Studies on the *Fusarium*-lily interaction:
interaction:
a breeding approach

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Studies on the *Fusarium*-lily interaction:
a breeding approach

Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 9 november 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1. De opmerking van Imle in 1942 dat bijna alle veredeling bij lelie is uitgevoerd zonder te letten op ziekteresistentie is nog steeds actueel.

    Imle, E.P., 1942. The basal rot disease of lilies. Ph.D. Thesis Cornell University: 79 pp. + figures.

2. Bepaling van de "ziektegevoeligheid" van cultivars door middel van enquêtes onder telers, zoals uitgevoerd door het Milieuplatform Bloembollensector in 1993, is een niet-wetenschappelijke benadering en leidt tot onjuiste conclusies.

    Dit proefschrift.

3. Het ontbreken van discontinuïteit in de resistentieniveaus in F_1-populaties zoals waargenomen door Bowes et al. (1992) betekent nog niet dat de eigenschap polygeen overerft.

    Bowes, S.A., R.N. Edmondson, C.A. Linfield & F.A. Langton, 1992. Screening immature bulbs of daffodil (*Narcissus* L.) crosses for resistance to basal rot disease caused by *Fusarium oxysporum* f.sp. *narcissi*. Euphytica 63: 199-206.

4. De bewering van Ben-Yephet et al. (1993) dat een kastoets ter bepaling van *Fusarium*-resistentie in anjers onbetrouwbaar is als voorspeller voor het resistentieniveau van deze anjers in veldexperimenten is niet bewezen.

    Ben-Yephet, Y., M. Reuven & Y. Mor, 1993. Selection methods for determining resistance of carnation cultivars to *Fusarium oxysporum* f.sp. *dianthi*. Plant Pathology 42: 517-521.

5. Ter voorspelling van de duurzaamheid van resistentie kan beter worden gekeken naar het aantal betrokken resistentiegenen dan naar het resistentieniveau.

    Rapportage werkgroep Duurzaamheid van resistentie. CPRO-DLO, 1994.

6. De kwaliteit van het onderzoek in Italië is omgekeerd evenredig met de kwaliteit van de outillage.

    Bezoek aan Italië. Projectgroep *Fusarium*-resistentie bij bolgewassen, 1991.

7. Het instellen van een leerstoel selectiemethoden zou niet alleen tot de plantenveredeling beperkt moeten blijven, daar objectieve selectiecriteria eveneens een rol spelen bij allerlei maatschappelijke vraagstukken.

8.   Indien we met de tulp nog 400 jaar vooruit willen dient de veredeling, op korte termijn, sterk te worden gestimuleerd.

9.   Er is blijkbaar nog onvoldoende druk van de maatschappij op de bloembollenveredelingsbedrijven om resistentie als belangrijk kenmerk bij de selectie te beschouwen.

10.   Het laten verschijnen van vier vakbladen voor de bloembollensector is pure geldverspilling, maar goed voor de publikatielijst.

11.   De huidige aandacht voor de natuur kan leiden tot vreemde situaties. Het vermeerderen en instandhouden van enkele roggelelies levert veel meer publiciteit en waardering op dan gedegen resistentie-onderzoek.

12.   De overeenkomst tussen biotechnologisch onderzoek en aandelen is dat je er op tijd in moet stappen, … en eruit.

13.   Ter bescherming van flora en fauna in natuurgebieden heeft het de voorkeur om mensen in hun vrijheid te beperken boven honden.

Th.P. Straathof
Studies on the *Fusarium*-lily interaction: a breeding approach
Wageningen, 9 november 1994

WONDERFUL
When all the world is quiet and still
And doubts run deep as the ocean
Through troubled years and broken dreams
To the place where all silence has spoken

RUNRIG, 1993: Amazing Things

VOORWOORD

Op 1 februari 1989 werd op het toenmalige IVT, het huidige CPRO-DLO, gestart met het project 'Resistentieveredeling tegen *Fusarium oxysporum* bij lelie en gladiool', waarvoor ik als projectleider werd aangesteld. Het *Fusarium*-onderzoek bij bolgewassen groeide, mede in het kader van het 'Urgentieprogramma Bollenziekte- en veredelingsonderzoek', de afgelopen vijf jaar uit tot een volwaardig wetenschappelijk onderzoekprogramma. Zeer velen hebben hiervoor een bijdrage geleverd waarvoor mijn hartelijke dank, een aantal van hen wil ik graag met name noemen.

Hein Inggamer, gedurende 3½ jaar heb jij mij geassisteerd bij het uitvoeren van vele experimenten. Met name jou nauwkeurigheid van werken heeft ertoe bijgedragen dat betrouwbare resultaten zijn verkregen. Huub Löffler, als fytopatholoog en initiator van het *Fusarium*-onderzoek bij lelie, was jij mijn directe begeleider en steunpunt gedurende het onderzoek. Naast het gezamenlijk uitvoeren van experimenten was jij met name betrokken bij het tot stand komen van de wetenschappelijke artikelen, die de basis vormen van dit proefschrift. Je niet aflatende kritiek op de concepten van de artikelen heeft een zeer belangrijke positieve invloed gehad op de uiteindelijke tekst. Jaap van Tuyl, niet alleen als sectiehoofd Bolgewassen verantwoordelijk voor de coördinatie van het bloembollenveredelingsonderzoek op CPRO-DLO, maar zeker als de leliespecialist zorgde jij, zowel praktisch als met ideeën, dat aan de plantzijde van de bestudeerde interactie alles voorspoedig verliep. Hein van Holsteijn, afdelingshoofd Siergewassen en programmaleider van het Urgentieprogramma, jou ben ik zeer erkentelijk voor de vrijheid van werken die ik kreeg en de interesse die jij altijd toonde tijdens de uitvoering van de experimenten en in de resultaten.

Evert Jacobsen, je was snel bereid om als promotor op te treden ondanks dat dit onderwerp niet direct je eigen terrein is. We hebben elkaar niet zoveel gesproken tijdens het onderzoek, je had er alle vertrouwen in dat de begeleiding goed verzorgd was. Wel heb je alle artikelen kritisch bekeken en vele suggesties gedaan ter verbetering, met name om mijn zendelingenwerk wat in te dammen.

Hans Sandbrink, jou komst als moleculair bioloog bij de sectie Bolgewassen betekende een nieuw tijdperk in het veredelingsonderzoek bij bolgewassen. Hoofdstuk 7 in dit proefschrift laat de eerste resultaten van dit onderzoek zien. Hans Jansen, veelvuldig ben ik aangelopen tegen jou statistische methoden voor het analyseren van waarderingscijfers. Gelukkig was jij altijd bereid om mijn (onduidelijke) vragen, soms onduidelijk, te beantwoorden. Ook Ritsert Jansen en Johan van Ooijen wil ik bedanken voor de hulp bij het maken van de lotingschema's en verwerking van gegevens.

CONTENTS

GENERAL INTRODUCTION

THE LILY

The lily (*Lilium* spp.) is a perennial bulb, that is used worldwide as an ornamental. The original centre of the genus *Lilium* L. is the Himalayan Region (De Jong, 1974). Currently, the genus is scattered over the Northern Hemisphere (North America, Europe and Asia). More than half of the approximately 100 species originate from Asia. The ancestors of the modern lilies such as the Asiatic and Oriental hybrid lilies originate mostly from China and Japan (Beattie & White, 1993). The taxonomic classification divides *Lilium* into seven sections (Comber, 1949; De Jong, 1974).

Cultivation of lily bulbs for ornamental purposes started at the end of the 19th century in Europe, Japan and the United States of America. Although the lily is a cross-breeding crop, genotypic characteristics are perpetuated by vegetative propagation. The perennial bulb exists of scale-like leaves attached to a compressed stem, the basal plate, and are used as storage organs. These scales are used to propagate the lilies (Griffiths, 1933).

An important advance in lily breeding was made with interspecific hybridisation. One of the important interspecific hybridisations was made in the 1940's by J. De Graaff at Oregon Bulb Farm. He selected the Mid-Century hybrids, e.g., 'Enchantment', by crossing *L. maculatum*, *L.* x *hollandicum* (*L. bulbiferum croceum* x *L. maculatum*) and *L. tigrinum*. The Mid-Century hybrids were crossed with other species of the *Sinomartagon* section, e.g., *L. davidii* and *L. cernuum*, to form the group called Asiatic hybrid lilies (De Graaff & Hyans, 1967; Feldmaier & McRae, 1982). The Asiatic hybrid lily 'Connecticut King' has been one of the most successful cultivars. The Oriental hybrid lilies were obtained by interspecific hybridisation of species of the Archelirion section, e.g. *L. auratum*, *L. speciosum*, and *L. rubellum* (McRae, 1976; Feldmaier & McRae, 1982). They are increasingly of importance. The first upfacing Oriental hybrid lily 'Star Gazer', bred by L. Woodriff, is the leading lily in the Netherlands. Cultivars of *L. longiflorum* (section *Leucolirion*) are important as cut flowers in Japan and they have been produced for over one hundred years (Van Tuyl, 1985). In the United States, *L. longiflorum* is mainly used as a pot plant (Miller, 1993). The Aurelian hybrids, obtained from crosses between species of the *Leucolirion* section (*L. regale*, *L. sulphureum*, *L. sargentiae*) and *L. henryi*, are used only in gardens and are, therefore, commercially less important.

New tissue culture techniques have permitted further hybridisations (Van Creij et al., 1990). Tetraploidisation techniques can restore fertility of sterile interspecific

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hybrids (Van Tuyl, 1990). This offers new opportunities for using the genetic variation in *Lilium* for important traits, like new flower colour and flower shape combinations, low-light tolerance, forcing characteristics, vase life, and disease resistance.

In the Netherlands, lily cultivation has increased from 227 ha in 1970 to 2896 ha in 1993 (Van Tuyl, 1992). Currently, the lily is the second bulb crop, after tulip. The Netherlands is the world leading country in the production of lily bulbs, followed by the United States and Japan. The Asiatic hybrid lilies are the most important group with ± 60 % of the area in 1993. The area with Oriental hybrid lilies has increased rapidly and comprised ± 33 % in 1993. In the past, the cultivation of *L. longiflorum* was not possible in the Netherlands because of summer sprouting and most bulbs were imported from Japan. Through breeding, new cultivars have been obtained with a lower sensitivity to summer sprouting (Van Tuyl, 1985; Van Tuyl, 1988; Van Tuyl, 1992) and *L. longiflorum* bulbs is now exported from the Netherlands to Japan (Matsuo, 1992). From the total Dutch lily bulb production, 560 million bulbs (65 %) are exported to other countries, mainly for cut flower production. Important countries for bulb export are Italy, Japan, the United States, France, and the United Kingdom. The remaining 35 % of the bulbs is used for year round flower production in the Netherlands. About 80 % of the flowers produced in the Netherlands are exported. Important countries for flower export are Germany, France, and the United States. The annual economic value of lily cultivation and flower production in the Netherlands is estimated to be at least 600 million Dutch guilders (Lommerse, 1992).

The cultivation of lily bulbs is threatened by a number of pathogens. In the Netherlands, the most important ones are viruses (Tulip Breaking Virus, Lily Symptomless Virus, Lily Virus X, Tobacco Rattle Virus), nematodes (*Pratylenchus penetrans*, *Aphelenchoides fragariae*), a leaf fungus (*Botrytis elliptica*), the soil fungi (*Fusarium oxysporum*, *Cylindrocarpon destructans*, *Rhizoctonia spp.*, *Pythium spp.*), and the fungi affecting during storage (*Penicillium spp.*, *Botrytis cinerea*) (Bergman et al., 1983).

In 1931, Van Hell investigated bulb and root rots found in lily bulbs cultivated in the Netherlands. *Cylindrocarpon radicola* Wr. (*Cylindrocarpon destructans*) was isolated and inoculation experiments on lilies resulted in typical symptoms of root and bulb rot. Besides *Cylindrocarpon*, *Rhizoctonia solani* and *Fusarium solani* were isolated, but inoculation experiments with these fungi did not lead to disease development. Imle first mentioned a bulb disease of lilies caused by *Fusarium spp.* in 1940. In 1942, Imle published his thesis on basal rot of lilies, caused by a fungus, classified as *Fusarium oxysporum* f.sp. *lilii*. Comparison of *Cylindrocarpon* and *Fusarium* in screening tests showed that *Fusarium* was more destructive than *Cylindrocarpon* (Smith & Maginnes, 1969; Bollen, 1972). At low temperatures (5 °C) the growth of *Fusarium* ceases, but *Cylindrocarpon* still produces some

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disease symptoms (H.J.M. Löffler, *personal communication*).

FUSARIUM

Fusarium belongs to the class of the Deuteromycetes (Fungi Imperfecti) and *F. oxysporum* Schlecht. is the only species in the section *Elegans* (Snyder & Hansen, 1940). The haploid fungus can produce three asexual spore forms, uni(bi-)cellular microconidia, multicellular 3-4-septate macroconidia, and chlamydospores. Genetic exchange by a parasexual cycle may possibly occur following cell fusion when heterokaryons are formed (Puhalla, 1981; Molnar et al., 1990). Under unfavourable conditions chlamydospores (cells with a thick cell wall) can be separated from the hyphae and they can survive for a long period in soil (Schipper & Van Eck, 1981). Infection occurs by germinated spores which penetrate the roots of a host (MacHardy & Beckman, 1981; Baayen, 1992).
Within *F. oxysporum* more than 75 formae speciales are defined which are distinct from each other by their host plant range (Armstrong & Armstrong, 1981). Most of these formae speciales cause wilting and colonize the vessels of the host plant (MacHardy & Beckman, 1981). In flower bulbs, however, the dominant symptom is rotting of the bulb or corm.
In lily, *F. oxysporum* f.sp. *lilii* causes scale and bulb rot (Imle 1942a; Imle 1942b; Linderman, 1981). The main infection court is probably through the bulb roots, but infection can also occur in scales via stomata, the basal plate, and wounded tissue. After infection, the root tissue between exodermis and endodermis is destroyed, but the fungus does not penetrate beyond the endodermis. Hyphae mostly grow intercellularly, and cells may be killed ahead of fungal hyphae after which the dead cells can be invaded (Imle, 1942a; Imle, 1942b; Baayen, 1992; Rijkenberg & Baayen, *in preparation*). Infection of the bulbs leads to brown or black necrotic lesions, and if the rot extends to the basal plate, to disintegration of the bulbs. Above-ground symptoms are yellowing and necrosis of leaves and dwarfing of the stems. These symptoms are not always visible, because stem roots can develop and survive, after bulb roots have been destroyed. (Imle, 1942a; Imle, 1942b).

FUSARIUM CONTROL IN LILY

Fusarium damage in lily occurs mainly during bulb propagation and cultivation. *Fusarium*-infected bulb lots have lower yields and can be rejected by the Inspection Service. Contaminated soil cannot be used for lily cultivation for several years and infected bulb material causes major problems for bulb exporters. Infected bulb material and intensive soil use can also cause major problems during flower forcing.

