1.5	1.0	1.0	1.0
Verne	--	1.0	5.2	2.0	2.0	1.5	1.0	1.0	1.0
Hermes	--	1.0	4.7	1.7	1.5	1.2	1.5	1.0	1.2
AC McDuff	--	1.0	4.8	2.2	1.0	1.2	2.5	1.0	1.0
Atalante	--	1.2	5.0	2.0	1.3	1.3	2.5	1.0	1.0
AC Linora	--	1.2	4.3	1.7	1.5	1.3	1.5	1.0	1.0
AC Emerson	--	1.0	4.8	2.5	2.2	1.3	2.5	1.0	1.0
Evelin	--	1.0	6.0	1.8	1.7	1.2	1.0	1.0	1.0
Mean	--	1.1	5.4	2.7	2.2	1.5	1.5	1.3	1.2

[1] for abbreviations see Table 6.1
-- = data not obtained

Table 6.5

Score 4, average level of attack 30 flax and linseed cultivars, determined at the fourth stage, the green boll stage, at nine flax wilt nurseries in Europe and North America, averaged across 1995 and 1996. A scale was used from 1 - 9 according to Rashid and Kenaschuk (1993). Cultivars are ordered towards increasing level of average resistance in the fourth stage, locations are ordered towards decreasing level of average attack of *Fusarium oxysporum* f.sp. *lini* in the different nurseries during the fourth stage.

	US[1]	FR	CM	CS	CZ	GE	RU	BE	NL	Mean
Ocean	7.5	6.5	7.7	8.2	7.8	7.7	7.0[2]	6.3	7.8	7.4
Barbara	7.8	5.3	7.8	8.5	8.2	7.2	3.8	5.8	5.7	6.7
Regina	6.2	8.7	4.8	5.5	7.3	6.0	7.0	5.0	6.8	6.4
Hera	7.0	5.8	5.5	5.5	7.7	6.0	5.3	5.0	7.0	6.1
Liflora	6.3	8.5	7.5	8.0	6.3	5.7	4.5	4.8	3.0	6.1
Slavny 82	7.3	6.0	5.2	5.4	5.0	4.0	5.2	5.0	5.8	5.4
K-5327	6.9	4.5	5.5	5.2	5.5	5.9	2.7	4.7	3.5	4.9
McGregor	7.2	6.0	4.3	4.2	6.0	5.2	4.5	3.7	2.7	4.9
Natasja	6.3	6.2	4.5	4.5	4.8	4.8	5.2	3.0	3.5	4.8
Elise	7.8	7.8	4.2	3.7	4.5	4.2	4.5	3.3	2.3	4.7
Ariane	5.7	7.8	4.3	3.7	4.5	4.5	4.0	2.7	2.3	4.4
Escalina	6.8	6.2	4.7	4.2	4.8	3.0	4.0	3.0	2.3	4.3
Raisa	4.7	5.3	4.8	5.0	5.2	3.5	2.5	3.0	3.0	4.1
Viking	6.7	5.7	3.8	3.0	3.2	3.8	5.3	2.5	2.2	4.0
Laura	6.3	6.3	3.8	3.7	3.5	4.0	3.2	2.8	2.0	4.0
Alexim	4.3	4.2	4.7	3.7	4.0	4.7	3.3	4.0	2.7	3.9
Texa	5.8	5.7	5.2	4.0	2.8	4.7	1.5	2.7	3.0	3.9
Linda	6.8	3.3	5.0	4.5	3.2	3.8	4.3	2.0	2.2	3.9
NorLin	6.8	3.3	4.2	3.7	4.3	5.0	3.2	2.5	2.2	3.9
Bison	6.7	4.5	2.8	3.8	3.8	4.7	3.8	2.5	1.7	3.8
Culbert	6.5	5.8	4.3	4.3	3.0	4.2	2.2	2.7	1.2	3.8
Marina	5.8	4.5	4.3	4.3	4.0	3.0	3.5	2.5	2.0	3.8
Nike	4.7	3.7	5.0	4.0	3.0	7.0	1.5	2.5	1.8	3.7
Verne	4.5	3.8	4.7	4.8	3.2	4.5	3.5	2.2	1.5	3.6
Hermes	4.7	4.8	4.5	3.7	4.0	3.5	2.2	2.8	2.2	3.6
AC McDuff	7.2	4.5	3.8	3.7	3.2	2.5	3.7	2.3	1.5	3.6
Atalante	3.5	5.0	4.5	4.0	3.0	3.3	3.7	2.2	1.7	3.4
AC Linora	5.3	2.8	3.3	4.2	3.5	3.7	3.3	2.3	2.0	3.4
AC Emerson	5.5	3.2	3.3	4.0	3.3	2.7	3.8	1.8	1.5	3.2
Evelin	4.5	3.5	4.0	3.5	3.2	2.0	2.3	1.8	1.8	3.0
Mean	6.1	5.3	4.7	4.6	4.5	4.5	3.8	3.3	3.0	4.4

[1] for abbreviations see Table 6.1
[2] estimated value, data missing

Table 6.6

Average development of the *Fusarium* flax wilt disease in nine flax wilt nurseries in 1995 and 1996 determined at four stages from young stage (10 - 15 cm tall, stage 1) to green boll stage (stage 4). The values are determined by taking the average level of attack from 30 cultivars in the three replicates, using a scale from 1 - 9 according to Rashid and Kenaschuk (1993).

Stage	Year	US[1]	FR	CM	CS	CZ	GE	RU	BE	NL
1	1995	--	1.2	6.5	2.8	2.2	1.2	2.0	1.1	1.0
2	1995	--	2.0	5.2	--	3.2	3.1	2.4	1.5	1.1
3	1995	5.1	4.3	4.9	4.1	3.9	3.7	2.5	3.1	1.9
4	1995	6.9	6.4	4.7	5.1	4.5	4.3	3.2	3.4	3.5
1	1996	--	1.0	4.3	2.6	2.2	1.7	1.0	1.4	1.3
2	1996	4.3	1.1	4.5	4.4	3.3	3.2	--	2.2	2.3
3	1996	5.3	1.7	4.8	4.3	4.2	4.2	--	2.8	2.5
4	1996	5.3	4.2	4.8	4.1	4.6	4.7	4.4	3.1	2.5

[1] for abbreviations see Table 6.1
-- = data not obtained

Development of the disease at the wilt nurseries

In the first stage the wilt scores for the Canadian nurseries and the Czech wilt nursery were relatively high compared with the other nurseries, where the disease developed mainly in the later stages. Especially the nursery in Morden had high levels of disease occurrence in the first stage.

In stage four the most severe damage was observed in the oldest flax wilt nursery, "Plot 30", in North Dakota, USA, but also in the French flax wilt nursery the disease developed to rather high levels, particularly in 1995. Remarkable were the observations in 1995 in Morden, Canada and in 1996 in Saskatchewan, Canada where the disease seemed to diminish during the season. The Russian, Belgian and Dutch flax wilt nurseries were the nurseries where the lowest average level of *Fusarium* attack was observed (Table 6.6).

In Belgium and The Netherlands the fungus was not equally dispersed

Table 6.7

Correlations between nine flax wilt nurseries in Europe and North America, from which the level of attack of 30 flax and linseed cultivars was determined at the fourth stage, the green boll stage, averaged across 1995 and 1996. A scale was used from 1 - 9 according to Rashid and Kenaschuk (1993).

	US[1]	FR	CM	CS	CZ	GE	RU	BE	NL
US	1.00**								
FR	0.37*	1.00**							
CM	0.28	0.36	1.00**						
CS	0.36	0.35	0.90**	1.00**					
CZ	0.50**	0.32	0.70**	0.79**	1.00**				
GE	0.34	0.41*	0.64**	0.66**	0.67**	1.00**			
RU	0.44*	0.36	0.50**	0.57**	0.53**	0.14	1.00**		
BE	0.49**	0.35	0.52**	0.43*	0.53**	0.74**	0.43*	1.00**	
NL	0.43*	0.44**	0.50**	0.39*	0.60**	0.67**	0.57**	0.88**	1.00**

[1] for abbreviations see Table 6.1
* Significant correlations at 5% level
** Significant correlations at 1% level

over the nursery (data not shown), which caused deviations in the observations. However, in these nurseries the differences between diseased and healthy cultivars were reasonably clear. In the Russian flax wilt nursery in 1995 the fungus caused hardly any wilt in all cultivars in 1995, while in 1996 the range between healthy and affected cultivars was more clear. The observations for the 30 cultivars grown in the flax wilt nurseries in Germany and The Czech Republic were similar to the average of all nurseries.

Correlations between scorings and between nurseries

Correlations between stage 1 and stage 4 were calculated for the nurseries which showed a reasonable wilt occurrence at the first stage, namely for Morden, Canada (CM, r = 0.78**), Indian Head, Canada (CS, r = 0.88**) and The Czech Republic (CZ, r = 0.94**), these correlations were significant at the 1% level.

Table 6.8

Analysis of variance (ANOVA) of wilt scores of 30 flax and linseed cultivars over 1995, from which the data were obtained in the nine flax wilt nurseries, in three replicates, during four stages from the young stage (10-15 cm tall, stage 1) to green boll stage (stage 4).

Stage	Source of variance	D.f.[1]		S.s.	M.s.	V.r.	F pr.
1	Environment (Env)	7	(1)	2134.9	305.0	388.9	<.001
	Residual	16	(2)	12.5	0.8	2.1	
	Cultivar (Cv)	29		174.9	6.0	16.4	<.001
	Cv * Env	203	(29)	407.6	2.0	5.5	<.001
	Residual	460	(62)	168.8	0.4		
	Total	715	(94)	2847.7			
2	Environment (Env)	6	(2)	1018.1	169.7	76.4	<.001
	Residual	14	4)	31.1	2.2	4.6	
	Cultivar (Cv)	29		437.1	15.1	31.3	<.001
	Cv * Env	174	(58)	389.8	2.2	4.7	<.001
	Residual	404	(118)	194.6	0.5		
	Total	627	(182)	1964.6			
3	Environment (Env)	8		800.6	100.1	54.7	<.001
	Residual	18		32.9	1.8	2.9	
	Cultivar (Cv)	29		1182.2	40.8	64.5	<.001
	Cv * Env	232		844.3	3.6	5.8	<.001
	Residual	519	(3)	327.9	0.6		
	Total	806	(3)	3164.9			
4	Environment (Env)	8		1211.0	151.4	51.6	<.001
	Residual	18		52.9	2.9	4.2	
	Cultivar (Cv)	29		940.7	32.4	46.2	<.001
	Cv * Env	232		928.5	4.0	5.7	<.001
	Residual	516	(6)	362.4	0.7		
	Total	803	(6)	3434.3			

[1] missing values in brackets

Correlations between the nurseries are shown in Table 6.7. The correlation coefficients for the Belgian, the German and the Dutch nurseries were acceptable, and between the two Canadian nurseries the correlation

Table 6.9

Analysis of variance (ANOVA) of wilt scores of 30 flax and linseed cultivars over 1996, from which the data were obtained in the nine flax wilt nurseries, in three replicates, during four stages from the young stage (10-15 cm tall, stage 1) to green boll stage (stage 4).

Score	Source of variance	D.f.[1]	S.s.	M.s.	V.r.	F pr.
1	Environment (Env)	7 (1)	756.2	108.0	54.3	<.001
	Residual	16 (2)	31.9	2.0	7.1	
	Cultivar (Cv)	29	148.7	5.1	18.2	<.001
	Cv * Env	200 (32)	171.9	0.9	3.1	<.001
	Residual	456 (66)	128.4	0.3		
	Total	708 (101)	1189.3			
2	Environment (Env)	7 (1)	909.8	130.0	45.3	<.001
	Residual	16 (2)	45.9	2.9	2.1	
	Cultivar (Cv)	29	561.9	19.4	14.1	<.001
	Cv * Env	197 (35)	637.4	3.2	2.4	<.001
	Residual	450 (72)	619.7	1.4		
	Total	699 (110)	2672.4			
3	Environment (Env)	7 (1)	978.6	139.8	25.5	<.001
	Residual	16 (2)	87.8	5.5	3.4	
	Cultivar (Cv)	29	754.3	26.0	16.2	<.001
	Cv * Env	197 (35)	750.1	3.8	2.4	<.001
	Residual	450 (72)	721.3	1.6		
	Total	699 (110)	3162.0			
4	Environment (Env)	8	565.2	70.7	14.9	<.001
	Residual	18	85.2	4.7	2.5	
	Cultivar (Cv)	29	1025.9	35.4	18.4	<.001
	Cv * Env	225 (7)	1244.1	5.5	2.9	<.001
	Residual	506 (16)	971.3	1.9		
	Total	786 (23)	3837.3			

[1] missing values in brackets

was good. A certain trend of higher correlations between nurseries which have a short geographical distance was noticeable, but did not hold true for the French nursery and for "Plot 30" in Fargo, USA. These nurseries had low

correlations with all other nurseries.

Interactions

For all stages the analysis of variance (ANOVA) showed large main effects for the environments and considerable cultivar effects. From stage 1 to stage 4 the effect of the cultivars increased, compared with the effect of the environments. The interaction effects were clearly present and the interaction variance was significant in all stages and in both years (Table 6.8 and 6.9), but always of a lesser magnitude than the main cultivar and environment effects.

Agglomerative clustering analysis

An agglomerative clustering procedure was carried out using the data of the first and the fourth stage (Fig. 6.1 and 6.2)

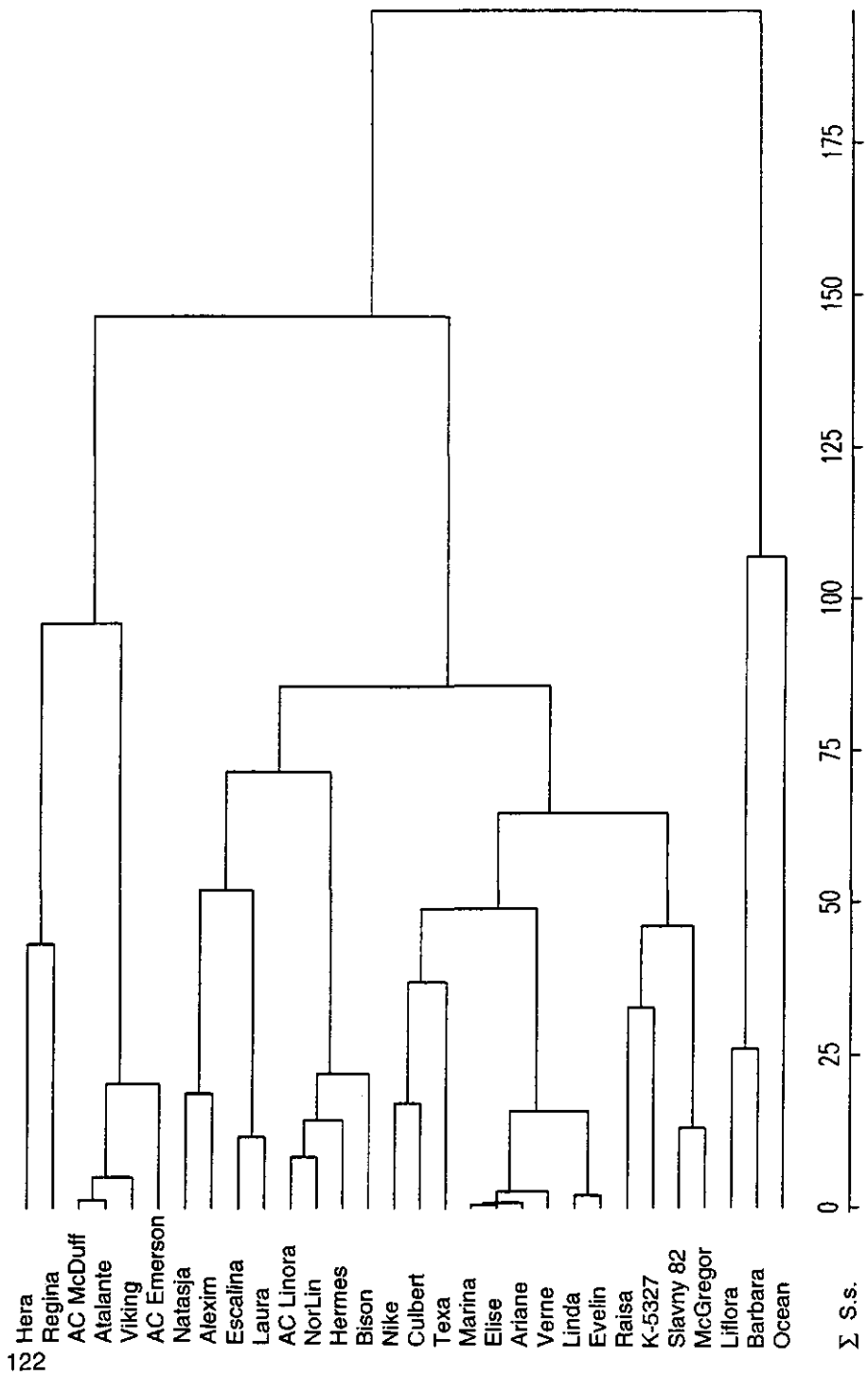
For the missing data of the first stage from US in 1995 and 1996, and for all other individual missing data, the averages per cultivar per year were used in the clustering procedure. In the first stage the susceptible cultivars 'Liflora', 'Barbara' and 'Ocean' were separated from a main group ($P < 0.05$). The second level of clustering did not occur on the base of susceptibility. The susceptible cultivars 'Hera' and 'Regina' were clustered in the same group as the resistant 'AC McDuff', 'Atalante' and 'AC Emerson'. The locations CS-1995 ($P < 0.05$) but also RU-1995 were isolated by clustering, which is difficult to explain. No clustering took place on the basis of year, soil type, level of attack, or climate.

By the clustering procedure for the fourth stage (Fig. 6.2) the susceptible cultivars 'Hera', 'Regina', 'Ocean', 'Barbara', 'Slavny 82' and 'K-5367' were separated from a more resistant main group ($P < 0.05$), although the relatively susceptible 'Liflora' was placed in the large, more resistant group. For the environments no pattern was recognizable for years, soil type, wilting, or climate. The environments US-1996, FR-1996 and RU-1995 were placed in a small group ($P < 0.05$), versus the other environments (the main group).

Tetrad analysis

To indicate multiple interactions between cultivars and environments, a tetrad analysis was carried out, using the two-way average tables, originating from the first and the fourth stage for both years. For the first stage 70 multiple outliers for cultivars and environments were found (numbers mentioned in brackets), 44 combinations in 1995 and 26 combinations in 1996. For this stage 'Hera' (9), 'Barbara' (6), 'Ocean' (5), 'Liflora' (5) and 'Raisa' (5) came forward as cultivars with the highest frequency of multiple outliers. In 1995 the environments in Russia (13), Saskatchewan, Canada (10), Manitoba, Canada (7) and The Czech Republic (6) showed the highest numbers of outliers, and in 1996 these were the environments in The Czech Republic (8) and Saskatchewan, Canada (6). For the fourth stage 30 multiple outliers were found, 9 in 1995 and 21 in 1996. For this stage 'Liflora' (3) came forward as the cultivar with the highest frequency of outliers, whereas 'Barbara', 'Regina', 'Hera', 'Slavny 82', 'K-5327', 'Alexim', 'Texa', 'NorLin' and 'Atalante' appeared twice in a tetrad, significantly deviating from additivity. In 1995 the environments in France (3), Russia (2), and The Netherlands (2) showed multiple outliers, and in 1996 these were the environments in France (10), North Dakota (5), Germany (3) and Russia (2).