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No survey of the financial damage caused by *Fusarium* in lily has been reported. This is due to the fact that most of the damage is indirect and difficult to determine. Prevention of infection by *Fusarium* depends on a combination of measures (Linderman, 1977; McRae, 1987). Besides cultivation practices like crop rotation, a low nitrogen supply, steaming of soil, and the use of healthy plant material, prevention is mostly based on chemical disinfestation of bulbs (e.g., benomyl, captan, prochloraz). However, resistance of *Fusarium* to fungicides can occur (Bollen, 1972). Furthermore, insufficient protection is often obtained by fungicides. Chemical disinfestation of soil used for control of nematodes in lily, also has a fungicide effect. In the Netherlands, a reduction of the application of chemicals for disease control is necessary to reduce environmental pollution. For soil disinfestation, with respect to 1985, a reduction of 60-85 % in the flower bulb industry and approximately 75 % in the bulb flower industry must be accomplished before the year 2000 (Van Aartrijk et al., 1990). A decrease in the use of nematocides in lily cultivation can lead to an increase in damage caused by *Fusarium*.

An environmental friendly alternative will be disease resistant cultivars. Breeding for resistance has proven to be successful in many crops and for many pathogens, including soil-borne diseases (Tinline et al., 1989) such as *F. oxysporum* (Shaner, 1981). In flower bulbs, breeding for *Fusarium* resistance is being investigated for the major crops, i.e., lily, gladiolus, tulip and narcissus.

BREEDING FOR *FUSARIUM* RESISTANCE IN FLOWER BULBS

Imle (1942a; 1942b) was the first to study *Fusarium* resistance in lily. He performed screening tests in greenhouses and in the field. A temperature range between 27 and 30 °C and a pH range between 5.6 and 7.4 was optimal for fungal growth. Soil or bulbs were artificially infested with the pathogen. He primarily used commercial bulbs for his experiments, but sometimes stem bulblets, stem bulbils, scale bulblets and scales were used. Bulblets gave greater losses than mature bulbs. Smith & Maginnes (1969) described a screening test using scales. This test was later optimized by Löffler & Mouris (1989). Van Tuyl (1980) described a screening test using scale bulblets. Variation in *Fusarium* resistance was detected, mainly between *Lilium* species (Imle, 1942a; Imle 1942b). Variation within species was not mentioned. No absolute resistance has been found in lily. Imle (1942a) found no evidence for the existence of physiological races of the fungus. Bald et al. (1971) showed that isolates of *F. oxysporum* f.sp. *lilii* from roots, bulbs and stems differed in tissue specificity. Development of a seedling test was unsuccessful, since direct sowing of seed in infested soil caused heavy losses by damping-off (Imle, 1942a). *F. oxysporum* f.sp. *gladioli* (Massey, 1926) Snyd. & Hans. (1940) causes corm rot

in many crops of the *Iridaceae*, such as gladiolus, crocus, iris and freesia (McClellan, 1945; Apt, 1958; Linderman, 1981). Besides rotting of corms and roots of the gladiolus, *Fusarium* is able to colonize the vascular tissue (Nelson et al., 1981). Screening tests were developed and variation in *Fusarium* resistance was found (McClellan & Pryor, 1957; Palmer & Pryor, 1958; Wilfret & Woltz, 1973; Jones & Jenkins, 1975; Chandra et al., 1985). New resistant cultivars were released (Wilfret & Magie, 1979; Wilfret, 1981; Wilfret, 1986). Our experiments showed that absolute resistance to *Fusarium* does exist in the species *Gladiolus dalenii* (*not published*). The occurrence of physiological races of the pathogen has been reported by Roebroeck & Mes (1992). Our results showed that selecting for *Fusarium* resistance is possible at seedling level (Straathof & Löffler, 1992). Seedlings showed less sensitivity than corms.

Breeding for resistance in tulip to *F. oxysporum* f.sp. *tulipae* Apt (1958) was carried out by Van Eijk and colleagues (Van Eijk et al., 1978; Van Eijk et al., 1979; Van Eijk & Eikelboom, 1983). Screening tests were developed, variation in resistance was retrieved and inheritance in terms of general and specific combining ability was studied. Seedling tests were also performed. Juvenile bulbs were less sensitive than adult bulbs. In tulip, the fungitoxic tulipalin A exerts a role in the resistance mechanism (Bergman, 1966; Baayen, 1992).

The pathogenesis of *F. oxysporum* f.sp. *narcissi* Snyd. & Hans. in narcissus has been described by Langerak (1985). Breeding for resistance has been mainly carried out in the United Kingdom. Screening tests were developed and variation in resistance was found (Linfield, 1986; Linfield & Price, 1986; Tompsett, 1986). In several species and cultivars absolute resistance was detected (Linfield, 1992). Furthermore, seedling tests were performed and inheritance in terms of general and specific combining ability studied (Bowes et al, 1992). Tests with several ages of bulbs suggested that sensitivity to *Fusarium* decreased with bulb age.

Although formae speciales can be distinguished by their host range, cross-infection is reported. Imle (1942a) reported that an isolate of *F. oxysporum* f.sp. *lilii* was pathogenic on crocus, and a *Fusarium* isolated from crocus was pathogenic to *L. formosanum*. Apt (1958) found that cross-infection only occurred within the *Iridaceae*. Valásková (1976) demonstrated that freesia could be infected by fusaria isolated from gladiolus, lily and tulip. Löffler & Mouris (1992) showed that *Fusarium* isolates from gladiolus and tulip were pathogenic for lily. This can have a large influence on the crop rotation system for bulb production.

Although breeding for *Fusarium* resistance in lily was investigated by Imle (1942a; 1942b), lily breeders do not use it. In 1989, the Royal General Bulbgrowers' Association (KAVB), the Dutch Bulb Exporters Association (BVB), and the Commodity Board for Ornamental Plants (PVS) together with the Dutch government decided to finance the Urgency Programme for Research on Diseases and Breeding of Flower Bulbs. The research on the possibilities for breeding for *Fusarium*

resistance in lily and gladiolus was part of this programme. This thesis presents results of the research programme on lily.

OUTLINE OF THE THESIS

Several steps are necessary in order to start a breeding programme for *Fusarium* resistance in lily. First, a useful screening test has to be developed. For flower bulbs, screening tests can be performed at clonal level and under standardized conditions, by which an accurate estimation of the resistance level can be obtained. Special attention has to be paid to resistance measurements, since the major symptoms are below the soil surface. The development of these techniques is described in Chapter 1. For lily bulb cultivation, different stages of development have to be passed. Comparison of *Fusarium* resistance in the various developmental stages is necessary, since resistance may differ between these stages (Chapter 2). To establish reliable results in a screening test, the influence of testing conditions on the cultivar ratings has to be investigated (Chapter 3).

Next, the occurrence of genetic variation for *Fusarium* resistance is required in order to start a successful breeding programme. Variation was determined in (old) cultivars or wild species (Chapter 4). The possibilities for interspecific hybridisation in lily allow for introgression of resistance genes from various species. Besides variation in resistance in the host, variation in virulence may exist in the pathogen. When different physiological races of the pathogen occur, resistance might not occur for all isolates. Before intensive and expensive interspecific breeding programmes are carried out, the durability of the resistance has to be estimated (Chapter 5).

Finally, screening tests that can preferably be applied at individual level in the seedling stage are required for selecting new resistant cultivars. The development of a seedling test is described in Chapter 6. Molecular markers can be used for indirect selection of a desired trait without disturbance by environmental variation. A first prerequisite of this technique is a close linkage between *Fusarium* resistance and molecular markers (Chapter 7). Inheritance of partial resistance is hard to evaluate. Based on a diallel analysis of the seedling test and using the molecular marker system, the inheritance of *Fusarium* resistance in lily was studied.

ACKNOWLEDGEMENT

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CHAPTER 1

Determination of resistance to *Fusarium oxysporum* in *Lilium*

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ABSTRACT

A screening test for determination of resistance to Fusarium oxysporum *f.sp.* lilii *in* Lilium *was developed. Under standardized conditions, 16 Asiatic hybrid lily cultivars were tested for resistance. Disease rating data were analyzed statistically using a threshold model for ordered categorical data. Bulb weight measurements were modeled as weight change of plants cultivated in* Fusarium-*infested soil relative to weight change of control plants, and further analyzed by ANOVA. Results of both observations were highly correlated. For disease ratings, six categories provided more information than two or three categories. Variation in resistance among the cultivars was demonstrated. The threshold analysis with six categories showed at least four groups of cultivars that differed in resistance. Disease ratings provided reproducible results and a practical experimental design.*

Keywords: Asiatic hybrid lilies, disease rating, *Fusarium* resistance, relative weight change, scale bulblets, threshold model.

INTRODUCTION

Lily (*Lilium* L.) is cultivated worldwide as a cut flower, pot plant, and garden plant. Lily bulbs are propagated vegetatively by inducing development of scale bulblets. Scale bulblets are grown for one or two years to obtain commercial bulbs. The culture of lily bulbs, especially at the scale bulblet stage, is often limited by the soilborne fungus *Fusarium oxysporum* f.sp. *lilii* Imle, which causes basal rot. Infection of the bulbs leads to brownish or black necrotic lesions, and, if the whole basal plate rots, the bulbs disintegrate (Imle, 1942; Bald et al., 1971).
Prevention of such damage depends mostly on chemical disinfestation of soil and plant material (Bald & Chandler, 1957; Boontjes, 1974; Bald et al., 1983; McRae, 1987). Resistance of the fungus to fungicides can occur (Bollen, 1972; Duineveld & Beijersbergen, 1975), however, and reduced application of chemicals is desirable to limit environmental pollution. Another way of controlling the disease would be the cultivation of resistant cultivars, which must be developed in breeding programs. In the *Fusarium*-lily interaction, only partial resistance has been reported (Imle, 1942; Smith & Maginnes, 1969; Maginnes & Smith, 1971; Van Tuyl, 1980; Löffler & Mouris, 1989). Therefore, a standardized screening test with sufficient sensitivity to discriminate between different resistance levels is needed.
Such a test requires that observations reflect the 'true' level of resistance in the plants considered. Furthermore, for a precise quantification of differences in resistance level, the observations must be analyzed statistically. This paper describes an approach to the analysis requirement.

Often the development of symptoms is considered a reflection of susceptibility. Usually, severity of symptoms is observed visually, and results are recorded as ratings on an ordinal scale. Consistent ratings may be difficult to achieve, however, especially between experiments. Moreover, analysis of disease ratings is not straightforward because the measurement scale may be nonlinear. A threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990; Jansen, 1991) may provide a suitable method for analysing disease ratings of basal rot in lily.

The difficulties associated with subjective observations may be avoided by a method to determine the decay of plants quantitatively. In this respect, weight change of plants infected by the pathogen relative to weight change of control plants may be used. Analysis of variance (ANOVA) may then be used to analyze the data.

Classification of cultivars with respect to resistance may provide a concise representation of the results of a screening test. The problem of classification of cultivars has been considered for quantitative data (Caliński & Corsten, 1985) and similar ideas can be applied in the case of disease ratings.

The objective of this research was to compare resistance levels of scale bulblets of Asiatic hybrid lily cultivars based on disease rating data with those based on relative weight change data. Effects of combining categories on the analysis of disease ratings were considered, and cultivars were classified in groups representing different levels of resistance.

MATERIALS AND METHODS

Plant material

Cultivars were chosen so that variation in resistance level could be expected (J.M. Van Tuyl, *personal communication*). The experiment was conducted twice (in 1989 and 1990). Commercial bulbs, which were used to induce scale bulblets in the 1989 experiment, were obtained from several growers. Bulbs used in the 1990 experiment were cultivated under standardized conditions and without fungicides. Scales of commercial bulbs of 16 Asiatic hybrid lily cultivars (Aristo, Connecticut King, Enchantment, Esther, Golden Melody, Hilde, Milano, Mont Blanc, Montreux, Napoli, Orlito, Pirate [1990 experiment only], Prominence, Snow Star, Sterling Star, and Yellow Blaze) were incubated at 25 °C in plastic bags with wet vermiculite to induce development of scale bulblets (Van Tuyl, 1983). After eight weeks at 25 °C, the temperature was reduced to 17 °C for another four weeks, followed by eight weeks at 5 °C. After the final incubation, newly formed scale bulblets were harvested and selected for uniformity of weight within each genotype. The resistance tests were then performed on the selected scale bulblets.

Fungus

Two highly aggressive isolates of *Fusarium oxysporum* f.sp. *lilii* (CPRO-Fol4 and CPRO-Fol11) (Löffler & Mouris, 1989; Löffler & Rumine, 1991) were used. These isolates are monospore cultures provided by the Bulb Research Centre (LBO, Lisse, The Netherlands) and the Department of Phytopathology (LUW, Wageningen, The Netherlands), respectively and were stored on Protect Bacterial Preservers (Technical Service Consultants LTD, Lancs, UK) at -80 °C for long term preservation. Before experimental use, fresh cultures were obtained by plating this stock on Czapek-Dox agar medium (Oxoid LTD, Hampshire, UK). For soil infestation, the fungus was incubated for three weeks at 23 °C in an autoclaved (120 °C, 2 h) oatmeal-soil mixture (1:4 w/w). The fully grown cultures were ground and mixed in a 1:100 ratio with soil (Löffler & Mouris, 1989). The number of propagules was determined by plating soil dilutions on a modified Komada medium (Komada, 1975; Löffler & Mouris, 1989) immediately after mixing ($\pm$ 150,000 propagules per gram of soil) and at the time of planting the scale bulblets, two weeks after infestation of the soil ($\pm$ 100,000 propagules per gram of soil).

Experimental design

Scale bulblets were planted in pots and placed in a temperature-controlled greenhouse at 18/14 °C (16 h day / 8 h night). Each pot contained four bulblets of the same genotype. The experiment was arranged in ten blocks each of an infested and a noninfested (= control) pot of each genotype. The 16 cultivars and the two treatments were randomly assigned to the pots. Observations were made six (1989) or eight (1990) weeks after bulblets were planted.

Disease measurement

Disease severity was measured in two ways. Decay of the infested bulblets was rated visually according to an ordinal scale with six categories: 1 = healthy; 2 = slightly rotten; 3 = moderately rotten; 4 = heavily rotten; 5 = very heavily rotten; and 6 = completely decayed. In addition, fresh weight of each plant (bulb + stem + leaves) was measured. Roots were removed to avoid inclusion of soil particles in the weight measurements.

Analysis of ordinal data

Disease rating data were analyzed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990). The threshold model assumes the presence of an underlying, continuous variable y that is related to disease resistance. The value of y of bulblet k (= 1...4) of cultivar j (= 1...16) in block i is given by

$$Y_{ijk} = \mu + \beta_i + \gamma_j + e_{ij} + e_{ijk}$$

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where μ is the grand mean, β_i is the effect of block i ($\beta_1 = 0$), γ_j is the effect of cultivar j ($\gamma_1 = 0$), e_{ij} is a random contribution related to the pot with cultivar j in block i and e_{ijk} is a random contribution related to bulblet k in the pot with cultivar j in block i. The random contributions e_{ij} and e_{ijk} are assumed to be independent and normally distributed with zero mean and variances σ_P^2 and σ_B^2, respectively. The variances σ_P^2 and σ_B^2 represent between-pot and within-pot variation, respectively. The disease severity score (DSS) of cultivar j is defined as $\mu + \beta_. + \gamma_j$ where $\beta_. = \Sigma_i \beta_i / 10$, i.e., the average value for cultivar j on the underlying scale.