Based on the results of the tetrad analysis and the agglomerative clustering analysis, the cultivars as well as the environments were divided into groups, the cultivars in groups with letter a and the environments in groups with letter b (Fig. 6.3). Cultivars and environments with no significant interactions at all and being placed in the main clustering groups were placed in group one, group two consisted of cultivars and environments which were indicated as multiple outlier more than once and which were clustered in the main groups. Group three consisted of cultivars and environments which were isolated from the main groups by agglomerative clustering, not of being a multiple outlier more than once, and group four consisted of cultivars and environments which were isolated from the main groups by agglomerative


Hera
Regina
AC McDuff
Atalante
Viking
AC Emerson
Natasja
Alexim
Escalina
Laura
AC Linora
NorLin
Hermes
Bison
Nike
Culbert
Texa
Marina
Elise
Ariane
Verne
Linda
Evelin
Raisa
K-5327
Slavny 82
McGregor
Liflora
Barbara
Ocean
Σ S.s.
0
25
50
75
100
125
150
175

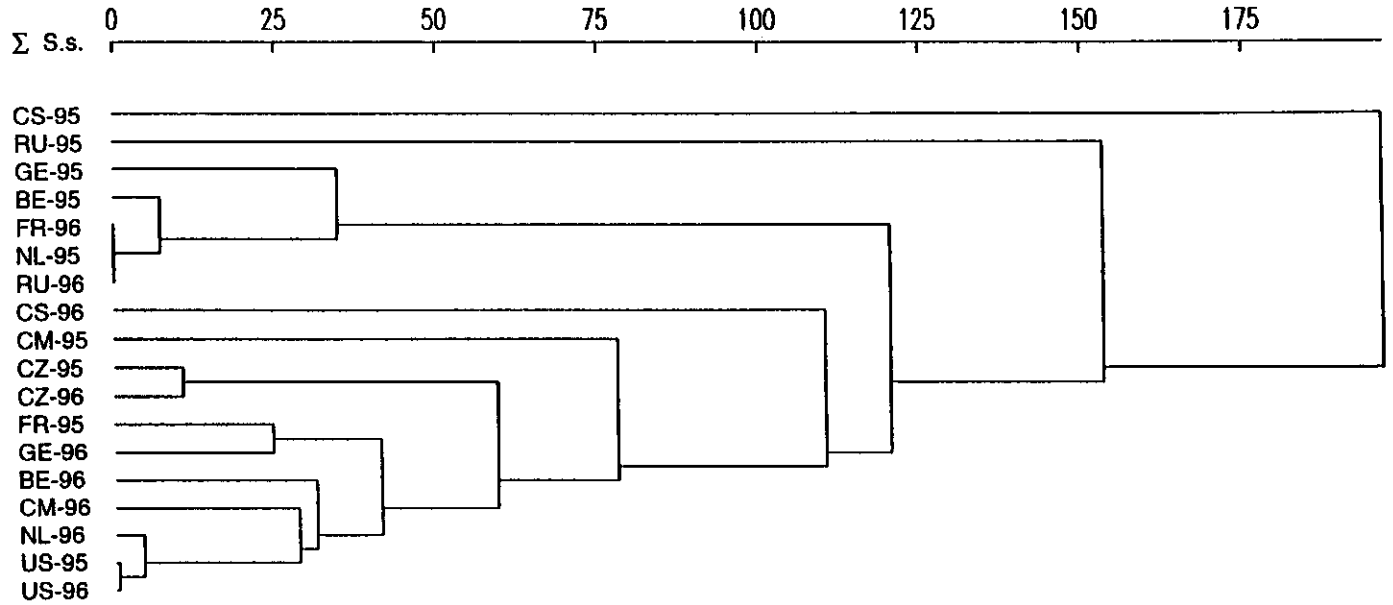
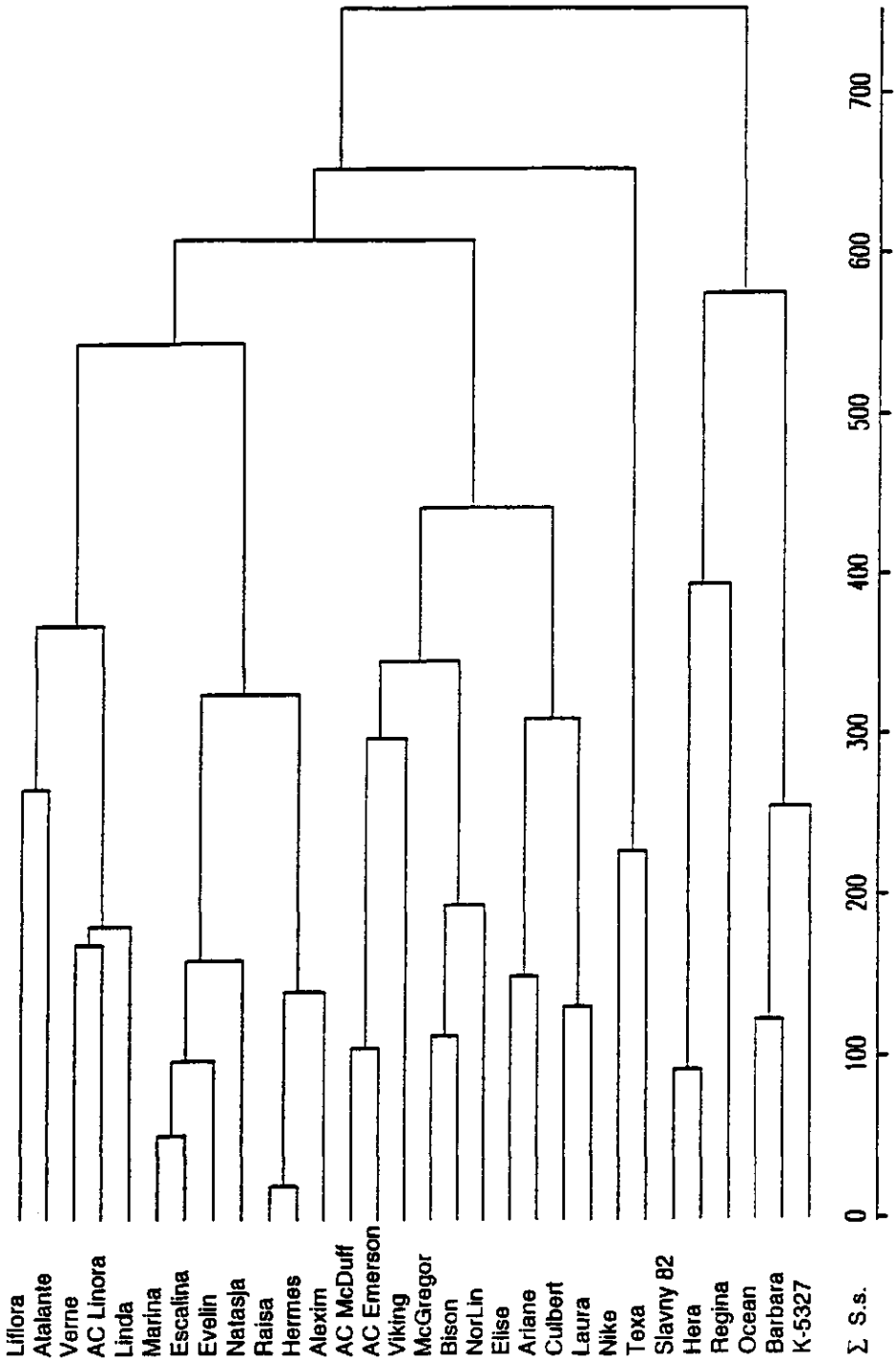


Figure 6.1

Dendrograms produced by cluster analysis of the data of nine flax wilt nurseries in Europe and North America in 1995 and 1996, and 30 flax and linseed cultivars according to Corsten and Denis (1990). The analysis was performed on the results regarding the first stage (plants were 10 - 15 cm tall). Cultivars and environments (locations per year) were clustered successively according to similarity, in terms of minimal contribution to interaction.


Liflora
Atalante
Varne
AC Linora
Linda
Marina
Escalina
Evelin
Natasja
Raisa
Hermes
Alexim
AC McDuff
AC Emerson
Viking
McGregor
Bison
NorLin
Elise
Ariane
Culbert
Laura
Nike
Texa
Slavny 82
Hera
Regina
Ocean
Barbara
K-5327
Σ S.s.
0
100
200
300
400
500
600
700

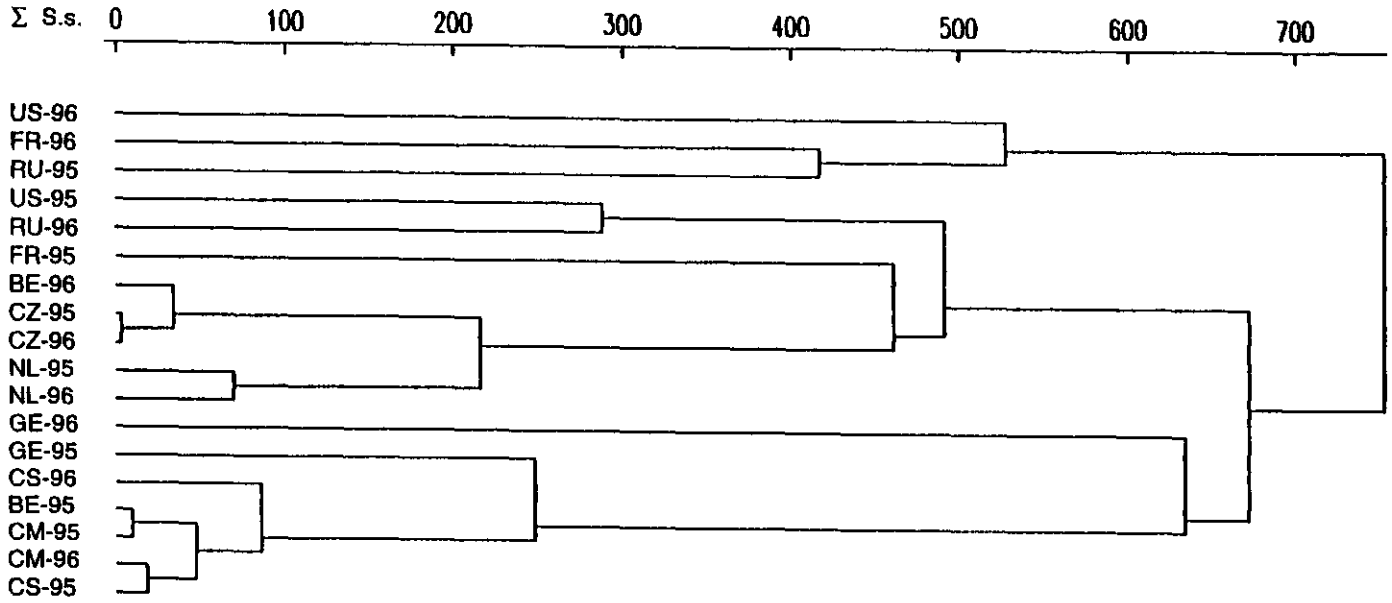


Figure 6.2

Dendrograms produced by cluster analysis of the data of nine flax wilt nurseries in Europe and North America in 1995 and 1996, and 30 flax and linseed cultivars according to Corsten and Denis (1990). The analysis was performed on the results regarding the fourth stage (green boll stage). Cultivars and environments (locations per year) were clustered successively according to similarity, in terms of minimal contribution to interaction.

clustering, and which were indicated as being a multiple outlier more than once.

Additive Main effects and Multiplicative Interaction effects (AMMI) model

An Additive Main effects and Multiplicative Interaction effects (AMMI) model was used to describe the interactions. AMMI-analyses were performed using the two-way average tables of wilt scores for cultivars and environments, from the data of the first and the fourth stage.

While for the analysis of the first score 31% of the variance was determined by the cultivars, 31% was determined by the environments, for the fourth score the variance was determined for 8% by the cultivars and for 76% by the environments.

The results of the analyses indicated that for both analyses two multiplicative terms were enough to describe the interaction. The cultivars as well as the environments can be represented in a two-dimensional co-ordinate system per AMMI-analysis, a so-called biplot, whereby the axes represent the two multiplicative terms needed to describe the interaction (Fig. 6.3 and 6.4) The grouping, which was based on the results from the tetrad analysis and the agglomerative clustering procedure was integrated in the biplots. The cultivars and environments were made visible by closed and open marks, respectively.

In the biplot of the first stage the data which were positioned on the most exterior of the biplot were two environments of group 4b (Saskatchewan, Canada-95 and Russia-95), indicating that in the first stage these environment differences were most predominant. Less predominant but still positioned on the exterior was an environment of group 2b (Canada, Morden-1995). Some cultivars of group 4a ('Hera', 'Regina', 'Ocean' and 'Liflora') were placed on the exterior of the biplot too (Fig. 6.3). The other groups of cultivars and environments clustered together in the biplot of the first stage, indicating no prominent interaction for these cultivars and environments.

In the fourth stage the most exterior positions were taken up by

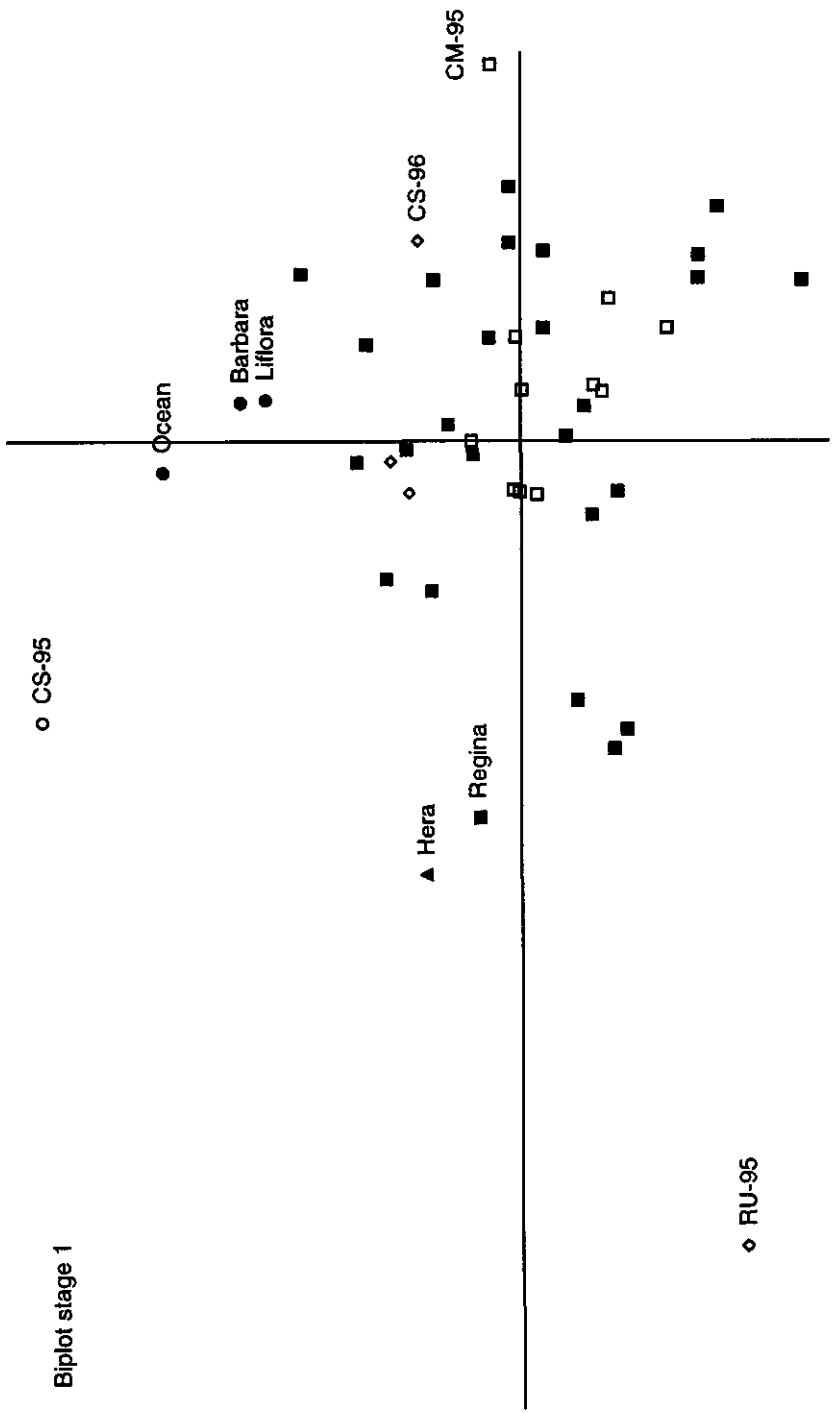
Figure 6.1 (page 128)