Categorization is thought to arise from partitioning of this underlying scale by five thresholds (θ_1, θ_2...θ_5) which are assumed to be the same for all cultivars. A plant is assigned to category 1 of the ordinal scale if the value of y is less than or equal to the threshold θ_1. A plant is assigned to category 2 if its value of y lies between θ_1 and θ_2 and so on. Finally, the plant is assigned to category 6 if its value of y is larger than the threshold θ_5. To estimate parameters two restrictions must be made. First, the origin of the underlying scale has to be fixed, which is done by taking $\theta_1 = 0$. Second, the scale parameter has to be fixed, which is done by taking $\sigma_B^2 = 1$. As a consequence, the probabilities that a plant of cultivar j in block i is assigned to categories 1, 2...6, respectively are given by

$$p_{ij}^1 = \Phi(-[\mu + \beta_i + \gamma_j + e_{ij}])$$
$$p_{ij}^c = \Phi(\theta_c - [\mu + \beta_i + \gamma_j + e_{ij}]) - \Phi(\theta_{c-1} - [\mu + \beta_i + \gamma_j + e_{ij}])$$
$$c = 2...5; \theta_1 = 0$$
$$p_{ij}^6 = 1 - \Phi(\theta_5 - [\mu + \beta_i + \gamma_j + e_{ij}])$$

where Φ denotes the probability distribution function of the standard normal distribution. The probabilities p_{ij}^c may be considered as areas under a normal probability density function with mean $y_{ij} = \mu + \beta_i + \gamma_j + e_{ij}$ and unit variance. A graphical representation of the model is given in Figure 1.1.

Estimates of all unknown parameters (μ, β_i, γ_j, θ_c, and σ_P) can be obtained simultaneously by maximum likelihood with the computer package Genstat (Payne et al., 1987). Furthermore, deviance statistics (McCullagh & Nelder, 1989) are calculated, which are similar to sum-of-squares used in ANOVA. Values of a deviance statistic have to be compared with the table of the chi-squared distribution with the appropriate number of degrees of freedom. (A Genstat procedure as well as a Fortran program can be obtained for a nominal fee from the second author.)

Analysis of weight changes
The relative weight change (RWC) per block of a cultivar was calculated as the ratio of the weight change (final weight minus initial weight) of the four plants cultivated in infested soil and the weight change of the four control plants. Values of RWC were analyzed by ANOVA.
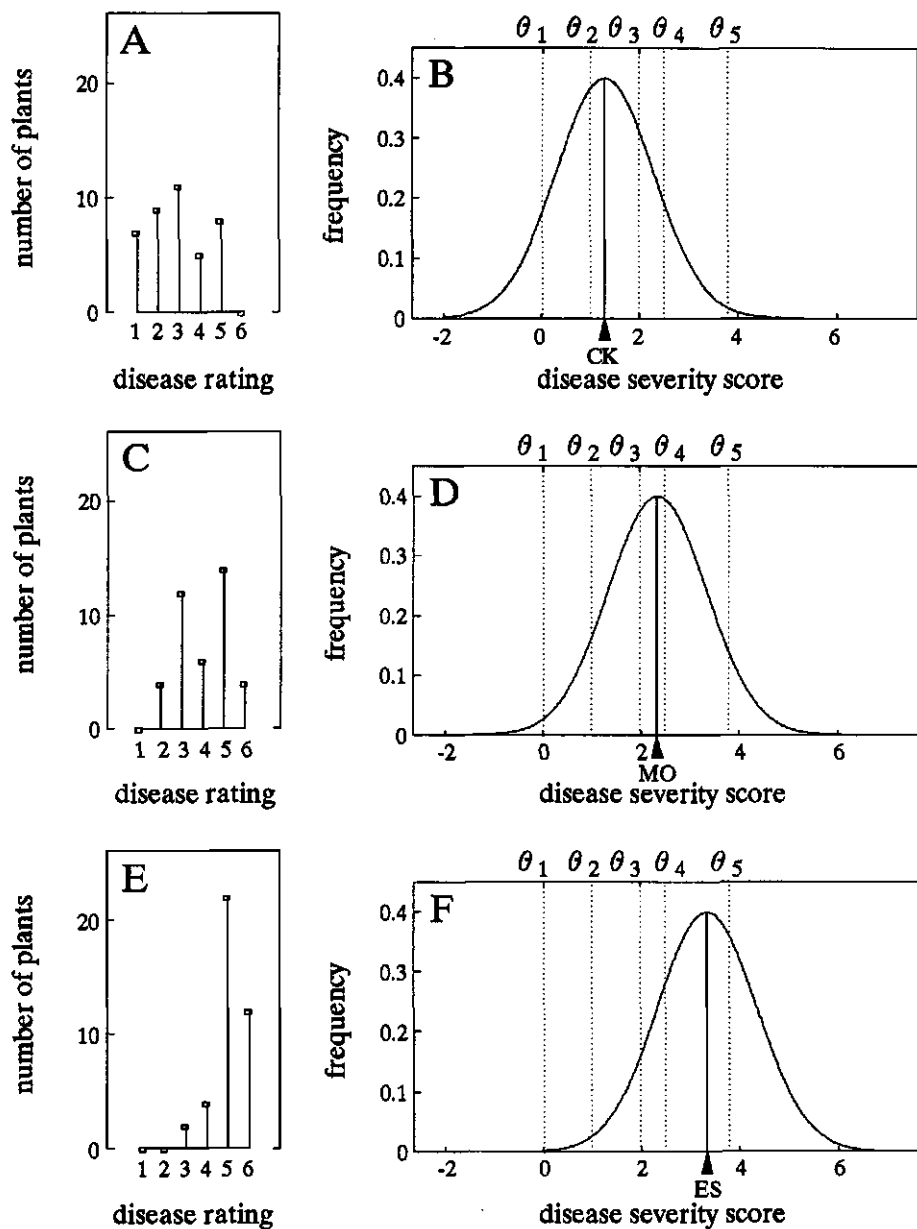
Figure 1.1 Representation of ordinal disease rating data for three lily cultivars infected by *Fusarium oxysporum* f.sp. *lilii*. Data were analyzed according to a threshold model for ordered categorical data. A, C, E, Distribution of disease ratings for resistance for 'Connecticut King' (CK), 'Montreux' (MO) and 'Esther' (ES), respectively. B, D, F, Corresponding normal distributions and disease severity scores according to the threshold model with five thresholds ($\theta_1 \ldots \theta_5$).

Correlations

Correlation diagrams were made to investigate the relation between DSS data and RWC data. To investigate reproducibility, correlations between the two independent experiments were calculated for both DSS and RWC data.

Combining categories of the ordinal scale

The disease rating data were regrouped into three new categories (i.e., old category 1+2, 3+4, and 5+6) or two new categories (i.e., old category 1+2+3 and 4+5+6) to determine whether or not a disease rating with fewer than six categories would lead to less discriminating power relative to a rating with six categories.

Classification of cultivars

Cultivars were classified with regard to resistance so that most of the variation among cultivars could be attributed to variation among groups rather than to variation within groups. The criterion for classification was the deviance for differences between cultivars within groups. This deviance reached a maximum when all the cultivars were classified in a single group and a minimum (zero) when the number of groups was equal to the number of cultivars. To obtain a concise representation of the results, the number of groups must be as small as possible. Results can be represented in a dendrogram similar to that proposed by Caliński & Corsten (1985) for continuous data.

RESULTS

Bulbs used in the 1990 experiment were cultivated under standardized conditions and without fungicides, so results of this experiment are assumed to be more reliable than those from 1989 and are emphasized here.

Results of the 16 cultivars with regard to disease rating with six categories, estimated DSS values, and corresponding standard error of differences (relative to 'Connecticut King') of the 1990 experiment are given in Table 1.1. The deviance for differences between cultivars is equal to 221.2 based on 15 degrees of freedom, which shows that large differences in resistance to *Fusarium* exist between the different cultivars ($P < 0.001$). Significant block effects did not occur (deviance equal to 4.7 with 9 degrees of freedom). The estimate of the between-pot variance (σ_p^2) is 0.17 (standard error = 0.124). Estimates and standard errors (s.e.) of the thresholds are 0 (fixed), 0.98 (s.e. = 0.117), 1.95 (s.e. = 0.132), 2.47 (s.e. = 0.139), and 3.76 (s.e. = 0.165).

Table 1.1 Number of bulblets in six disease categories and disease severity score (DSS) obtained by the threshold model, after planting scale bulblets of 16 Asiatic hybrid lily cultivars in *Fusarium*-infested soil (1990 experiment).

cultivar	disease category						DSS	
---	1	2	3	4	5	6	estimate	s.e.d.[z]
Connecticut King (CK)	7	9	11	5	8	0	1.29	-
Mont Blanc (MB)	0	12	15	8	5	0	1.52	0.245
Prominence (PR)	1	8	17	9	3	2	1.65	0.245
Orlito (OR)	2	6	16	8	6	2	1.75	0.245
Napoli (NA)	0	5	19	9	7	0	1.79	0.245
Hilde (HI)	1	4	11	11	11	2	2.10	0.247
Milano (MI)	0	4	13	5	17	1	2.21	0.247
Montreux (MO)	0	4	12	6	14	4	2.33	0.248
Yellow Blaze (YB)	1	6	8	7	11	7	2.35	0.249
Golden Melody (GM)	2	2	8	5	20	3	2.43	0.249
Sterling Star (ST)	0	3	4	8	20	5	2.72	0.252
Snow Star (SN)	1	1	8	7	11	12	2.85	0.254
Enchantment (EN)	0	0	0	3	29	8	3.33	0.261
Esther (ES)	0	0	2	4	22	12	3.37	0.261
Aristo (AR)	0	0	1	1	8	30	4.39	0.293
Pirate (PI)	0	0	1	0	3	36	4.99	0.338

[z]Standard error of differences, and refer to differences with 'Connecticut King'.

The weight data (average initial and average final weight) per cultivar per treatment in the 1990 experiment are given in Table 1.2. The initial weight of bulblets within cultivars was similar. Among cultivars, the average initial weight per bulblet ranged from 0.27 ('Pirate') to 0.89 g ('Esther') with an overall average of 0.55 g. In the control, the absolute increase of weight ranged from 0.34 ('Pirate') to 5.77 g ('Mont Blanc'). Variation in resistance level, calculated as RWC among cultivars was observed (Table 1.2). The analysis of variance indicated highly significant differences among cultivars ($P < 0.001$) with no block effects detected.

To investigate the relationship between the RWC data and the DSS data, correlation diagrams were made (Figure 1.2). A high correlation between DSS, obtained from the threshold model with six categories, and RWC was observed. Correlations between the 1989 and 1990 experiments for both DSS and RWC data are presented in Figure 1.3. Results of both years were correlated for DSS as well as RWC.

Analysis of regrouped data in two or three categories still gave highly significant differences between cultivars ($P < 0.001$). The deviance, however, was smaller (189.1 for three categories and 148.4 for two categories) than the deviance obtained with six categories (deviance $= 221.2$). This indicates that discrimination was reduced by combining categories.

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Table 1.2 Initial weight, final weight, and relative weight change (RWC) of scale bulblets of 16 Asiatic hybrid lily cultivars planted in control and *Fusarium*-infested soil (1990 experiment).

cultivar	initial weight (g)^y		final weight (g)^y		RWC^z
	control	infested	control	infested	
Connecticut King (CK)	0.45	0.44	5.78	1.96	0.34
Napoli (NA)	0.54	0.57	5.12	1.65	0.24
Milano (MI)	0.56	0.54	6.24	1.65	0.24
Prominence (PR)	0.52	0.54	5.45	1.49	0.21
Mont Blanc (MB)	0.58	0.56	6.35	1.62	0.20
Orlito (OR)	0.54	0.52	5.09	1.13	0.14
Golden Melody (GM)	0.61	0.56	4.79	1.15	0.14
Hilde (HI)	0.67	0.56	5.40	1.08	0.12
Montreux (MO)	0.56	0.46	5.97	0.93	0.09
Enchantment (EN)	0.36	0.37	5.10	0.68	0.07
Sterling Star (ST)	0.57	0.62	5.46	0.93	0.07
Yellow Blaze (YB)	0.70	0.72	4.72	0.86	0.04
Snow Star (SN)	0.59	0.59	4.60	0.65	0.02
Esther (ES)	0.89	0.76	4.06	0.53	-0.08
Aristo (AR)	0.59	0.59	2.27	0.13	-0.36
Pirate (PI)	0.27	0.28	0.62	0.01	-0.69
Standard error of differences					0.07

^{y}Each value is the mean of 40 plants.
^{z}Each value is the mean of 10 blocks.
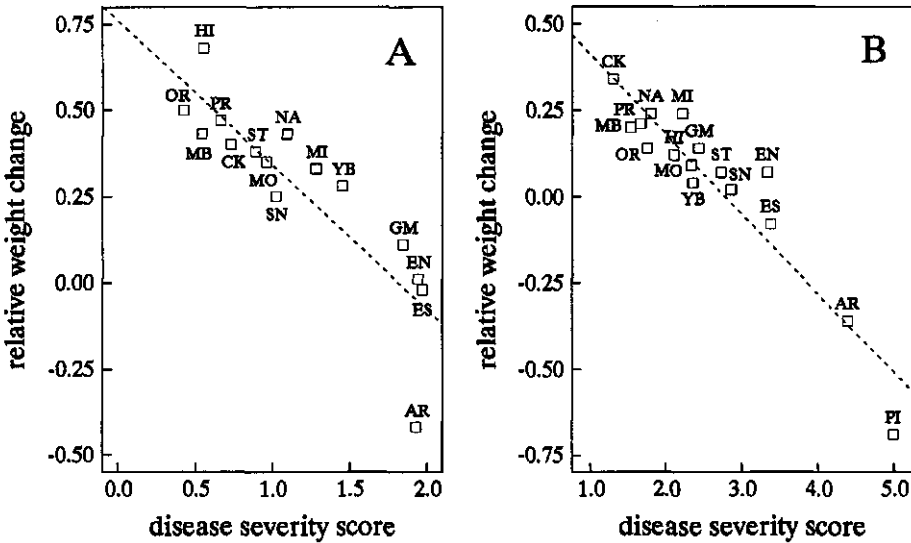
Figure 1.2 Correlation diagram between disease severity score obtained by the threshold models and the relative weight change of scale bulblets of 16 Asiatic hybrid lily cultivars planted in *Fusarium*-infested soil. See Table 1.1 for abbreviations of cultivar names. A, 1989 (r = 0.87). B, 1990 (r = 0.94).

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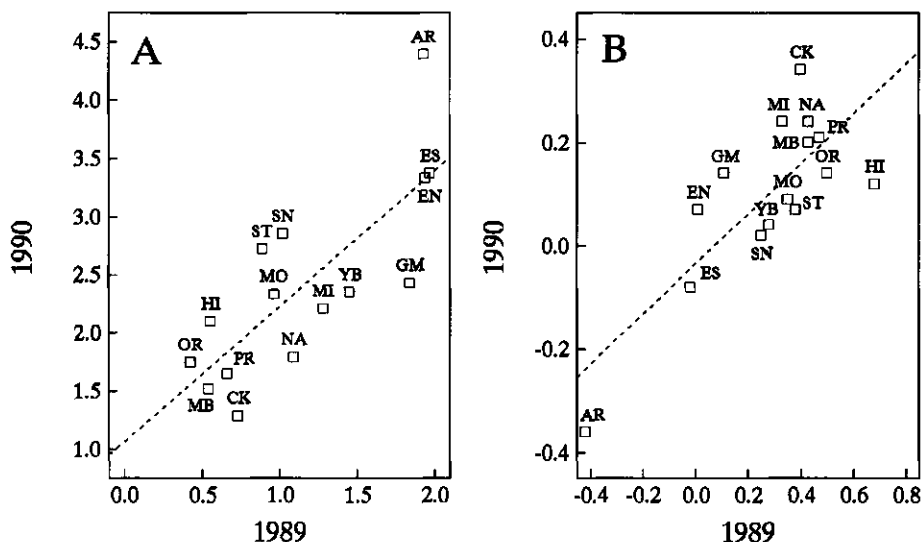
Figure 1.3 Correlation diagram between results of the 1989 and 1990 experiments with scale bulblets of 15 Asiatic hybrid lily cultivars planted in *Fusarium*-infested soil. See Table 1.1 for abbreviations of cultivar names. A, Disease severity scores obtained by the threshold model (r = 0.78). B, Relative weight change (r = 0.79).