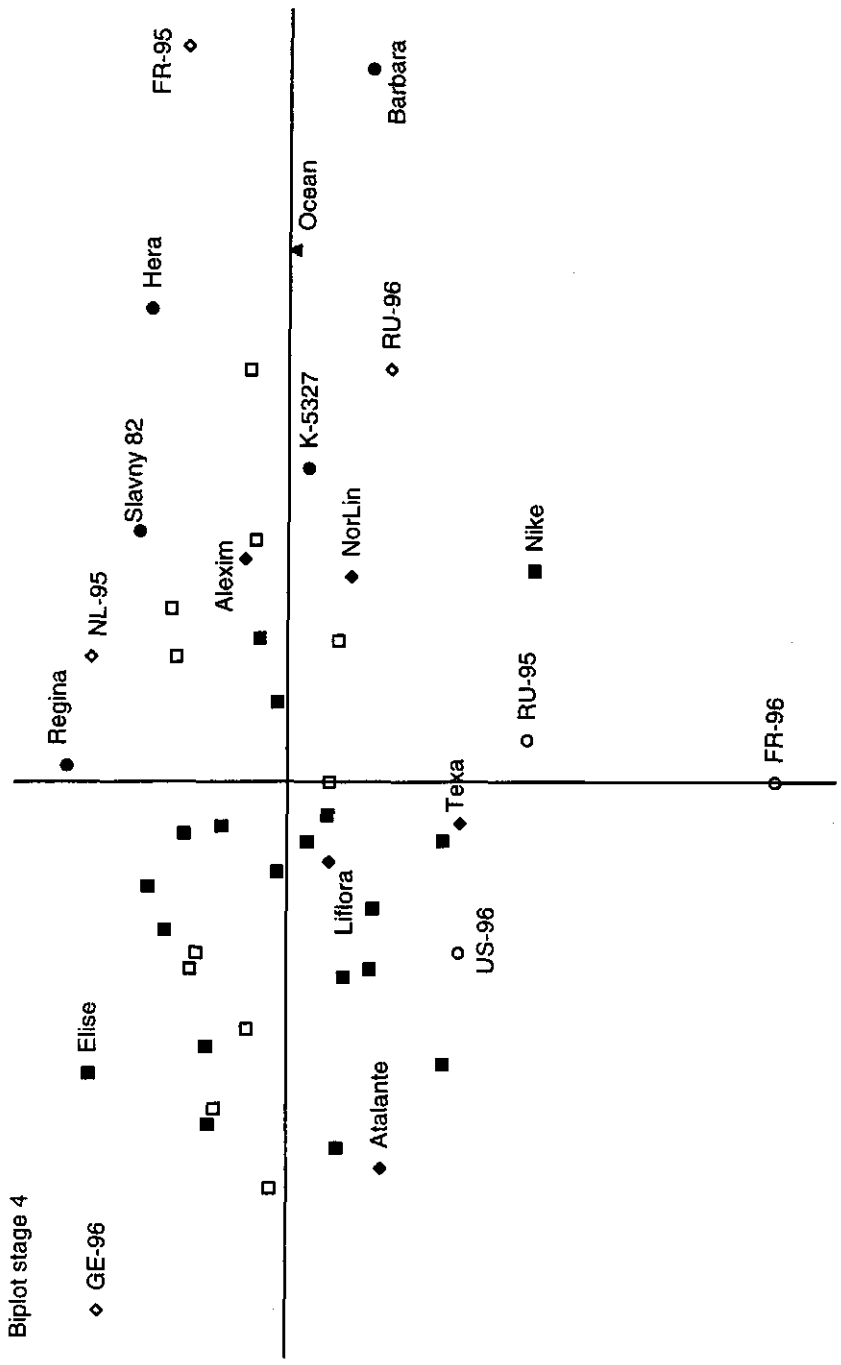
Adapted biplot, produced from the AMMI-analysis according to Van Eeuwijk (1996); 30 flax and linseed cultivars, grown at the nine flax wilt nurseries in 1995 and 1996. The analysis was performed on the first stage (plants were 10 - 15 cm tall). The cultivars are presented as a closed mark, the locations as an open mark.
■ = group 1a, cultivars not indicated as being outliers by the tetrad analysis, and clustered in the main group by the clustering procedure.
□ = group 1b, locations not indicated as being outliers by the tetrad analysis, and clustered in the main group by the clustering procedure.
◆ = group 2a, cultivars indicated as being outliers by the tetrad analysis, and clustered in the main group by the clustering procedure
◇ = group 2b, locations indicated as being outliers by the tetrad analysis, and clustered in the main group by the clustering procedure.
▲ = group 3a, cultivars not indicated as being outliers by the tetrad analysis, and clustered in the small group by the clustering procedure.
△ = group 3b, locations not indicated as being outliers by the tetrad analysis, and clustered in the small group by the clustering procedure.
● = group 4a, cultivars indicated as being outliers by the tetrad analysis, and clustered in the small group by the clustering procedure.
o = group 4b, locations indicated as being outliers by the tetrad analysis, and clustered in the small group by the clustering procedure.

Figure 6.2 (page 129)

Adapted biplot, produced from the AMMI-analysis according to Van Eeuwijk (1996); 30 flax and linseed cultivars, grown at the nine flax wilt nurseries in 1995 and 1996. The analysis was performed on the fourth stage (green boll stage). Legend as in Fig. 6.3.

environments of group 4b (France-95) and group 3b (France-96 and Germany-96), while less exterior but still rather extreme positions were found for environments of group 4b (Russia-95 and USA-96). All cultivars of group 4a ('Regina', 'Slavny 82', 'Hera' and 'Barbara') and one of group 2a ('Ocean') were placed on the exterior of the biplot. Most striking was the difference in behavior between the two years of the French flax wilt nursery. They were most extreme compared with all others, but while France-95 was placed on an extreme place near the x-axis of the biplot, France-96 was placed on an extreme position at the y-axis.


CM-95
CS-96
Barbara
Liflora
Ocean
CS-95
Regina
Hera
RU-95
Biplot stage 1


Biplot stage 4
Regina
NL-95
Slavny 82
Alexim
Hera
FR-95
Barbara
Ocean
RU-96
K-5327
NorLin
Nike
RU-95
FR-96
Teka
Liflora
US-96
Atalante
Elise
GE-96

DISCUSSION

Field trials

The main reason to perform a wide-ranging field experiment was the difficulty in the past to fully understand the flax-*Fusarium* interaction. Despite more than 50 years of research, it is not known whether races exist and how environmental factors may affect this host-pathogen interaction (Kommedahl *et al.*, 1970). The scale of the experiment was chosen as wide as possible, in order to include as many possible interactions. It covered a wide range of cultivars, locations across two continents, and observations at four plant stages. Such a wide-scale experiment is expected to have an increased frequency and magnitude of interactions, but also an increased error variance. This is caused by the differential effects of climate, weather, soil type and also pathogen populations between locations, years and cultivars. To keep the error variance as small as possible the seed sources were standardized as well as the treatment of the seeds before sowing. A well defined protocol for scoring was used to diminish the effect of differences due to in the staff carrying out the scoring.

Emergence

In general the emergence was fairly similar for all fields, the differences between most locations being small, so small that possible differences in emergence are not expected to influence the scores to a measurable extent in most cases. 'Hera' and 'Ocean' emerged poorly in the first year, in all nurseries. The replacement of the seeds of 'Hera' in the second year gave a better emergence but not clearly different wilt scores for this cultivar, so the data of the first year were not eliminated. The drought in the spring of 1996 in the Belgian field resulted in two waves of emergence and germination, resulting in a very low emergence in 1996. This low emergence did not seem to have influenced the scores of the Belgian nursery during the first stage, as the wilt scores in 1996 did not deviate much from those in 1995, when the emergence was good.

Disease screening

Development of the disease in the cultivars

The level of *Fusarium* attack of the cultivars observed in the fourth stage agreed quite well with the level of resistance, as indicated by the source of origin of the cultivars (Table 6.2). A remarkable deviation of the expected resistance was shown by 'Ariane'. In earlier French trials (Beaudoin, 1991) this cultivar was assessed to be susceptible, but in the present experiments it was moderately resistant to resistant, except in the French nursery.

The cultivars, assessed as being susceptible during the first stage, were also susceptible during the other stages. There was no cultivar susceptible for early wilt, which was not susceptible for late wilt too. Kommedahl *et al.* (1970) observed cultivars which differed in resistance against early and late wilt, but in the present experiment no evidence was found to justify a difference between early and late wilt. In the more susceptible cultivars the disease was observed earlier.

Development of the disease at the locations

Clear differences in disease severity were observed between the locations for the two years, but in all environments the disease did develop eventually. Only at the Russian nursery in 1995 the disease was hardly visible.

In Canada the disease developed very early in both years. In 1995 in Manitoba there were indications that the plots had more dead plants in the early stages. The same trend was also visible after the second observation in 1996 in Saskatchewan. Because in the more resistant cultivars *Fusarium* wilt is expressed as late wilt, and because wilt occurrence is promoted by high temperatures (Milikan, 1945; Tochinai and Takee, 1950), which mainly occur later in the season, it is not very likely that *Fusarium* wilt occurrence in the early stage will be followed by a recovery of the plants in later stages. The diseased plants responsible for the high disease scores in the early stage could die and disappear, while wilt symptoms and disease severity may differ

on surviving plants at the end of the season.

The plants in the American nurseries generally were shorter than those in the European trials. The sowing date in the nurseries in North America was on average more than one month later than in the nurseries in Europe, but the last observations were about one month later as well. The use of the standard cultivar helped to compensate for this difference.

Interactions

The ANOVAs showed a significant cultivar * environment variance in all stages ($P < 0.001$). The data of the first stage averaged over both years (Table 6.4) did not give an indication for clear identifiable interactions, but in the fourth stage, four cultivars seemed to cause interactions with locations (Table 6.5). 'Linda', 'NorLin' and 'AC McDuff' were much more susceptible in the North Dakota wilt nursery, "Plot 30", compared to all other nurseries. 'Ariane' appeared to be much more susceptible in the French nursery, compared to all other nurseries. However, for all four cultivars the trend was not statistically significant for the combined results of both years. The clustering analysis did not separate these cultivars, and the tetrad analysis did not trace them as multiple outliers for both years. Also the AMMI-analysis did not place these cultivars apart in the biplot. 'McGregor' showed unstable reactions in the nurseries of St. Paul, Minnesota and Fargo, North Dakota ("Plot 30"), which can not be seen from Table 6.5. In the Czech wilt nursery the expected resistance level of 'McGregor' was less than the observed one, but this difference was not significant.

Clustering took place in the first stage for some of the susceptible cultivars, whereas in the fourth stage clustering occurred for most of them. Also the tetrad analysis indicated the most susceptible cultivars as outliers, causing interaction with environments. For the environments no pattern could be found, neither in the first stage nor in the fourth stage. In the fourth stage two environments from 1996 and one from 1995 were separated, two continental and one maritime, two severely diseased and one only slightly

diseased, so no recognizable parameter was responsible for the environment part of the interaction.