A dendrogram (Figure 1.4) based on disease ratings in six categories indicates that a division of the 16 cultivars into two groups led to a deviance of 94.1 (with 14 degrees of freedom) for differences between cultivars within groups. Further division into three, four, or five groups led to deviances of 53.0 (with 13 degrees of freedom), 17.0 (with 12 degrees of freedom) and 6.2 (with 11 degrees of freedom), respectively. A classification with four groups provided an adequate summary of the variation among cultivars, yet kept the number of groups acceptably small. Such a classification accounted for 92 % of the differences among cultivars. Borders between groups were found where large intervals of DSS existed between cultivars, i.e., 'Napoli' and 'Hilde', 'Snow Star' and 'Enchantment', and 'Esther' and 'Aristo' (Table 1.1).

DISCUSSION

Disease severity in lily scale bulblets was measured and analyzed in two different ways. In the first analysis, disease rating data were used, in which plants were assigned to the categories of an ordinal scale according to the severity of symptoms. This method of evaluating disease severity is relatively easy and, therefore, commonly used. Analysis of these data is difficult, however. The threshold model

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for analysing ordered categorical data is a fairly new approach that involves an underlying linear scale (McCullagh, 1980; Jansen, 1990; Jansen, 1991).
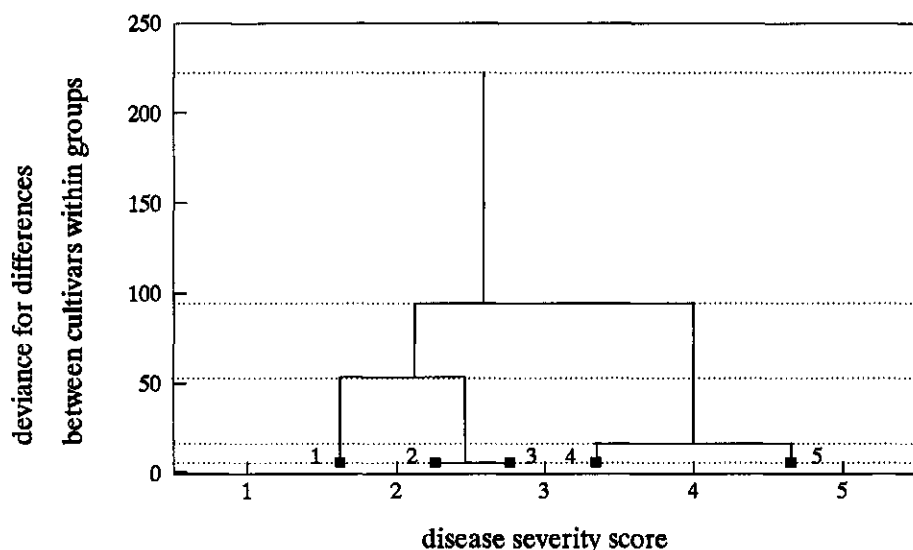
Figure 1.4 Deviances for differences between cultivars within groups and corresponding disease severity scores obtained by the threshold model, after classification of cultivars in different groups (1990 experiment). Group 1 = CK, MB, PR, OR, NA; group 2 = HI, MI, MO, YB, GM; group 3 = ST, SN; group 4 = EN, ES and group 5 = AR, PI. See Table 1.1 for abbreviations of cultivar names.

Categories of the disease rating scale must be well-defined to obtain consistent results. A larger number of categories improves discrimination between disease severity levels but increases the difficulty of assigning plants to specific categories. Moreover, the chance of rating errors or empty categories increases. On the DSS scale, thresholds mark the borders of the disease rating categories. The distance between thresholds determines how precisely categories are chosen. In this study, six categories were used, and fewer plants were assigned to category 4 than to either categories 3 or 5. This conforms with the relatively small estimated difference between thresholds 3 and 4 and may be due to the fact that symptom development in the bulbs progresses at a variable rate after infection.

Adjacent categories were combined to study the effect of using a reduced number of categories. When two or three categories were used instead of six, however, deviances were reduced and led to less discrimination between the cultivars. When only two categories were used, the model was reduced to a binomial model in which plants were classified only as healthy or diseased. The reduction of the deviance with two categories is due to the fact that a part of the information is discarded, i.e., severity of symptoms is not considered.

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Although almost all scale bulblets were infected in the experiments, large cultivar effects occurred. The cultivars Aristo and Pirate both had a rather high standard error of differences compared to the other cultivars, which means that the DSS estimates of 'Aristo' and 'Pirate' is less reliable than the DSS estimates of the other cultivars. This occurred because both 'Aristo' and 'Pirate' were severely diseased and received disease ratings mainly in the high categories.

Disease ratings, although rated on a progressively coarser scale with a limited number of categories, are often analyzed by analysis of variance. An analysis of variance on the disease rating data presented in this paper provided results similar to those obtained with the threshold model (not shown). Standard errors of differences between cultivars as provided by analysis of variance were relatively underestimated compared to those obtained by the threshold model, however, especially if extremely susceptible or resistant cultivars were involved. The threshold model acknowledges the fact that discrimination becomes more difficult if cultivars are extremely resistant or extremely susceptible. In complex experiments involving several factors, analysis of variance may lead to inclusion of interactions that are caused by the limitations of the ordinal scale rather than the biology of the system. Another way of estimating the disease severity was by calculation of the relative weight change of lily scale bulblets. Normally, bulblets gain weight during cultivation by developing shoots and by growing of the bulbs. Infection causes bulb rot, wilting, and stunting, and all are reflected in diminished weight increase or even weight decrease. Therefore, weight change of infected bulbs relative to weight change of control bulbs can express disease severity. Differences in RWC reflect the difference in disease severity of the cultivars used. As with DSS estimates, RWC data analyzed by analysis of variance revealed a large cultivar effect.

The RWC values apparently were not influenced by the initial weight of the bulblets because RWC values and initial weight were not correlated. Results might be influenced, however, by the vigour of the cultivars. The two most slowly growing cultivars also had the lowest RWC. Whether this slow growth biases the test results or low vigour implicates a high *Fusarium* disease severity is unclear.

The 1989 and 1990 experiments gave reproducible results for both DSS and RWC. Differences between the data sets might be due to differences in the origin of the bulbs. Both methods described to estimate disease severity (DSS and RWC) gave clear results. The high correlation between results from those two methods in both years means that both methods adequately describe the same phenomenon, i.e., disease severity. In this case, however, both resistance and tolerance of cultivars must be considered. Both methods are based on symptoms, so neither method can discriminate between tolerance and resistance. Histological research indicated that genotypes with a low level of disease retard colonization (R.P. Baayen, *personal communication*), which suggests that resistance rather than tolerance is involved.

We expected that the weight measurements would reflect disease severity more

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precisely than the visible symptom scale because weight measurements are more objective than the disease ratings. The high correlation between RWC and DSS, however, indicates that the disease rating, if analyzed properly, can be used. Both methods provided similar precision. The weight measurement is labour intensive and needs a large experimental design (equal number of infested and control pots), which are important practical considerations. If disease ratings are used, only a small number of control plants have to be analyzed to determine whether or not bulbs were infected prior to the experiment. Although weight measurements can be analyzed easily compared to disease ratings, labour and greenhouse capacity involved in obtaining these measurements make their application in large screening tests impractical.

As expected from the choice of cultivars for this study, variation in resistance to *Fusarium oxysporum* f.sp. *lilii*, although partial, was found in Asiatic hybrid cultivars of *Lilium*. Classification of cultivars into phenotypic groups would provide an adequate representation of the results of a screening test. In this study, at least four groups of cultivars, differing in resistance, could be distinguished by the disease rating analysis with six categories. Cultivars within groups can be treated as equivalents. Results can be used for purposes of practical breeding and variety research.

Whether the resistance level observed for some cultivars in this study is sufficient to justify reduced use of fungicides in commercial bulb cultivation is not yet clear. Cultivars that were resistant in this test (e.g., 'Connecticut King' and 'Mont Blanc') did not exhibit disease symptoms when cultivated in the field. Cultivars that were susceptible in this test (e.g., 'Pirate' and 'Esther') often have been susceptible under field conditions. Our results were also comparable with other reports (Van Tuyl, 1980; Löffler & Mouris, 1989).

The *Fusarium* test under standardized conditions combined with disease ratings, analyzed using the threshold model as applied in this study proved to be very suitable for detecting differences in resistance level among cultivars. Disease ratings provided reproducible results and a practical experimental design. This test will be used to compare *Fusarium* resistance of different developmental stages of lily bulbs in further research.

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Resistance to *Fusarium oxysporum* at different developmental stages of Asiatic hybrid lilies

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ABSTRACT

Tests for determining resistance to Fusarium oxysporum *f.sp.* lilii *in different developmental stages of* Lilium *were developed. Under standardized conditions, commercial bulbs, yearling bulbs, scale bulblets, and scales of 16 Asiatic hybrid lily cultivars were tested for* Fusarium *resistance. Disease ratings were analyzed statistically using a threshold model for ordered categorical data. For each cultivar in each stage the resistance level was calculated as the disease severity score (DSS). Disease severity score values of cultivars in the four stages were reproducible between experiments, although some variation in cultivars was found. This variation may be attributed to the origin of plant material. Disease severity score values among cultivars in each developmental stage were correlated with the average DSS over all four stages, although some specific cultivar-stage deviations were found. The scale test is recommended for early selection while the scale bulblet test is recommended as a final check.*

Keywords: commercial bulbs, disease rating, *Lilium*, resistance breeding, scale bulblets, scales, threshold model, yearling bulbs

INTRODUCTION

Production of the bulbous ornamental lily (*Lilium* L.) involves vegetative propagation followed by cultivation over several years. Bulb production is reduced by bulb rot, caused by *Fusarium oxysporum* f.sp. *lilii* Imle.

During propagation and growth, lily bulbs pass through three developmental stages. The first stage involves the induction of scale bulblets (Griffiths, 1933), whereby scales of commercial bulbs are incubated in soil, soilless substrate (e.g., vermiculite), or artificial media in vitro. Scale bulblets are then grown in the field for one season to form yearling bulbs. Yearling bulbs can also form during cultivation of commercial bulbs on the below-ground portion of the flower stem. The stem bulblets that develop are considered to be yearling bulbs by the end of the season. Yearling bulbs are grown for a further season in order to form commercial bulbs (Blaney & Roberts, 1966). Commercial bulbs are used in the production of cut flowers, pot, and garden plants.

Fusarium can cause severe losses, especially in scale bulblet and yearling bulb stages. Preventing bulb rot depends mostly on fungicide application (Bald & Chandler, 1957; Boontjes, 1974; Bald et al., 1983; McRae, 1987). Increasingly the reduction of fungicide application is considered desirable in order to limit environmental pollution. An alternative method of disease management is the cultivation of resistant cultivars. A reliable screening test is needed to breed for

resistant varieties.

A screening test requires that observations reflect the 'true' level of resistance in the plants considered. Field experiments can provide this information, but results can vary greatly because of widely ranging environmental conditions. Greenhouse tests are preferred because environmental conditions can be more closely controlled and monitored. This is the more important since standardisation is required to test with sufficient accuracy between genotypes because partial resistance is involved (Imle, 1942; Smith & Maginnes, 1969; Maginnes & Smith, 1971; Van Tuyl, 1980; Löffler & Mouris, 1989; Straathof et al., 1993).

Van Tuyl (1980) described a *Fusarium* screening test using lily scale bulblets; this test was optimized by Straathof et al. (1993) and was carried out under standardized conditions. Resistance levels were determined using a disease rating in combination with statistical analysis based on a threshold model (Jansen, 1990). There is no information in the literature concerning tests with yearling and commercial bulbs. Disease resistance between developmental stages may differ, so the levels of resistance in different stages must be compared.

Smith & Maginnes (1969) and Maginnes & Smith (1971) described a screening test using lily scales; this test was optimized by Löffler & Mouris (1989). A scale is a bulb organ and might be useful in screening for resistance, because of a more efficient use of greenhouse space.

The objective of this research was to determine resistance to *Fusarium* in a range of Asiatic hybrid lily cultivars, and to compare cultivar resistance in the four stages of development. Tests were also evaluated for their efficiency.

MATERIALS AND METHODS

Plant material

Commercial bulbs of 16 Asiatic hybrid lily cultivars (Aristo, Connecticut King, Enchantment, Esther, Golden Melody, Hilde, Milano, Mont Blanc, Montreux, Napoli, Orlito, Pirate, Prominence, Snow Star, Sterling Star, and Yellow Blaze) were obtained from Dutch growers in Autumn 1988. Plant material (Table 2.1) was cultivated under standardized conditions without using fungicides. For each experiment, bulbs or scales were selected for uniformity of weight within each cultivar (coefficient of variation $\pm$ 25 %). 'Pirate' was not used in the 1989 experiments, neither was 'Orlito' except for the scale bulblet test.

Commercial bulbs obtained from growers were used directly in a screening test (1989), or scales were broken from the commercial bulbs and incubated in plastic bags with wet vermiculite in order to obtain scale bulblets. Scale bulblets were cultivated for a further two years to produce new commercial bulbs. Those new commercials were screened for *Fusarium* resistance in 1991.

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Yearling bulbs were produced by the cultivation of scale bulblets for one year, or were harvested from the stems of cultivated commercial bulbs. Both groups of yearling bulbs were screened for *Fusarium* resistance in 1990.

Scale bulblets were induced on scales from commercial bulbs obtained from growers (1989 test) and from cultivation at CPRO-DLO location (1990 test) as described by Straathof et al. (1993).

Scales were broken from commercial bulbs obtained from growers (1989 test) and from cultivation at CPRO-DLO location (1990 test) and further treated according to Löffler & Mouris (1989).

Fungus

Two highly aggressive isolates of *Fusarium oxysporum* f.sp. *lilii* (CPRO-Fol4 and CPRO-Fol11) (Löffler & Mouris, 1989; Löffler & Rumine, 1991) were used to evaluate cultivar resistance. For soil infestation, the fungi were incubated for three weeks at 23 °C in an autoclaved (120 °C, 2 h) 1 oatmeal : 4 soil mixture (w/w). The fully grown cultures were ground and mixed in a 1:100 ratio with nonsterile potting soil (Löffler & Mouris, 1989). The number of propagules in soil was determined by dilution plating on a modified Komada medium (Komada, 1975; Löffler & Mouris, 1989) at planting time, which was two weeks after soil infestation ($\pm$ 100.000 propagules per gram of soil).

Experimental design

Plant material of different developmental stages were tested in separate experiments. Commercial bulbs, yearling bulbs, and scale bulblets were planted in 7.0-, 1.9-, and 1.1-litre pots, respectively. Several bulbs (Table 2.1) of one cultivar were planted in one pot per stage. Scales of all 16 cultivars were planted in a 45 x 30 cm flat tray containing 12 litres of soil. Per tray, ten scales of each cultivar were planted in a row. Experiments were laid out using a randomized block design, in which each block (Table 2.1) consisted of one pot or one row of all 16 cultivars. The 16 cultivars in a block were randomly assigned to the pots or rows. Several noninfested pots and trays were included as controls. Pots and trays were placed in a temperature-controlled greenhouse at 18/14 °C (16 h day / 8 h night). Because of the partial resistance of the cultivars, and to achieve optimal discrimination between resistance levels in each stage, bulbs were dug up and observations were carried out 6-22 weeks after planting (Table 2.1).

Disease measurement

Cultivars were evaluated for decay of the infested plant material visually according to an ordinal scale with six categories: 1 = healthy; 2 = slightly rotten; 3 = moderately rotten; 4 = heavily rotten; 5 = very heavily rotten; and 6 = completely decayed (Straathof et al., 1993).

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Table 2.1 Origin, year of the experiment, experimental design (number of blocks and number of plants per block) and duration of the experiment (weeks) used in *Fusarium* screening tests at different developmental stages of Asiatic hybrid lilies.

developmental stage	origin	year	experimental design		weeks
			blocks	plants per block	
commercial bulbs	growers	1989	4	3	22
commercial bulbs	cpro-dlo	1991	5	4	20
yearling bulbs (stem)	cpro-dlo	1990	5	4	18
yearling bulbs (scale)	cpro-dlo	1990	5	4	18
scale bulblets	growers	1989	10	4	6
scale bulblets	cpro-dlo	1990	10	4	8
scales	growers	1989	4	10	12
scales	cpro-dlo	1990	8	10	12

Statistical analysis

Disease ratings were analyzed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990), using a probit link function. The between-pot/row variation was estimated from the data (Jansen, 1990). For each experiment, the disease severity score (DSS) of a cultivar was calculated as the mean of disease ratings of that cultivar on an underlying linear scale (Straathof et al., 1993).

Conclusions concerning block and cultivar effects were based on deviance statistics (McCullagh & Nelder, 1989), which have to be compared with the table of the chi-squared distribution. The computer package Genstat (Payne et al., 1987) was used for all calculations.

To investigate reproducibility within developmental stages and to compare between stages, correlation diagrams of DSS values were made.

RESULTS

Disease severity score values for the 16 cultivars, corresponding standard error of differences (relative to the resistant 'Connecticut King'), deviances, and corresponding degrees of freedom for each experiment are given in Table 2.2. The analysis of deviance shows that cultivars significantly differ in their resistance to *Fusarium* ($P < 0.001$). Significant block effects ($P < 0.05$) occurred in three tests. The estimated between-pot/row variances were small except for the scale test in 1989. For the 'Esther', DSS could not be calculated using the threshold model in three of the tests because almost all plants had the same disease rating. There were no plants in the disease rating category six (completely decayed) in the commercial bulb test of 1989 and categories one (healthy) and six in scale tests of 1989 and 1990, leaving either four or three thresholds to be estimated. Disease severity score

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Table 2.2 Disease severity score (DSS), corresponding standard error of differences (s.e.d.; relative to 'Connecticut King'), deviances and corresponding degrees of freedom (df) obtained by the threshold model for different developmental stages of 16 Asiatic hybrid lily cultivars planted in *Fusarium*-infested soil.

Column groups — **commercial bulbs** (1989, 1991); **yearling bulbs 1990** (stem bulbs, scale bulbs); **scale bulblets** (1989, 1990); **scales** (1989, 1990).

cultivar	comm. 1989 DSS	s.e.d.	comm. 1991 DSS	s.e.d.	stem bulbs DSS	s.e.d.	scale bulbs DSS	s.e.d.	scale bulblets 1989 DSS	s.e.d.	scale bulblets 1990 DSS	s.e.d.	scales 1989 DSS	s.e.d.	scales 1990 DSS	s.e.d.
Connecticut King (CK)	1.73	-	1.26		1.91		1.77		0.73		1.29		1.73		-0.03	
Ortito (OR)	-[z]		-0.30	0.4	2.50	0.4	1.64	0.3	0.42	0.2	1.75	0.2	-		0.50	0.2
Prominence (PR)	3.53	0.6	1.37	0.4	2.60	0.4	1.58	0.3	0.66	0.2	1.65	0.2	1.30	0.4	-0.92	0.2
Yellow Blaze (YB)	1.68	0.5	1.70	0.4	3.25	0.4	0.91	0.3	1.45	0.2	2.35	0.2	2.99	0.4	0.76	0.2
Napoli (NA)	2.43	0.6	1.51	0.4	3.04	0.4	2.37	0.3	1.09	0.2	1.79	0.2	3.04	0.4	0.58	0.2
Mont Blanc (MB)	2.75	0.6	1.48	0.4	1.71	0.4	1.95	0.3	0.54	0.2	1.52	0.2	3.11	0.4	2.31	0.2
Hilde (HI)	4.67	0.6	1.99	0.4	3.71	0.4	2.14	0.3	0.55	0.2	2.10	0.2	3.50	0.4	2.61	0.2
Snow Star (SN)	1.66	0.5	1.99	0.4	3.57	0.4	3.37	0.3	1.02	0.2	2.85	0.3	2.09	0.4	0.67	0.2
Golden Melody (GM)	3.93	0.6	3.22	0.4	3.47	0.4	4.43	0.4	1.84	0.2	2.43	0.2	1.97	0.4	0.06	0.2
Montreux (MO)	4.25	0.6	3.85	0.5	2.88	0.4	2.96	0.3	0.96	0.2	2.33	0.2	4.83	0.5	3.80	0.2
Milano (MI)	4.66	0.6	3.11	0.4	4.84	0.4	5.96	0.6	1.28	0.2	2.21	0.2	4.44	0.4	2.41	0.2
Sterling Star (ST)	5.08	0.6	4.08	0.5	5.33	0.5	4.03	0.4	0.89	0.2	2.72	0.3	4.43	0.5	3.39	0.2
Enchantment (EN)	4.03	0.6	5.44	0.5	4.62	0.4	4.96	0.4	1.94	0.2	3.33	0.3	4.14	0.5	3.83	0.2
Pirate (PI)	-		4.89		5.60	0.5	4.70	0.4	-		4.99	0.3	-		3.66	
Aristo (AR)	7.04	0.8	5.49	0.5	4.80	0.4	5.09	0.4	1.93	0.2	4.39	0.3	3.87	0.4	3.73	0.2
Esther (ES)	+[y]		7.04	0.5	+[y]		5.96	0.6	1.97	0.2	3.37	0.3	4.54	0.5	+[y]	
block — deviance / df	1.1[ns]	3	10.3[*]	4	3.2[ns]	4	10.4[*]	4	3.7[ns]	8	4.7[ns]	9	1.3[ns]	3	17.9[*]	7
cultivars — deviance / df	115.2[***]	13	170.3[***]	15	145.4[***]	15	188.4[***]	15	128.0[***]	14	221.2[***]	15	91.3[***]	13	414.5[***]	15

[z]Not included in the experiment.

[y]DSS not calculated.

ns,*,***Nonsignificant or significant at $P = 0.05$, or 0.001, respectively, by $\chi2$ test.

values for a cultivar and the deviance for cultivar effects differed from test to test and from year to year. For all developmental stages, the second experiment gave a larger deviance for cultivar differences.

To study the reproducibility of the screening tests, results from replicate experiments for each stage were compared (Figure 2.1). The DSS values for cultivars in the two experiments were correlated in all four stages but correlation was highest for the scale test. In the three bulb tests some cultivars showed variation in DSS values between replicates.

Figure 2.1 Correlation diagrams between disease severity scores (DSS) of two years of data obtained by the threshold model for commercial bulbs (A, r=0.76), yearling bulbs (B, r=0.79), scale bulblets (C, r=0.78), and scales (D, r=0.92) of 16 Asiatic hybrid lily cultivars planted in *Fusarium*-infested soil. See Table 2.2 for abbreviations of cultivar names.

To compare DSS values of cultivars between the four developmental stages, the replicate with CPRO-DLO cultivated bulbs was chosen from each stage. For the yearling bulbs the replicate with the largest deviance for cultivar effect was used. Disease severity score values of cultivars at each stage were compared with the DSS values averaged over all four stages (Figure 2.2). Cultivar resistance at each stage was correlated with the average over all stages. Correlations were highest for commercial bulbs and some specific cultivar-stage deviations occurred.

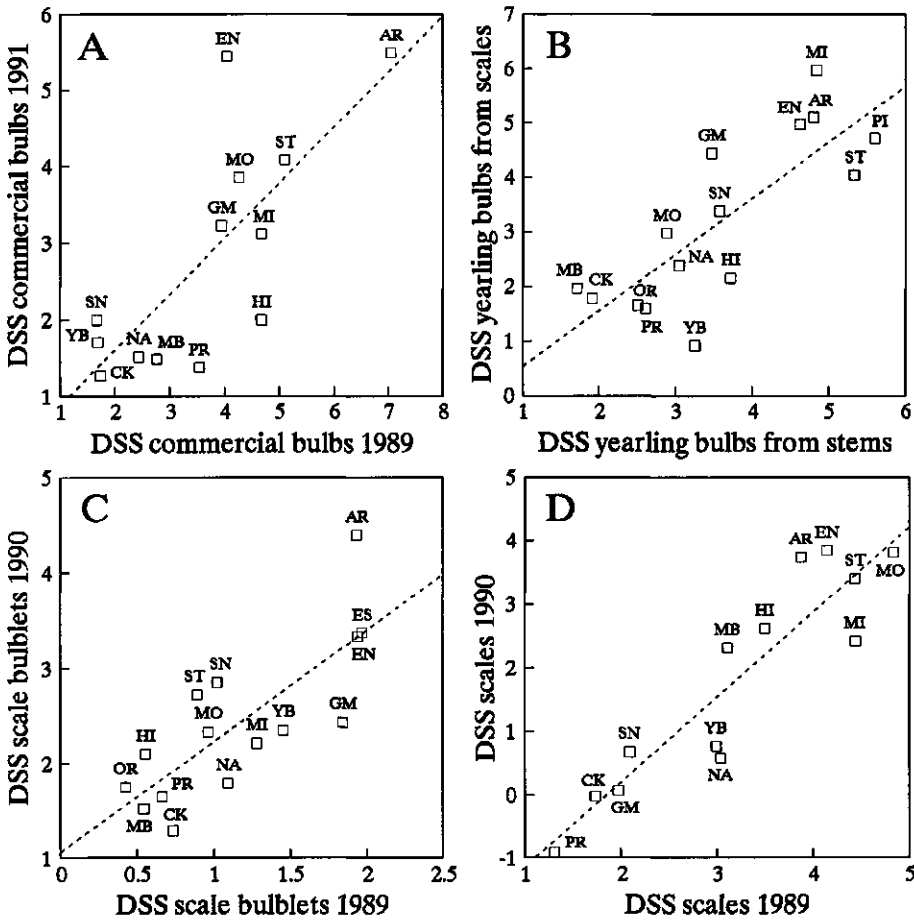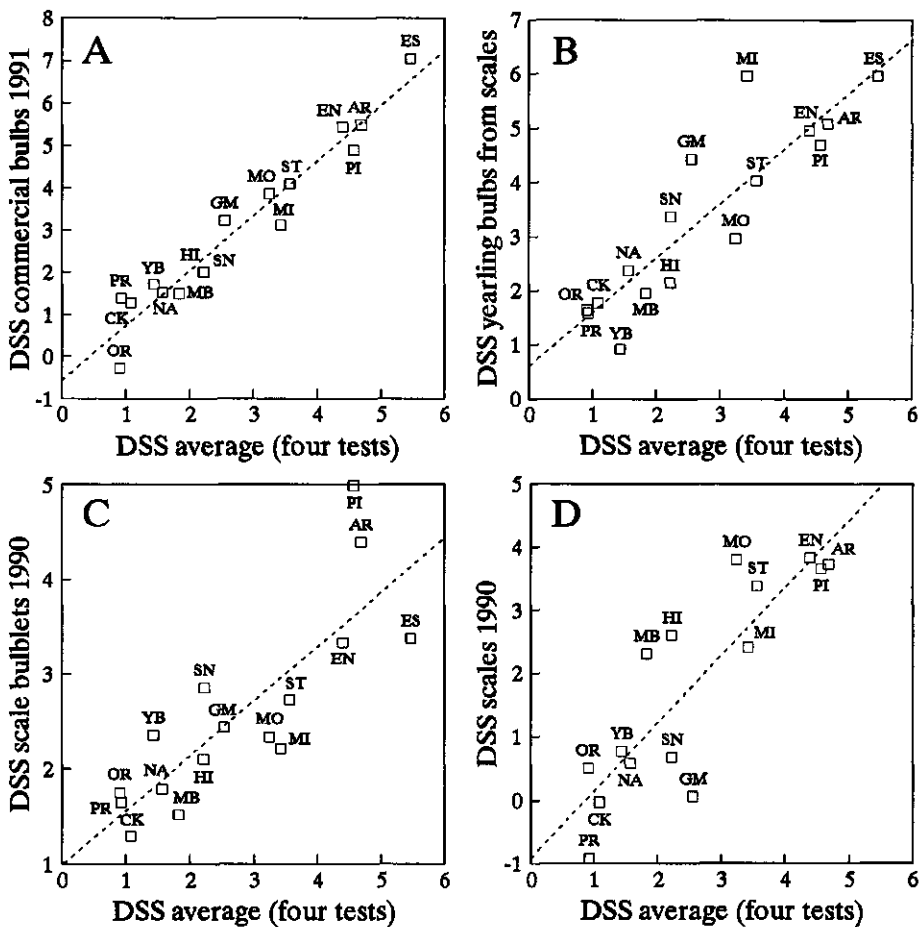**Figure 2.2** Correlation diagrams between disease severity scores (DSS) obtained by the threshold model between commercial bulbs (A, r=0.97), yearling bulbs (B, r=0.89), scale bulblets (C, r=0.82), and scales (D, r=0.86) and the DSS value averaged over those four stages of 16 Asiatic hybrid lily cultivars planted in *Fusarium*-infested soil. See Table 2.2 for abbreviations of cultivar names.

DISCUSSION

The standardized methods developed to screen for *Fusarium* resistance in scale bulblets (Straathof et al., 1993) and scales (Löffler & Mouris, 1989) were used to determine and compare resistance in different developmental stages of lily cultivars. Between replicates, similar results were obtained for all four stages, indicating that the tests are reproducible. Some variation between the replicates was, however, detected. The experiments with yearling bulbs were not replicates in time; bulbs differed in their derivation. Using scales and commercial bulbs, a higher number of plants were tested in the second experiment. Especially in the second scale test, a high level of discrimination between the cultivars was found.

The commercial bulbs used in the 1989 experiments to induce the scale bulblets and to obtain the separate scales originated from different growers. Plant material for the other experiments was cultivated under identical conditions without fungicides. An increased nitrogen supply during the growing season or fungicide treatment (e.g., Benomyl, Captan, Prochloraz) used by some growers could have influenced resistance of the bulbs in the 1989 tests (Linderman, 1977). Because the cultivation conditions were standardized the year before the second tests, results of those experiments are assumed to be more reliable.

If the DSS values of the four lily stages are compared with the average DSS, in general a high correlation is found. This is in agreement with results of testing daffodils and tulips for resistance to *Fusarium oxysporum*. Daffodil bulbils derived from chips or twin scales and one-year-old bulbils (Linfield & Price, 1986) and young and old daffodil bulbs (Bowes et al., 1992) showed similar levels of resistance. In tulips resistance in adult bulbs corresponded with the resistance identified in juvenile bulbs (Van Eijk & Leegwater, 1975; Van Eijk et al., 1979; Van Eijk & Eikelboom, 1983).