The tetrad analysis of the first stage showed a very high frequency of outliers, indicating a high number of small but significant interactions, whereby most of the interactions were found between the susceptible cultivars and the environments with the highest wilt occurrence. The same holds true for the fourth stage, but to a lesser extend. Also in stage four most of the interactions were found between the susceptible cultivars and environments with the highest wilt occurrence. The highest amounts of multiple outliers were found in the French nursery and in "Plot 30". These results indicate that on the one hand a high level of occurrence of flax wilt in the nursery and on the other hand susceptibility of the cultivar seem to cause interaction.

The AMMI-analysis showed that the interaction could be described in two imaginary additive environmental terms, and that the main differences could be found in the environments. The environments, separated by the clustering analysis were not the same as the environments which were indicated as interacting highly significantly in the tetrad analysis. Only the AMMI-analysis placed all these interactive environments in the extreme positions of the biplots.

CONCLUSIONS

- No evidence was found for a separate status of early wilt and late wilt, neither in susceptibility patterns, nor in interaction patterns.

- The cultivar * environment interaction effects were clear and significant, but mostly visible by differences in environments of the susceptible cultivars.

- In the AMMI model the cultivar * environment interaction scores were described in two terms, which indicated a relatively simple interaction pattern.

- The French flax wilt nursery showed up most strongly in interaction patterns.

- Although the interaction variance was significant no consistent cultivar

* environment interactions came forward. Therefore, there is no evidence of identifiable races. If race-specific effects, causing cultivar * environment interaction exist, they must to be small, too small to be recognized by this type of test.

ACKNOWLEDGEMENTS

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CHAPTER 7

Summary and concluding remarks

SUMMARY

Flax is a crop which currently is the subject of revived attention of agriculture, media and industry, since promising new and environmental friendly utilizations of flax fibers have been developed. Although the crop can be grown with relatively low amounts of agrochemicals, it may suffer from various diseases of which flax wilt is among the most serious ones. Flax wilt, caused by the host-specific fungus *Fusarium oxysporum* f.sp. *lini,* is a major problem in flax and linseed (*Linum usitatissimum*), and breeding for resistance to this fungus is one of the most important selection targets of flax and linseed breeders. High levels of partial resistance exist in some flax and linseed cultivars. Conventional screening methods for resistance involve field trials at strongly infested fields, and wilt development is visually assessed. The results are highly variable and therefore require replicated observations, preferably over a few years. More knowledge about the defense mechanisms and disease processes of flax and linseed to *Fusarium* wilt is a prerequisite for the improvement of the current selection techniques. However, next to nothing is known about the molecular and biochemical background of the resistance mechanisms, and little is known about the inheritance of the resistance. Neither races of the pathogen nor race-specific resistance have been unambiguously reported. About the course of infection and colonization some knowledge has been accumulated, but unsufficient for a full insight in these processes. Also the influence of environmental factors such as temperature and soil type have proven to be very difficult to assess in the *Fusarium* - flax pathosystem.

The aim of this research project was to study the flax and linseed interaction with *Fusarium oxysporum* f.sp. *lini.* Two *in vitro* screening methods were developed to determine more accurately the wilt resistance of flax and linseed genotypes and to study the infection and colonization processes. The first method consisted of growing seedlings in test tubes, filled with vermiculite and a 10% MS-solution. After six days a selection for equal sprout length was carried out and the seedlings were inoculated with a spore suspension of the

fungus. After 16 days disease severity was assessed, using sprout length reduction as a scale. The second method consisted of growing flax and linseed seedlings in two liter preserving jars from which the inner walls were covered with filter paper and seeds of flax and linseed were sown between glass and paper. After six days a spore suspension was added onto the seedling roots. Disease severity was assessed after three weeks, again using sprout length reduction as a scale. The first method was most suitable for a more accurate and quantitative determination of resistance. The second method proved to be especially useful for the study of root infection and colonization processes. Both methods proved to be more accurate for screening for resistance in breeding programs than field trials, and can be carried out in earlier generations than field trials. Therefore they can be considered an improvement on the present screening methods (Chapter 2).

A histological study of the colonization and development of the fungus in a resistant and a susceptible flax cultivar, gave detailed insight in the development of the pathogen and the resulting damage in seedlings. From the second day after inoculation root infection occurred, mainly through intercellular invading of the root cap by fungal hyphae. The root defense consisted of the development of distinct appositions next to penetrating hyphae, while other cells next to penetrating hyphae collapsed. Four days after inoculation the fungus had reached and penetrated the cortex intercellularly, which was followed by a rapid and massive colonization of the entire root tip. The fungus did not show a rapid growth towards the stem. Heavily colonized roots were hollowed out, followed by fungal penetration of the protoxylem. In this experiment the macroscopic disease symptom (sprout length reduction) was not very different between the resistant and the susceptible cultivar. Also microscopically, disease development in both cultivars was similar, although occlusion of intercellular spaces with gum-like components and cell wall enforcement with phenolics seemed to be more prominent in the resistant cultivar (Chapter 3).

Quantitative measurements of the fungal sterol ergosterol, and of the

toxin of *Fusarium oxysporum* f.sp. *lini*, fusaric acid, *in planta*, could be a way to assess the presence and the aggressiveness of the fungus in this host - pathogen system. Ergosterol measurements confirmed the presence of the fungus in an *in vitro* experiment and in a field experiment, although the correlation between the amount of ergosterol and disease symptoms was low, except for sprout length reduction in the *in vitro* experiment. In the susceptible 'Regina' the fungus was present in considerably higher amounts than in the four resistant cultivars, indicating resistance, i.e. reduced amounts of the fungus, more than tolerance (reduced symptoms). However, the amounts of ergosterol measured in 'Regina' were very variable between the plants, suggesting that the fungus was present in variable amounts in plants of the diseased cultivar. Fusaric acid could not be detected in any of the plant materials and no indication was obtained that increased breakdown of fusaric acid is related to resistance (Chapter 4).

The possible existence of races in *Fusarium oxysporum* f.sp. *lini* was studied with help of the *in vitro* test tube method. The first experiment included 12 single spore cultures of the fungus, mainly originating from the same flax wilt nursery and 14 flax and linseed cultivars. In the second experiment 14 single spore cultures from six countries, and 15 cultivars of flax as well as linseed were used. Analyses of variance and an agglomerative clustering analysis indicated the presence of a significant but small interaction variance between pathogen isolates and the flax cultivars. There was no clear indication for race-specificity (Chapter 5).

The influence of locations and years (environments) on the development of flax wilt were studied in an international field experiment. Thirty flax and linseed cultivars were grown at flax wilt nurseries over a two year period at six locations in Europe (Belgium, The Czech Republic, France, Germany, The Netherlands and Russia) and three locations in North America (Canada, Manitoba and Canada, Saskatchewan as well as USA, North Dakota). To make the experiments comparable a protocol was developed for disease scoring, which has been used by all collaborators in both years. Resistance

levels of flax and linseed were measured four times during the growing season. The measurements were analyzed by an ANOVA, and showed significant main effects for cultivars and environments, and significant cultivar by environment interaction for all scorings. A tetrad module, an agglomerative clustering procedure and an Additive Main effects and Multiplicative Interaction effects (AMMI) model were used to structure the interactions in a more manageable form. The interactions showed no correlation with resistance and were not reproducible. Thus, no indication was obtained for different races in the different locations, nor for race-specificity in flax and linseed (Chapter 6).

Concluding remarks

The host - pathogen system described in this thesis is an outstanding example of a system whereby the assessment of resistance has to be performed through indirect effects, explaining why the results of resistance screening show considerable variations. Even the attempts to measure the amount of fungus directly, through ergosterol measurements, proved to be very variable. The development of the two *in vitro* screening methods yielded a better tool to study the plant - pathogen system and the resistance mechanisms involved compared with standard field studies. Although some questions remained it could be concluded from the work presented in this thesis that resistance rather than tolerance plays a role in the relationship between flax and linseed and *Fusarium oxysporum* f.sp. *lini,* and that, if race-specificity exists, it only may work at minor gene level so that the resistance appears to be a quantitative characteristic. This knowledge and the *in vitro* tests, represent a significant contribution to improved breeding strategies for resistance.

Many questions, however, remained unanswered. More knowledge is needed about the molecular and biochemical aspects of the resistance. The role of fusaric acid remained unclear. The fungus was present in low or variable amounts, maybe to low to produce fusaric acid in detectable

amounts. However, fusaric acid is a non-specific toxin of *Fusarium oxysporum*, while the host - pathogen relationship is very specific. Flax and linseed have good defense mechanisms against all other, fusaric acid producing *formae speciales* of *Fusarium oxysporum*. It would be of great advantage to know the specific biochemical factor(s) of the flax - *Fusarium* interaction to understand this complex host - pathogen system. Also the identification of molecular markers, for instance PCR or AFLP markers, in flax and linseed as well as in the pathogen is a great challenge. Molecular markers form a powerful tool to identify genes for resistance in flax and linseed and might give more insight in the inheritance of the resistance.

SAMENVATTING

Vlas (*Linum usitatissimum* L) is een gewas dat momenteel opnieuw in de belangstelling staat van landbouw, media en industrie doordat recentelijk veelbelovende en milieuvriendelijke toepassingen van natuurlijke vezels zijn ontwikkeld. Het gewas kan milieuvriendelijk geteeld worden en heeft weinig bestrijdingsmiddelen en voedingsstoffen nodig. Echter, bij de teelt kunnen een aantal ziekten, waarvan de *Fusarium* verwelkingsziekte een van de belangrijkste is, tot aanzienlijke schade leiden.