The results from commercial lily bulbs were in close agreement with the average values. In the other developmental stages, however, some specific cultivar-stage deviations were found. The yearling stage of the cultivar Milano was more susceptible than at other stages. In the repeat experiment, however, 'Milano' was found to be less susceptible. Since this deviation is not consistent it may be ascribed to variation in the plant material. Scale bulblets of the most susceptible cultivars (e.g., 'Pirate' and 'Aristo') gave results that deviated from the average. This, however, did not affect the ranking of those cultivars.

In the scale test some cultivars (e.g., 'Golden Melody', 'Mont Blanc') differed from the average DSS. Those deviating results were reproducible (Figure 2.1). This might be explained by differing mechanisms of infection and/or defense in scales compared with other developmental stages. A scale is broken from the basal plate of a bulb, resulting in a large wound. This may facilitate entrance of the fungus (Roebroeck et al., 1987). Scales planted in *Fusarium*-infested soil never formed any scale

bulblets in contrast with control scales, which produced scale bulblets with roots and leaves within six to eight weeks. Due to the high inoculum concentration, the basal part of the scales was always rapidly infected in all cultivars, preventing the formation of new bulblets (Straathof & Inggamer, 1992). Wounding is absent in the other stages, thereby biasing the results from the scale tests. To determine whether a different infection and/or defense mechanism is involved in scales, further investigation is required.

Clear cultivar differences were noticed when scales, scale bulblets, yearling bulbs, and commercial bulbs were tested. *Fusarium* resistance in cultivars ranged from partially resistant to completely susceptible. Although most bulbs were affected at harvest time, only commercial bulbs of the most susceptible cultivars were heavily diseased. All commercial bulbs except 'Aristo' and 'Esther' gave normal flower production when compared with the control. In practice, flower production using commercial bulbs is less affected by *Fusarium* than using scale bulblets or yearlings for bulb production.

Scale bulblets were much more sensitive to *Fusarium* than larger bulbs. Scale bulblets had to be harvested within eight weeks to be able to discriminate between partial resistant and susceptible cultivars. Imle (1942) has noted that lily seedlings are much more sensitive to *Fusarium* than larger bulbs. In daffodils, one-year-old bulbs had a lower survival rate against *Fusarium* than two- and three-year-old bulbs (Bowes et al., 1992). In tulips, however, Van Eijk & Leegwater (1975) found that juvenile bulbs were less easily infected than adult bulbs. Most of the scale bulblets were infected in our experiments. The resistance found in some of the cultivars, however, is highly valuable to breeders and growers. The high levels of disease development after six to eight weeks on scale bulblets is partly due to the high inoculum concentration used to obtain optimal discrimination in a short period of time.

Determination of *Fusarium* resistance in lily using disease ratings in combination with a threshold model (Jansen, 1990) is very useful (Straathof et al., 1993). The calculated disease severity score is not an absolute value, but depends on the experiment. Disease severity score values cannot be calculated if most of the ratings for a certain genotype fall in the same category. This occurs if a certain cultivar is completely healthy or, conversely, heavily diseased (e.g., 'Esther'). Disease severity score values are more useful in screening tests if the performance of new genotypes is compared with a group of standard cultivars.

In conclusion, the standardized tests described can be used to detect levels of *Fusarium* resistance in Asiatic lily cultivars at different stages of development. For early selection of resistant genotypes in breeding programs, a scale test can be very useful because it is efficient in time, space, and labour, and only a small amount of plant material is needed. Because of some deviations in the scale tests compared with evaluations of bulbs, a final check of selected bulbs at the scale bulblet stage

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(which is also efficient in time) is suggested.

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CHAPTER 3

Influence of temperature, inoculum concentration and time course in a scale test for *Fusarium* resistance in *Lilium*

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ABSTRACT

The influence of temperature, inoculum concentration and time course on Fusarium *disease development in a lily scale test is described. Five Asiatic hybrid lily cultivars were tested for their resistance level, using a disease rating in combination with a statistical analysis based on a threshold model for ordered categorical data. The order of the cultivars with respect to resistance was identical for all combinations of treatments. It is shown that within the limits set by the experiment a higher temperature, a higher inoculum concentration and a longer time course will increase disease development in lily scales.*

Keywords: Asiatic hybrid lilies, disease rating, *Fusarium oxysporum* f.sp. *lilii*, scales, threshold model.

INTRODUCTION

Bulb rot caused by the soil-borne pathogen *Fusarium oxysporum* f.sp. *lilii* Imle is one of the major problems in *Lilium* cultivation (McRae, 1987). Resistant cultivars can play an important role in the control of this disease. In 1989 a program to develop *Fusarium* resistant lily cultivars was started in the Netherlands. One of the aims of this program is to develop screening tests at clonal and seedling level. Those tests can be used to detect resistance in cultivars, new selections and species, and for the study of the inheritance of resistance in segregating populations and the occurrence of races of the pathogen.

So far only partial resistance is found in *Lilium* (Imle, 1942a; Imle, 1942b; Smith & Maginnes, 1969; Maginnes & Smith, 1971; Van Tuyl, 1980; Löffler & Mouris, 1989, Straathof et al., 1993). In order to detect small differences in resistance level, any screening method should have a high discriminatory power. Therefore standardization of the screening test is necessary. To detect differences in resistance level between genotypes, the disease development has to be determined. By using a disease rating in combination with a statistical analysis based on a threshold model (Jansen, 1990), the level of resistance in scale bulblets of 16 cultivars of Asiatic lilies was established (Straathof et al., 1993).

Several developmental stages (i.e. scale bulblets, yearling bulbs and commercial bulbs) occur in bulb propagation and can be used for screening tests. Van Tuyl (1980) used lily scale bulblets to detect the level of resistance and this test was optimized by Straathof et al. (1993). Screening tests with yearling bulbs and commercial bulbs have been studied by Straathof & Löffler (1994). In addition to testing bulbs, separate scales can be used. Smith & Maginnes (1969) and Maginnes & Smith (1971) used lily scales to screen for Fusarium resistance. This method was

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optimized by Löffler & Mouris (1989). Because a scale test requires less space, time and bulb material, it is ideal for studying the influence of environmental conditions. For standardization of a screening test, knowledge of parameters which influence the disease development is necessary. this is especially true if temperature and/or inoculum concentration cannot be controlled completely. Compensating by changing the duration of the experiment is desirable in order to maintain discrimination between genotypes with different resistance levels. In which case it is important to know if the resistance ratings of the genotypes interact with those parameters. The relationship therefore between temperature, inoculum concentration and time course were studied for five Asiatic hybrid lily cultivars with known levels of *Fusarium* resistance using the bulb scale assay.

MATERIALS AND METHODS

Plant material
Five Asiatic hybrid lily cultivars (Connecticut King, Esther, Hilde, Snow Star and Sterling Star) with a well known level of *Fusarium* resistance were used (Straathof et al., 1993; Straathof & Löffler, 1994). Commercial bulbs were grown from scale bulblets in two years without using fungicides. After harvest, bulbs were stored at 2 °C for 2 months. Scales were broken from the commercial bulbs and were disinfected in 0.5 % hypochlorite solution for 10 minutes, rinsed three times with tap water and dried overnight (Löffler & Mouris, 1989). Scales were selected for uniformity in size within each cultivar.

Fungus
Two highly aggressive monospore isolates of *Fusarium oxysporum* f.sp. *lilii* (CPRO-Fol4 and CPRO-Fol11) (Löffler & Mouris, 1989; Löffler & Rumine, 1991), were used. Stock material, stored on Protect Bacterial Preservers (Technical Service Consultants LTD, Lancs, UK) at -80 °C, was propagated on Czapek-Dox agar medium (Oxoid LTD, Hampshire, UK) before experimental use. For large multiplication of the fungus, mycelium was transferred to an autoclaved (120°C, 2 h) oatmeal-soil mixture (1:4 w/w) and incubated for two weeks at 23 °C. The fully grown cultures were ground and mixed with soil at three concentrations (0.01, 0.1 or 1.0 % w/w). The number of propagules was determined by plating soil dilutions on a modified Komada medium (Komada, 1975; Löffler & Mouris, 1989), immediately after mixing and again at time of planting the bulb scales, two weeks after infestation of the soil.

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Experimental design

The experiment was carried out in an experimental design with 27 treatments. The treatments comprised all possible combinations of three temperature regimes (18, 22 and 26 °C), three inoculum concentrations (0.01, 0.1 and 1.0 % w/w), and three durations (4, 8 and 12 weeks after planting). The experiment was carried out in a phytotron under controlled conditions. For each temperature, 9 trays with 3 inoculum concentrations were prepared. Scales of the five lily cultivars were planted in trays, each tray containing 10 scales of each cultivar in a row. Within each tray the cultivars were randomly assigned to the rows. At each observation time, scales from one tray per combination of inoculum concentration and temperature were evaluated for their disease severity. For each temperature one control tray, without the pathogen, was prepared and observed after 12 weeks.

Disease measurement and statistical analysis

The disease severity among the cultivars was observed visually using a disease rating according to the following ordinal scale: 1 = 'healthy', 2 = 'slightly rotten', 3 = 'moderately rotten', 4 = 'heavily rotten', 5 = 'very heavily rotten' and 6 = 'completely decayed'.

For statistical analysis, the disease rating data was analyzed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990; Jansen, 1991) using a probit link function. An analysis of deviance was obtained with main effects and two-factor interactions only. For each combination of treatment and cultivar, a disease severity score (DSS; Straathof et al., 1993) was calculated. This score may be considered as the average value of the disease ratings on a new underlying linear scale. Conclusions concerning disease ratings were based on the deviance ratio's (McCullagh & Nelder, 1989) which were compared with the F-distribution (Jansen, 1990). The computer package Genstat (Payne et al., 1987) was used to carry out calculations.

In vitro growth

The influence of the temperature on mycelial growth was determined in vitro by culturing the *Fusarium* isolates CPRO-Fol4 and CPRO-Fol11 on Potato Dextrose Agar in 90 mm diameter plates at three temperatures (18, 22, and 26 °C). The experiment was carried out in five replicates. The diameters of the colonies were measured daily, with a final measurement after 9 days.

RESULTS

Directly after mixing, the inoculum density in soil infested with 0.01, 0.1 and 1.0 % oatmeal stock was 6.000, 55.000 and 400.000 propagules per gram soil respectively.

Two weeks later, at planting time, the density had fallen to 2.000, 18.000 and 140.000 propagules per gram soil. A similar decrease was observed by Löffler & Mouris (1989) and is probably due to lysis of propagules in combination with the formation of chlamydospores.

In Table 3.1, the analysis of deviance is given. All main effects appeared to be highly significant. Differences in time course (4, 8 and 12 weeks) had the largest effect on disease development, followed by the effect of the cultivar. The effect of temperature (18, 22, and 26 °C) and of inoculum concentration (0.01, 0.1, and 1.0 %) was similar. Apart from the interaction between temperature and duration, and duration and cultivars the two-factor interactions were not-significant. Disease severity scores calculated for the 5 thresholds were 0.0, 3.82, 6.38, 8.13 and 9.63.

Table 3.1 *Analysis of deviance calculated from disease ratings in a Fusarium-lily scale test. Disease ratings were analyzed using a threshold model with temperature (T), inoculum concentration (IC), time course (TC), cultivar (C) effects and two-factor interactions.*

effect	d.f.	deviance	mean	ratio	P-value
T	2	241	121	90	<0.001
IC	2	279	140	104	<0.001
TC	2	1536	768	573	<0.001
C	4	1008	252	188	<0.001
T x IC	4	3.6	0.9	0.7	nonsignificant
T x TC	4	26.5	6.6	4.9	<0.001
IC x TC	4	3.6	0.9	0.7	nonsignificant
T x C	8	14.1	1.8	1.3	nonsignificant
IC x C	8	16.0	2.0	1.5	nonsignificant
TC x C	8	81.4	10.2	7.6	<0.001
residual	88	118	1.3		

Table 3.2 shows the DSS of each cultivar-treatment combination. Resistance levels ranged from resistant ('Connecticut King', 'Snow Star'), to moderately resistant ('Hilde') and susceptible ('Sterling Star', 'Esther'). In all treatments, the order of the resistance of the cultivars was similar. Although discrimination between cultivars could be found in all treatments, the best discrimination between the cultivars was observed after 8 weeks at 26 °C and 1.0 % inoculum and after 12 weeks at 18 °C and 1.0 % inoculum.

The relationship between time course, temperature and inoculum concentration is given in Figure 3.1. Disease severity scores increased with increasing temperature, inoculum concentration and duration. It can be seen that different combinations of treatments can result in the same disease severity score. For example, at a given time, DSS at a low temperature in combination with a high inoculum concentration

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are comparable to DSS at a higher temperature in combination with lower inoculum concentration.

Table 3.2 Disease severity scores (DSS) of each cultivar-treatment combination in a Fusarium-lily scale test. DSS were calculated from disease ratings using a threshold model with temperature, inoculum concentration, time course and cultivar effects (n = 10).

time course (weeks)	temperature (°C)	inoculum (% w/w)	'Connecticut King'	'Snow Star'	'Hilde'	'Sterling Star'	'Esther'
4	18	0.01	1.3	1.3	2.8	3.2	2.8
		0.1	1.3	1.3	2.8	3.8	3.8
		1.0	2.0	2.8	3.2	3.8	4.1
	22	0.01	1.3	2.0	2.0	3.2	3.8
		0.1	3.2	3.2	3.2	3.6	4.6
		1.0	3.2	3.6	3.8	4.3	6.1
	26	0.01	2.0	2.0	3.6	3.6	5.2
		0.1	3.6	3.8	4.1	4.6	6.2
		1.0	3.8	4.8	4.1	5.8	6.4
8	18	0.01	2.8	2.8	3.6	6.4	5.9
		0.1	3.2	3.2	5.2	6.7	7.5
		1.0	4.1	4.1	5.9	7.1	7.7
	22	0.01	2.8	3.2	3.2	6.9	5.9
		0.1	3.8	3.6	4.1	6.4	6.7
		1.0	3.8	3.8	5.5	7.5	7.9
	26	0.01	3.6	3.2	3.6	6.9	6.9
		0.1	4.7	4.9	5.5	8.8	8.3
		1.0	4.8	4.6	6.9	8.6	10.2
12	18	0.01	4.8	4.6	5.9	8.7	9.2
		0.1	5.9	5.2	6.4	9.0	9.9
		1.0	6.2	6.2	7.7	10.4	11.8
	22	0.01	5.9	5.8	6.2	10.2	9.7
		0.1	7.1	7.7	7.7	9.7	10.4
		1.0	7.5	7.9	9.2	10.6	10.8
	26	0.01	7.2	7.1	7.1	9.9	10.2
		0.1	7.5	7.3	8.8	9.9	11.1
		1.0	8.1	7.7	8.1	11.4	11.4

time course (weeks)