De *Fusarium* verwelkingsziekte wordt veroorzaakt door de bodemschimmel *Fusarium oxysporum* f.sp. *lini*. In veredelingsprogramma's is resistentie tegen deze ziekte één van de belangrijkste selectiedoelen. Er bestaan genotypen met een goede resistentie tegen de schimmel, maar van de moleculaire en biochemische achtergrond van deze resistenties is nagenoeg niets bekend. Ook is er weinig bekend over de genetica van de resistentie. Het is nooit ondubbelzinnig vastgesteld of er fysio's van de schimmel bestaan en daaraan gekoppeld, of de resistenties in vezel- en olievlas tegen de schimmel fysio-specifiek zijn. Er is op het gebied van infectie- en kolonisatiepatronen wel onderzoek verricht, maar dit bleek onvoldoende om volledig inzicht te krijgen in deze processen. De gebruikelijke methode van selectie vezel- en olievlas cultivars met resistentie tegen *Fusarium* geschiedt door gebruik te maken van proefvelden die met *Fusarium oxysporum* f.sp. *lini* zijn besmet. Hierbij wordt de schade visueel vastgesteld. Deze methode heeft als nadeel dat de resultaten vaak erg variabel zijn en om die reden is het noodzakelijk dat beproevingen met veel herhalingen en bij voorkeur over meer jaren worden uitgevoerd. De invloed van omgevingsfactoren op het vlas-*Fusarium* ziekteproces, zoals temperatuur en bodemsoort is zeer moeilijk vast te stellen. Meer kennis over afweermechanismen en over het ziekteproces van de *Fusarium* verwelkingsziekte in vezel- en olievlas was nodig om de huidige selectie technieken te verbeteren.

Het doel van het onderzoek dat in dit proefschrift wordt beschreven was het uitvoeren van onderzoek naar de interacties tussen vezel- en olievlas en *Fusarium oxysporum* f.sp. *lini*. Teneinde resistenties nauwkeuriger te kunnen vaststellen en de mogelijkheid te creëren om het infectie- en kolonisatieproces in detail te bestuderen, zijn twee *in vitro* toetsmethoden ontwikkeld. Bij de eerste methode werd gebruik gemaakt van testbuizen gevuld met vermiculiet en een 10% MS-voedingsoplossing. Hierin werden vlaszaden tot kieming gebracht. Na zes dagen werden de zaailingen op gelijke spruitlengte geselecteerd, en vervolgens geïnoculeerd met een sporesuspensie van de schimmel. Na 17 dagen werd de mate van aantasting bepaald door middel van het bepalen van de lengtereductie ten opzichte van een gezonde controle. De tweede methode bestond uit het opkweken van vlaszaailingen in twee liter weckflessen. Tegen de binnenwand van de weckflessen werd filtreerpapier geplaatst zodanig dat de onderrand van het papier in een oplossing van 10% MS voedingsoplossing stond. De zaden werden geplaatst tussen papier en glas. Na zes dagen werd een sporesuspensie van de schimmel aan het systeem toegevoegd, vlakbij de vlaswortels. Na drie weken werd de mate van aantasting bepaald, door middel van het bepalen van de lengtereductie. De eerste methode bleek zeer geschikt voor het nauwkeurig en kwantitatief bepalen van het resistentieniveau. De tweede methode lijkt specifiek bruikbaar voor het bestuderen van infectie- en kolonisatie processen. Beide methoden bleken goed toepasbaar voor selectie op *Fusarium*-resistentie omdat ze minder variabele resultaten opleverden dan veldtoetsen en in vroegere generaties van het veredelingsprogramma kunnen worden uitgevoerd. Ze kunnen om die reden als een aanwinst worden beschouwd ten opzichte van de huidige toetsmethoden (Hoofdstuk 2).

In hoofdstuk 3 wordt de kolonisatie en ontwikkeling van de schimmel in een vatbare en een resistente cultivar beschreven, alsmede de veroorzaakte schade in de zaailingen als gevolg hiervan. Vanaf de tweede dag na inoculatie was er sprake van intercellulaire penetratie van de schimmelhyfen,

hoofdzakelijk in de calyptra. Het verdedigingsmechanisme van de calyptracellen bestond uit het vormen van plaatselijke celwandverdikkingen, zgn. "appositions", direct naast de penetrerende schimmelhyfen. Van andere calyptracellen die in contact kwamen met de schimmel werd de wand dusdanig beschadigd dat de cellen lyseerden. Na vier dagen had de schimmel de cortex bereikt, gevolgd door snelle en massieve intercellulaire invasie van de cortex en vervolgens kolonisatie van de gehele worteltop. Er was geen sprake van een snelle verplaatsing van de schimmel in de richting van de stengel. De wortels werden in hevige mate gekoloniseerd zodat op de plaats van de cortex holtes ontstonden. In dit stadium werd tevens penetratie van het protoxyleem waargenomen. Hoewel er in dit experiment zowel macroscopisch als microscopisch weinig verschil in reactie te zien was tussen de vatbare en de resistente cultivar, werd in de resistente cultivar toch vaker waargenomen dat intercellulaire ruimten gevuld werden met gom-achtige verbindingen. Ook werden er in de resistente cultivar wat vaker celwandverdikkingen gevonden waarbij fenolen gevormd werden (Hoofdstuk 3).

Het kwantitatief bepalen van de hoeveelheid van het schimmelspecifieke sterol ergosterol, en van het toxine van *Fusarium oxysporum* f.sp. *lini,* fusaarzuur, *in planta,* is een directe manier om de mate van aanwezigheid en aggressiviteit van de schimmel of zijn toxine te meten. Door middel van ergosterolmetingen aan plantmateriaal afkomstig van een met *Fusarium oxysporum* f.sp. *lini* geïnfecteerd vlasveld, en aan plantmateriaal verkregen uit een *in vitro* experiment, kon de aanwezigheid van de schimmel *in planta* worden aangetoond. De correlatie met waargenomen ziekte symptomen bleek laag, behalve in het geval van de lengtereductie bij het *in vitro* experiment. In de vatbare 'Regina' was de schimmel in aanzienlijk grotere hoeveelheden aanwezig dan in de resistente cultivars, hetgeen duidt op echte resistentie (minder schimmel), in plaats van tolerantie (minder symptomen). De gemeten hoeveelheden schimmel in 'Regina' waren echter nogal variabel, wat tot de conclusie leidt dat de schimmel in variabele hoeveelheden in

planten van de vatbare cultivar aanwezig was. Er werd in het geheel geen fusaarzuur aangetoond en er is dus geen aanwijzing gevonden dat een versnelde afbraak van fusaarzuur gerelateerd is aan resistentie (Hoofdstuk 4).

Met behulp van de *in vitro* testbuizentoets is het bestaan van fysio's bij *Fusarium oxysporum* f.sp. *lini* onderzocht. Er zijn twee experimenten uitgevoerd. Bij het eerste experiment zijn 12 monospore cultures van de schimmel gebruikt, hoofdzakelijk afkomstig uit één en hetzelfde geïnfecteerde proefveld, waarmee 14 vezel- en olievlas cultivars zijn geïnoculeerd. Bij het tweede experiment zijn 14 monospore cultures gebruikt afkomstig uit zes landen, en zijn 15 vezel- en olievlas cultivars geïnoculeerd. Uit een ANOVA en een agglomeratieve clustering procedure bleek dat er kleine maar significante interacties bestonden tussen isolaten en cultivars. Er was echter geen concrete aanwijzing voor fysiospecificiteit (Hoofdstuk 5).

Het effect van locaties en jaren op de *Fusarium*-vlas interactie is bestudeerd door het uitvoeren van een internationale veldtoets. Dertig cultivars van vezel- en olievlas zijn getest over een periode van twee jaren, op zes, met *Fusarium oxysporum* f.sp. *lini* geïnfecteerde proefvelden in Europa (België, Duitsland, Frankrijk, Nederland, Rusland en Tsjechië) en verder op drie geïnfecteerde proefvelden in Noord Amerika (Canada, Manitoba en Canada, Saskatchewan en de Verenigde Staten, Noord Dakota). Om de onderlinge verschillen in waarneming zo klein mogelijk te maken werd een protocol geschreven dat door alle medewerkers is gebruikt. Op vier momenten tijdens het groeiseizoen werd de aantasting beoordeeld. Vervolgens zijn de scoringsdata geanalyseerd met behulp van een ANOVA, waarbij significante hoofdeffecten werden waargenomen voor de cultivars en de omgevingsfactoren, maar ook significante interactie effecten. Met behulp van een tetrade module, een agglomeratieve clustering analyse en een AMMI-model werden de interacties meer gedetailleerd onderzocht. De interacties bleken geen correlaties te vertonen met resistentie en waren slecht reproduceerbaar. Uit de analyses is geen aanwijzing verkregen voor het bestaan van verschillende fysio's in de verschillende locaties, noch voor het

bestaan van fysiospecificiteit in vezel- en olievlas (Hoofdstuk 6).

RÉSUMÉ

Le lin (*Linum usitatissimum* L.) est sujet à un renouveau d'attention de la part du monde agricole, des médias et de l'industrie depuis le développement de nouvelles applications non polluantes des fibres de lin. Bien que la culture du lin ne nécessite que peu d'intrants (fertilisants, pesticides), cette culture est sensible à différents parasites parmi lesquels *Fusarium oxysporum* f.sp. *lini*, champignon du sol responsable de la fusariose du lin.