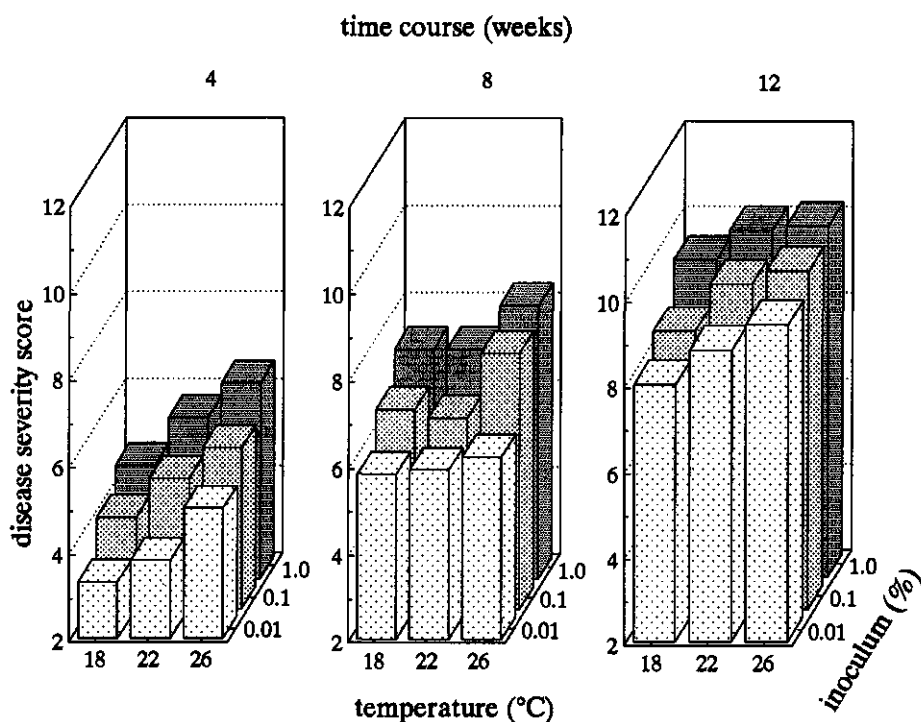**Figure 3.1** Relationship between inoculum concentration, temperature and time course in a Fusarium-lily scale test. Disease severity scores were calculated from disease ratings using a threshold model with temperature, inoculum concentration and time course effects (n = 50).
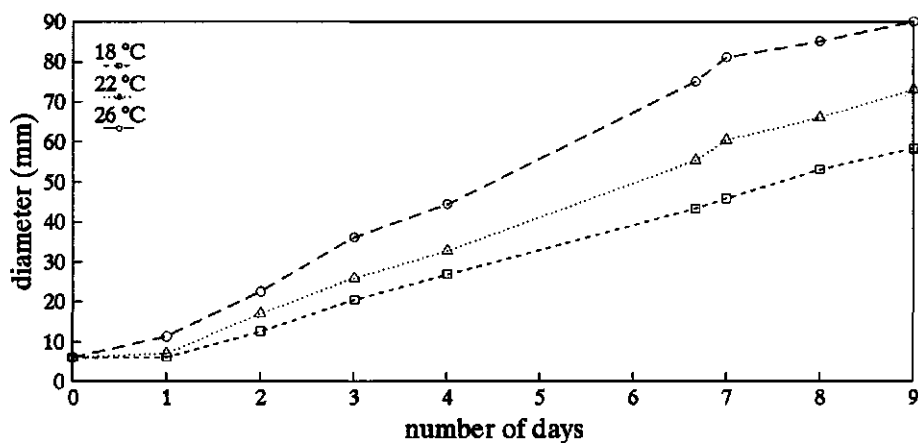

Figure 3.2 Growth (diameter) in time of Fusarium oxysporum f.sp. lilii isolate CPRO-Fol4 in vitro at three different temperatures.

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In vitro growth
The in vitro mycelial growth of the isolates CPRO-Fol4 and CPRO-Fol11 was comparable. The increase in diameter of CPRO-Fol4 is presented in Figure 3.2. An almost linear increase in diameter was found between one and nine days, at all three temperatures. Higher temperatures increased growth rate. Optimum temperature of growth could not be established as temperatures above 26°C were not included.

DISCUSSION

In this experiment, cultivars that were chosen possessed large differences in resistance level, and so 10 scales resulted in good discrimination. In general, more scales per cultivar have to be used in order to detect smaller differences. To obtain optimal discrimination between the resistance levels of cultivars with only small differences in resistance level standardization of screening tests is desirable. Resistance in the *Fusarium*-lily interaction is defined as a retardation of the disease process (Baayen, 1992). This is because absolute resistance is not found, therefore all bulbs or scales will eventually become infected.
The disease development is not only influenced by the resistance level of the host plant, but also by environmental conditions, like temperature, inoculum concentration and time course. A higher incubation temperature speeds up the disease development in the scales. This was to be expected since a temperature rise from 18 to 26 °C shifts the temperature towards the optimum for the fungus as found in the in vitro growth study and the literature (Imle, 1942b). In addition a higher inoculum concentration resulted in more disease. This is probably due to a larger number of infections in the first weeks after planting the scales. Most of the scales displayed symptoms of disease after four weeks. Only in treatments with a low temperature, a low inoculum concentration and a resistant cultivar were scale bulblets found. Because the disease proceeds continuously, a long incubation period will increase the disease development until all bulb scales, even those of resistant cultivars, will be completely rotten.
In all combinations of treatments the order of resistance levels among the cultivars was similar. The two factor interactions can be explained by the fact that after 12 weeks many scales were completely diseased. In this situation the possibilities for further enhancing the disease by changing parameters are limited. Therefore the cultivars will show differences to parameters at 8 weeks but not at 12 weeks. This results in the two-factor interactions. This interaction does not affect the disease rating of the cultivars, but rather the discrimination of the test. Because of this phenomenon the best discrimination between the cultivars, in this experiment was found after 8 weeks at a temperature of 26 °C and 1.0 % inoculum and after 12 weeks at 18 °C and 1.0 % inoculum.

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The results show that a higher temperature, a higher inoculum concentration and a longer time course result in a greater disease development. This is in agreement with studies of *Fusarium* disease in cyclamen (Rattink, 1986). If the different treatments are compared, it can be seen that periods of four weeks have a large influence on the disease development. This means that if small differences in resistance level between cultivars are to be detected the time course is of great importance. If inoculum concentration or temperature cannot be controlled completely, comparable results can be obtained by varying the duration of the experiment. A tenfold increase in the inoculum concentration resulted in a similar increase of the DSS to a temperature increase of 4 °C. If a short test is desired a high temperature and a high inoculum concentration can be used.

The scale test used in this experiment is efficient in terms of greenhouse space, time and bulb material. Results from those tests, however, not necessarily corresponds to results from tests with whole bulbs. Straathof & Löffler (1994) detected some cultivar-stage deviations with regard to the cultivar resistance. This may be due to the fact that scales are wounded when broken from the basal plate of the bulb which facilitates entry of the fungus. Furthermore scales do not have active growing organs such as roots. Therefore the results from the presented experiment cannot be extrapolated directly to screening tests using whole bulbs. Since, however, the defense mechanism of lily to *Fusarium* is due to active retardation or localization of the fungus in the intercellular cortical tissue in roots as well as in scales (Baayen, 1992; R.P. Baayen, *personal communication*) and since our own *Fusarium* tests with lily bulbs indicate that high temperatures, inoculum concentrations and longer time course indeed increase the disease development (results not shown); we are confident that the conclusions are also valid for screening tests with whole bulbs.

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Genetic variation in resistance to *Fusarium oxysporum* f.sp. *lilii* in the genus *Lilium*

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ABSTRACT

Genetic variation in Fusarium *resistance in 82* Lilium *genotypes was studied. A high level of resistance was found in the Asiatic hybrid lilies (section* Sinomartagon*) and to a lesser extent in cultivars of* L. longiflorum *(section* Leucolirion*). The Oriental hybrid lilies (section* Archelirion*) showed only moderate resistance. In the sections* Sinomartagon, Leucolirion *and* Martagon *species were detected with high levels of resistance. An accession of* L. dauricum *(section* Sinomartagon*) was the most resistant source. Possibilities for introgression of* Fusarium *resistance into cultivars by interspecific hybridisation, using the resistance found in* Lilium *genotypes, are discussed.*

Keywords: Asiatic hybrid lilies, *Fusarium oxysporum* f.sp. *lilii, Lilium longiflorum*, genetic variation, interspecific hybrids, *Lilium* species, Oriental hybrid lilies, resistance breeding

INTRODUCTION

The genus *Lilium* L. comprises seven sections (De Jong, 1974) with more than 75 species. Species within three of the sections are the ancestors of the three modern cultivar groups. Within the *Sinomartagon* section, interspecific hybridisation resulted in the Asiatic hybrid lilies. The Asiatic hybrid lilies are worldwide the economically most important group of lilies for cut flower production. The second important group comprises the Oriental hybrid lilies, which originate from interspecific crosses within the *Archelirion* section. In the section *Leucolirion*, cultivars of *L. longiflorum* represent the third group.

The soilborne fungus *Fusarium oxysporum* f.sp. *lilii* Imle is the most serious threat to bulb and flower production of all three groups. An environmentally safe method of preventing damage would be the cultivation of resistant cultivars. Breeding of resistant cultivars requires the availability of a screening test and the existence of genetic variation. Screening tests for *Fusarium* resistance in lily have been developed using commercial and yearling bulbs (Straathof & Löffler, 1994), scale bulblets (Van Tuyl, 1980; Straathof et al., 1993) and scales (Smith & Maginnes, 1969; Maginnes & Smith, 1971; Löffler & Mouris, 1989; Straathof & Inggamer, 1992). Partial resistance was found in some hybrid cultivars from the *Sinomartagon* section such as the Patterson hybrid lilies (Smith & Maginnes, 1969; Maginnes & Smith, 1971) and the Asiatic hybrid lilies (Straathof et al., 1993). Furthermore, *Fusarium* resistance was found in several *Lilium* species such as *L. callosum, L. maximowiczii* and *L. tigrinum* (section *Sinomartagon*), *L. hansonii* (section *Martagon*), *L. canadense* and *L. pardalinum* (section *Pseudolirium*), and *L. regale* and

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L. sargentiae (section *Leucolirion*) (Imle, 1942a; Imle 1942b). No absolute resistance has yet been reported.

The objective of this research was to evaluate the genetic variation in *Fusarium* resistance in the genus *Lilium*. Variation within the Asiatic hybrid lilies, the Oriental hybrid lilies and cultivars of *L. longiflorum* can lead to direct resistance breeding programs within these groups. If resistance is absent in one or more of the cultivar groups, it has to be introduced by interspecific hybridisation. Information about the level of *Fusarium* resistance in *Lilium* genotypes, in relation to the usefulness of these genotypes in interspecific hybridisation programmes, is a prerequisite for future breeding strategies.

MATERIALS AND METHODS

Plant material
In the autumn of 1990, bulbs of a group of genotypes selected from the CPRO-DLO *Lilium* collection were harvested and used for scale bulblet induction. Scale bulblets of all genotypes were stored for 10 weeks at 5°C to fulfil cold requirements. Uniform scale bulblets of each genotype were screened for *Fusarium* resistance in 1991. The rest of the scale bulblets were cultivated for one year to produce yearling bulbs. After selection for uniformity, the yearling bulbs were screened for *Fusarium* resistance in 1992.

During the two years a total of 82 genotypes consisting of 27 Asiatic hybrid lilies (including the standard cultivars Connecticut King, Orlito, Snow Star, Sterling Star, Esther and Pirate) (Straathof et al., 1993), 11 genotypes of *L. longiflorum*, 8 Oriental hybrid lilies, 16 *Lilium* (sub)species (with a total number of 25 accessions) and 11 interspecific hybrids (Table 4.1) were tested. All cultivars, except the Asiatic hybrid lily 'Spreepokal', are registered in The International Lily Register (Leslie, 1982). All genotypes are diploid, except the Asiatic hybrid lilies 'Hornback's Gold' and 'Theseus', *L. tigrinum splendens* (CPRO-35/1), the Longiflorum-Asiatic (LA) hybrid 'Lomonta' and the Oriental-Henryi hybrid lilies CPRO-26/1, CPRO-52/2, CPRO-56/1, CPRO-66/3 and CPRO-76/1 which are triploid. In Figure 4.1 taxonomical relationships and crossability of the species, interspecific hybrids and cultivar groups, used in the *Fusarium* tests, are given (after Van Creij et al., 1990).

Experimental design
Scale bulblets and yearling bulbs were planted in pots, with five bulblets or four bulbs of one cultivar per pot. The genotypes were randomly assigned to the pots. Experiments were laid out in six (scale bulblets) or three blocks (yearling bulbs), each of which consisted of pots of all genotypes. Several noninfested pots were included as controls. Pots were placed in a temperature-controlled greenhouse at

18/14 °C (16 h day / 8 h night).
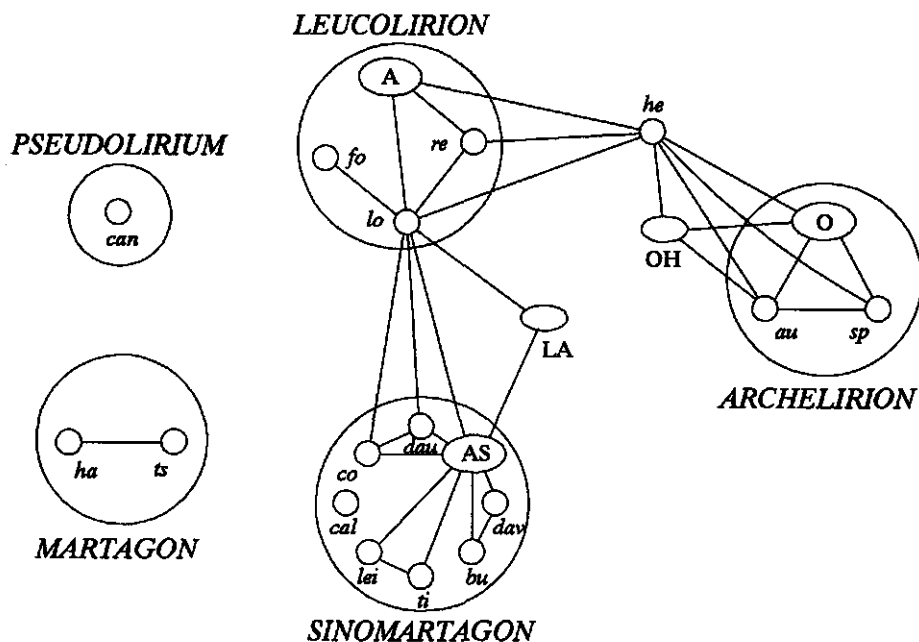
Figure 4.1 Crossing polygon of the genus *Lilium*. Large circles denote sections; circles: species; large ellipses: hybrid groups; small ellipses: species hybrids. Lines represent parentage of hybrids and successful cross combinations. Abbreviations: A: Aurelian hybrid lilies; AS: Asiatic hybrid lilies; *au*: *L. auratum*; *bu*: *L. bulbiferum*; *cal*: *L. callosum*; *can*: *L. canadense*; *co*: *L. concolor*; *dau*: *L. dauricum*; *dav*: *L. davidii*; *fo*: *L. formosanum*; *ha*: *L. hansonii*; *he*: *L. henryi*; *lei*: *L. leichtlinii*; LA: Longiflorum-Asiatic hybrid lilies; *lo*: *L. longiflorum*; O: Oriental hybrid lilies; OH: Oriental-Henryi hybrid lilies; *re*: *L. regale*; *sp*: *L. speciosum*; *ti*: *L. tigrinum*; *ts*: *L. tsingtauense*.