La fusariose étant une des principales limitation de la culture du lin, la recherche de génotypes résistants est l'un des principaux objectifs de l'amélioration variétale du lin. Certains cultivars de lin présentent de bons niveaux de résistance partielle à la fusariose mais les bases biochimiques et moléculaires de ces resistances n'étant pas connues il est nécessaire de recourir aux essais en champs pour déterminer le niveau de résistance des nouveaux cultivars. Ces vatiétés sont ainsi cultivées dans des parcelles contaminées par *F. oxysporum* et les dégâts observés visuellement. Les résultats étant relativement variables, les essais nécessitent de nombreuses répétitions et doivent être reconduit sur plusieurs années. Très peu de données sont disponibles quant à l'héritabilité de ces resistances. Peu de connaissances sont également disponibles concernant le champignon puisque l'existence de races physiologiques du pathogène et par la même de résistances race-spécifiques ne sont pas établie. Bien que l'épidémiologie de la fusariose du lin aie fait l'objet de quelques travaux, ceux-ci restent insuffisants. Il faut de plus ajouter que les facteurs environnementaux tels que la température ou la nature du sol peuvent modifier de façon importante l'établissement de la fusariose. Une meilleure connaissance du développement de la fusariose et des mécanismes de défense dont dispose la plante est donc un prérequis à l'amélioration des techniques de sélection du lin.

L'objectif du travail de recherche décrit dans cette thèse de doctorat est l'étude des interactions entre le lin (fibre et graine) et *Fusarium oxysporum*

f.sp. *lini*. Deux méthodes de criblage *in vitro* ont été développées afin de tester avec précision la résistance du lin vis-à-vis de la fusariose et d'étudier le processus d'infection et de colonisation par le champignon des tissus de l'hôte. Dans la première méthode des graines de lin sont mises à germer en tube à essais sur vermiculite additionnée d'une solution nutritive (10% MS). Après six jours de culture les plantules présentant un développement homogène sont sélectionnées et inoculées avec une suspension de spores du pathogène. Seize jours après inoculation la longueur des pousses est mesurée, la réduction de croissance étant alors prise comme symptôme de la maladie. Dans la deuxième méthode, des bocaux à conserve de deux litres contiennent une feuille de papier filtre adhérente à la paroi intérieure du bocal et imbibée d'une solution nutritive MS 10%. Les graines de lin sont mises à germer entre la paroi du bocal et le papier filtre. Six jours après semis, une suspension de spores est inoculée à proximité des racines. La longueur des pousses est mesurée après trois semaines de culture. La première méthode est particulièrement adaptée à la quantification du niveau de résistance du lin à la fusariose. La deuxième méthode s'avére plus adéquate pour étudier les processus d'infection et de colonisation des racines par le champignon. Les deux méthodes se révélent plus efficaces que les essais aux champs pour déterminer les niveaux de résistance du lin à la fusariose, du fait, d'une part d'une moindre variabilité des essais *in vitro*, et, d'autre part, de la possibilité de les utiliser plus tôt au cours des schémas d'amélioration variétale. Pour ces raisons ces tests peuvent être considérés comme un progrès par rapport aux méthodes classiques d'essais aux champs (Chapitre 2).

Dans le Chapitre 3, l'étude histologique de la colonisation et du développement du pathogène chez un cultivar sensible et chez un cultivar résistant est présentée. Deux jours après inoculation les hyphes mycéliens pénétrent la coiffe racinaire tout en restant localisés entre les cellules. Les cellules de la coiffe proches des hyphes réagissent alors par apposition de dépôts sur les parois cellulaires, alors que les cellules environnantes collapsent. Quatre jours après inoculation, les hyphes atteignent le cortex

racinaire et envahissent les cellules. Ceci est alors suivi par une colonisation rapide et massive de l'extrémité de la racine mais non de la tige. Le dévelopement du champignon dans la racine se traduit par l'apparition de cavités et par la colonisation du protoxylème. La réduction de croissance des pousses des cultivars résistants n'est que peu différentes de celles des cultivars sensibles. L'étude microscopique montre que le champignon se développe de façons semblables chez les deux types de cultivars. Cependant, chez les cultivars résistants il est possible d'observer plus tôt l'accumulation de gommes dans les lacunes intercellulaires et le renforcement des parois cellulaires par des composés de nature phénolique (Chapitre 3).

La teneur en un stérol spécifique du champignon, l'ergostérol, et en une toxine du champignon, l'acide fusarique, permet de quantifier, *in planta*, le degré de colonisation et l'agressivité du pathogène. La mesure des teneurs en ergostérol de plantes cultivées aux champs ou cultivées *in vitro* confirme la présence du champignon, bien que la corrélation entre teneur en stérol et symptômes soit faible (excépté pour la réduction de croissance des pousses *in vitro*). Chez le cultivar sensible "Regina", le champignon est présent en quantité plus importante que chez les quatre cultivars résistants, indiquant le développement de résistances (réduction de la quantité de pathogène), plutôt que de tolérances (réduction des symptômes). Cependant les teneurs en ergostérol observées dans les plants infestés du cultivar "Regina" sont très variables de plante à plante. L'acide fusarique n'a pu être mis en évidence dans aucune des plantes testées, et aucune indication quant à une possible dégradation de l'acide fusarique en relation avec la résistance n'a été mise en évidence (Chapitre 4).

L'existence de races physiologiques de *Fusarium oxysporum* f.sp. *lini* a été recherchée grâce au premier test *in vitro* décrit. Douze cultures monospores du champignon, majoritairement originaires du même champ, ont été inoculées à 14 variétés de lin (graine et fibres). Dans une deuxième étude, 14 cultures monospores du champignon, originaires de six pays

différents, ont été inoculées à 15 variétés de lin à graines et de lin à fibres. L'analyse de variances et l'analyse de groupes mettent en évidence la présence d'une interaction faible mais significative entre isolats du pathogène et cultivars de lin. Aucune indication ne permet d'assurer l'existence de résistance race-specifique (Chapitre 5).

L'effet des interactions entre lieu de l'essai et année de l'essai sur le développement de la fusariose du lin a été étudié au cours d'un essai aux champs réalisé au niveau international. Trente variétés de lin (graine et fibre) ont été cultivées durant deux années dans six pays européens (Allemagne, Belgique, France, Pays Bas, République Tchèque et Russie) et trois localisations en Amérique du Nord (Manitoba et Saskatchewan, Canada, et Dakota du Nord, Etats-Unis). Un même protocole écrit d'évaluation des dégâts a été utilisé par tous les collaborateurs au cours des deux années. Les niveaux de résistance étaient évalués quatre fois au cours de la période de croissance. L'analyse de variances des résultats montre un effet significatif du cultivar, de l'environnement et de l'interaction cultivar - environnement. Les donnés ont été soumises à différentes analyses telles que le module des tétrades, l'analyse de groupes et l'AMMI. Les interactions ne s'avérent pas corrélées à la résistance et sont non reproductibles. Nos résultats ne mettent en evidence ni l'existence de races physiologiques de *Fusarium oxyxporum* f.sp. *lini*, ni l'existence de résistance race-specifique chez les lins à graine et à fibre (Chapitre 6).

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Ik heb veel nut gehad van de onderzoeksschool Experimental Plant Sciences, waarvan ik de ontwikkeling van dichtbij mocht meemaken. De contacten met de leden van de AIO-raad waren misschien niet direct van nut voor mijn project, maar mensen als Peter Wittich, Ingeborg van Scheurwater en Enrico van Lieshout, de kern van de club-in-ontwikkeling, werkten wel mee aan het verkrijgen van een echte AIO-mentaliteit, althans zo voelde ik dat.

Het schrijven viel mij in het begin niet mee, maar Wouter Lange bleek in dit stadium van onschatbare waarde, altijd veel kritiek, maar altijd positief. Ook bleek mij toen pas goed dat ik een zeer bekwame promotor had getroffen in Jan Parlevliet. Van hetzelfde laken een pak, soms veel kritiek maar altijd op een prettige manier geserveerd.

Familie, vrienden en kennissen hebben me in de drukte wel eens vaker moeten missen dan hun lief was. Ik kan helaas niet beloven dat daar verandering in komt.

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CURRICULUM VITAE

Gezina Maria Leonien Wilhelmina Kroes werd geboren op 26 mei 1955 in Dalfsen waar ze de lagere school doorliep. Het HAVO-diploma behaalde ze in 1974 aan het Thomas à Kempis Lyceum in Zwolle. Na de geboorte van haar drie kinderen in 1974, 1976 en 1980, pakte ze de draad van het studeren weer op en behaalde in 1984 het VWO-diploma aan het Veluws Avond College te Harderwijk. Hierna volgde een studie biologie in deeltijd aan de Universiteit Utrecht. De propedeuse werd behaald in 1986. Met een specialisatie in biochemie en oeco-fysiologie, en met een praktijkstage bij het Consulentschap voor Natuur Bos Landschap en Fauna in Lelystad, werd het doctoraal examen afgelegd in 1992. Een aanstelling als onderzoeker aan het Centrum voor Plantenveredelings- en Reproductie Onderzoek (CPRO-DLO) volgde, waarbij ze de taak kreeg identificatiemethoden te ontwikkelen voor aardappelrassen met behulp van elektroforese en image-analysis. In 1993 kreeg ze een aanstelling als AIO bij de vakgroep Plantenveredeling (Landbouw Universiteit Wageningen), gedetacheerd op het CPRO-DLO, alwaar ze gewerkt heeft aan *Fusarium* resistentie in vlas. Enkele nevenfuncties waren het lidmaatschap van het dagelijks bestuur van de AIO-raad van de onderzoeksschool Experimentele Plantenwetenschappen en redactielid van de EPS-newsletter. Vanaf 1 januari 1998 zal ze als post-doc werkzaam zijn op het Morden Research Centre, Morden, Manitoba, Canada (Agri Food and Agriculture Canada) waar ze zal gaan werken aan moleculaire merkers bij *Fusarium* resistentie in vlas.