Screening assay

Inoculum was prepared and the soil infested as described by Straathof et al. (1993). The number of propagules was determined at the time of planting, two weeks after infestation of the soil ($\pm$ 30.000 propagules per gram of soil in both experiments). Bulb observations were carried out 8 weeks (scale bulblets) or 20 weeks (yearling bulbs) after planting (Straathof & Löffler, 1994). The resistance levels of genotypes were measured using a disease rating. Decay of the infested plant material was rated visually according to an ordinal scale with six categories: 1 = healthy; 2 = slightly rotten; 3 = moderately rotten; 4 = heavily rotten; 5 = very heavily rotten; and 6 = completely decayed.

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Statistical analysis

Disease rating data were analyzed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990), using a probit link function. The between-pot variation was estimated from the data (Jansen, 1990). For each cultivar within an experiment, a disease severity score (DSS) was calculated by the threshold model. This score may be considered as a transformed average value of the disease ratings of each genotype on an underlying linear scale (Straathof et al., 1993). Conclusions concerning block and genotype effects were based on deviance statistics (McCullagh & Nelder, 1989), which have to be compared with the table of the chi-squared distribution. The computer package Genstat (Payne et al., 1987) was used to carry out calculations.

RESULTS

From the 82 genotypes tested over two years, 71 were tested in both experiments. Five of the 82 genotypes did not produce enough uniform scale bulblets and were excluded from the 1991 experiment. In 1992, six genotypes were excluded since the quality of the yearling bulbs produced was not high enough.

The analysis of deviance gave highly significant differences ($P < 0.001$) between genotypes in both experiments. No significant block effects were detected. The estimate of the between-pot variance was 0.22 (s.e. $= 0.05$) and zero respectively for the 1991 and 1992 experiment. The DSS values of each genotype and corresponding standard error of differences (relative to 'Connecticut King') of both experiments are given in Table 4.1. Genotypes are arranged by origin and DSS value of 1991. In the 1992 experiment, all bulbs of some genotypes were completely decayed; in that case no DSS values could be calculated. A significant correlation between DSS of genotypes tested in both years was found (Figure 4.2), although some deviations (e.g. Asiatic hybrid lily 'Svetlana', *L. tsingtauense* CPRO-11/1, *L. concolor* CPRO-28/1) occurred. Genotypes with a level of resistance comparable with the standard cultivars Connecticut King and Orlito are called highly resistant, genotypes comparable with 'Esther' and 'Pirate' are called susceptible, and genotypes between these standards moderately resistant.

The variation in resistance level within the Asiatic hybrid lilies is given in Figure 4.2A. The level ranged from highly resistant to susceptible. The level of resistance within the cultivars of *L. longiflorum* was lower (Figure 4.2B) compared with the most resistant Asiatic hybrid lilies. Only moderate resistance was found in the Oriental hybrid lilies (Figure 4.2C), but the number of cultivars tested was small compared with the other two cultivar groups.

Table 4.1 Disease severity score (DSS) and standard error of differences (s.e.d.; relative to 'Connecticut King') of 82 *Lilium* genotypes after planting scale bulblets (1991) and yearling bulbs (1992) in *Fusarium*-infested soil.

genotype	origin	1991 DSS	s.e.d.	1992 DSS	s.e.d.
'Hornback's Gold'	Asiatic hybrid lily	0.24	0.30	1.74	0.50
'Vonnie'	Asiatic hybrid lily	0.29	0.30	0.77	0.50
'Orlito'	Asiatic hybrid lily	0.38	0.30	1.40	0.50
'Yellow Star'	Asiatic hybrid lily	0.39	0.30	1.97	0.50
'Snow Star'	Asiatic hybrid lily	0.53	0.30	1.20	0.50
'Connecticut King'	Asiatic hybrid lily	0.65	-	0.36	-
'Svetlana'	Asiatic hybrid lily	0.79	0.30	3.27	0.49
'Banga'	Asiatic hybrid lily	0.92	0.30	0.77	0.50
'Malinovka'	Asiatic hybrid lily	0.95	0.30	2.33	0.49
'Yellito'	Asiatic hybrid lily	*[y]	*	2.33	0.49
'Citronella'	Asiatic hybrid lily	1.03	0.30	1.97	0.50
'Grace Marshall'	Asiatic hybrid lily	1.26	0.30	2.33	0.49
'Brenda Watts'	Asiatic hybrid lily	1.36	0.38	1.90	0.58
'Sterling Star'	Asiatic hybrid lily	1.45	0.30	4.12	0.49
'Nocka'	Asiatic hybrid lily	1.54	0.30	3.17	0.49
'Zolotaja'	Asiatic hybrid lily	1.71	0.30	2.55	0.49
'Esther'	Asiatic hybrid lily	1.71	0.31	4.56	0.51
'Aelita'	Asiatic hybrid lily	1.84	0.31	*	*
'Mountaineer'	Asiatic hybrid lily	1.85	0.31	2.68	0.49
'Amalia'	Asiatic hybrid lily	1.90	0.30	4.11	0.49
'Brandy Wine'	Asiatic hybrid lily	1.99	0.31	2.56	0.49
'Spreepokal'	Asiatic hybrid lily	2.00	0.31	3.48	0.49
'Theseus'	Asiatic hybrid lily	2.36	0.32	>[z]	>
'Festival'	Asiatic hybrid lily	2.59	0.33	3.18	0.49
'Laura'	Asiatic hybrid lily	2.64	0.33	5.27	0.56
'Pirate'	Asiatic hybrid lily	2.71	0.33	4.16	0.49
'Prima'	Asiatic hybrid lily	2.72	0.44	*	*
'Nellie White'	L. *longiflorum* Thunb.	0.81	0.30	2.13	0.50
'White American'	L. *longiflorum* Thunb.	0.87	0.30	2.62	0.50
CPRO-19/1	L. *longiflorum* Thunb.	0.93	0.30	3.15	0.49
'Hinemoto'	L. *longiflorum* Thunb.	0.94	0.30	2.61	0.49
'White Europe'	L. *longiflorum* Thunb.	1.00	0.30	1.98	0.50
'White Diamond'	L. *longiflorum* Thunb.	1.56	0.30	4.23	0.50
'Snow Queen'	L. *longiflorum* Thunb.	1.77	0.31	5.13	0.59
'Gelria'	L. *longiflorum* Thunb.	1.78	0.31	4.49	0.50
'Ace'	L. *longiflorum* Thunb.	1.85	0.31	4.22	0.50
'Longivetta'	L. *longiflorum* Thunb.	2.36	0.32	4.59	0.51
'Flevo'	L. *longiflorum* Thunb.	2.80	0.34	4.15	0.50

Table 4.1 Continued.

genotype	origin	1991 DSS	s.e.d.	1992 DSS	s.e.d.
'Journey's End'	Oriental hybrid lily	1.18	0.30	3.45	0.49
'Laura'	Oriental hybrid lily	1.34	0.30	2.97	0.49
'Le Rêve'	Oriental hybrid lily	1.53	0.30	3.16	0.49
'Casa Blanca'	Oriental hybrid lily	2.20	0.31	4.48	0.50
'Stargazer'	Oriental hybrid lily	2.41	0.32	3.98	0.49
'White Mountain'	Oriental hybrid lily	2.51	0.32	5.51	0.59
'Capitol'	Oriental hybrid lily	2.96	0.35	5.09	0.54
'Dame Blanche'	Oriental hybrid lily	3.12	0.36	>	>
CPRO-01/1	*L. dauricum* Ker-Gawler	-0.52	0.33	-0.44	0.51
CPRO-02/1	*L. hansonii* Leichtlin	*	*	0.93	0.56
CPRO-09/1	*L. davidii* Duchartre	0.67	0.30	2.51	0.49
CPRO-10/1	*L. tigrinum* Ker-Gawler	0.68	0.30	1.59	0.50
CPRO-11/1	*L. tsingtauense* Gilg	0.72	0.30	3.33	0.49
CPRO-12/1	*L. henryi* Baker	*	*	1.97	0.54
CPRO-16/1	*L. regale* Wilson	0.85	0.34	1.22	0.56
CPRO-22/2	*L. davidii* Duchartre	0.96	0.30	1.42	0.50
CPRO-28/1	*L. concolor* Salis	1.15	0.30	4.61	0.56
CPRO-32/2	*L. henryi* Baker	1.26	0.30	2.57	0.49
CPRO-35/1	*L. tigrinum splendens* Leichtlin	1.37	0.30	1.92	0.50
CPRO-40/3	*L. henryi* Baker	1.57	0.32	*	*
CPRO-41/1	*L. leichtlinii maximowiczii* (Regel) Baker	1.62	0.30	2.98	0.49
CPRO-42/1	*L. speciosum* Thunb.	1.66	0.30	4.10	0.50
CPRO-51/4	*L. henryi* Baker	1.87	0.35	*	*
CPRO-57/1	*L. callosum* Siebold & Zucc.	2.15	0.31	>	>
CPRO-59/2	*L. concolor* Salis	2.21	0.31	5.49	0.59
CPRO-61/1	*L. canadense rubrum* L.	2.30	0.31	4.05	0.49
CPRO-65/5	*L. henryi* Baker	*	*	4.43	0.55
CPRO-67/1	*L. bulbiferum croceum* (Chaix) Persoon	2.46	0.32	>	>
CPRO-70/1	*L. formosanum* Wallace	2.57	0.38	>	>
CPRO-72/2	*L. regale* Wilson	2.61	0.43	*	*
CPRO-78/2	*L. bulbiferum croceum* (Chaix) Persoon	2.84	0.34	>	>
CPRO-81/2	*L. formosanum* Wallace	3.45	0.43	*	*
CPRO-82/1	*L. auratum* Lindle	3.45	0.40	>	>
CPRO-14/1	*L. henryi* x *L. regale*	0.80	0.30	1.20	0.50
'Golden Splendor'	Aurelian hybrid lily	1.79	0.30	3.68	0.50
'Loblanca'	*L. longiflorum* 'White Europe' x Asiatic 'Mont Blanc'	1.13	0.30	1.80	0.50
'Lomonta'	LA-hybrid 'Loblanca' x Asiatic 'Mont Blanc'	1.26	0.30	2.13	0.50

Table 4.1 Continued.

genotype	origin	1991 DSS	s.e.d.	1992 DSS	s.e.d.
CPRO-26/1	Oriental 'Journey's End' x (Oriental-Henryi CPRO-68/1)	1.09	0.30	2.48	0.49
CPRO-52/2	Oriental 'Journey's End' x (Oriental-Henryi CPRO-68/1)	1.89	0.30	4.47	0.50
CPRO-56/1	Oriental 'Stargazer' x (Auratum-Henryi CPRO-60/1)	2.12	0.31	5.02	0.53
CPRO-60/1	*L. auratum* x *L. henryi*	2.26	0.31	2.94	0.49
CPRO-66/3	Oriental 'Journey's End' x (Oriental-Henryi CPRO-68/1)	2.45	0.32	4.05	0.49
CPRO-68/1	Oriental 'Shikayama' x L. henryi	2.46	0.32	3.39	0.49
CPRO-76/1	*L. speciosum* 'Uchida' x (Oriental-Henryi CPRO-68/1)	*	*	4.95	0.53

'Not included in the experiment.
²DSS not calculated.

Species from the *Sinomartagon* section ranged from highly resistant (*L. dauricum*, *L. davidii* and *L. tigrinum*) to susceptible (*L. bulbiferum croceum*) (Table 4.1). The resistance of *L. dauricum* was not absolute: some small infection lesions were found at the basal plate. In the *Leucolirion* section, one of the two accessions of *L. regale* (CPRO-16/1) showed a high level of resistance, while both accessions of *L. formosanum* were very susceptible. In the *Archelirion* section both species (*L. speciosum* and *L. auratum*) were susceptible. Variation in resistance between the five accessions of *L. henryi* ranged from moderately resistant (CPRO-12/1) to susceptible. *L. hansonii* of the *Martagon* section was highly resistant in 1992 (not tested in 1991). *L. tsingtauense* of the *Martagon* section displayed a high level of resistance in the test of 1991 but in 1992 *L. tsingtauense* was more diseased. *L. canadense rubrum*, the only species tested from the *Pseudolirium* section, was susceptible.
Of the interspecific hybrids, the descendant of *L. henryi* x *L. regale* showed a high level of resistance (Table 4.1). The LA-hybrid lilies did not display a higher level of resistance than the most resistant cultivars of *L. longiflorum*. One of the seven Oriental-Henryi hybrid lilies (CPRO-26/1) was moderately resistant while the others were susceptible.

DISCUSSION

After 50 years (Imle, 1942a; Imle 1942b) a new extensive study was performed to investigate the genetic variation in *Fusarium* resistance in the genus *Lilium*. Knowledge of this variation within cultivar groups in combination with screening tests can lead directly to resistance breeding programmes. Furthermore, knowledge of the level of resistance in present-day cultivars makes it possible for growers to make a specific choice for resistant ones.
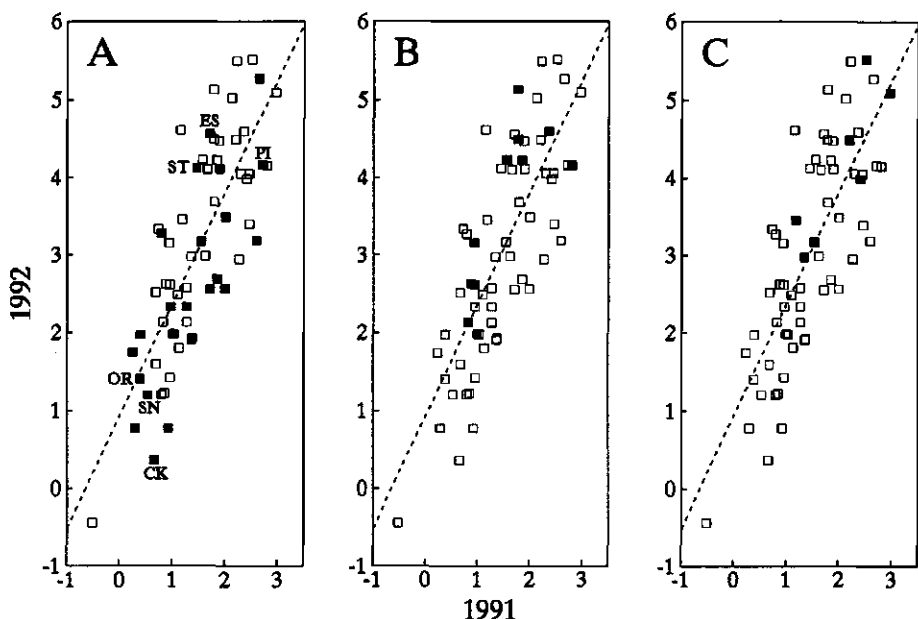
Figure 4.2 Correlation diagram between disease severity scores of the 1991 (scale bulblet) and 1992 (yearling bulb) experiments of 64 lily genotypes planted in *Fusarium*-infested soil (r = 0.79). ■ gives position of the Asiatic hybrid lilies including six standard cultivars (A), *L. longiflorum* cultivars (B) and Oriental hybrid lilies (C).

So far, interspecific hybridisation techniques have been focused mainly to exploit genetic variation in plant and flower morphology. Interspecific hybridisation can also be helpful to enrich cultivar groups with *Fusarium* resistance found in non-related species. Such programs, however, require considerable time because of the need for several back crossing generations for introgression by meiotic recombination, and moreover, crossing barriers have to be overcome (Asano, 1980b; Van Creij et al., 1990; Van Tuyl et al., 1991; Okazaki et al., 1992).

The six standard Asiatic hybrid lilies showed resistance levels comparable with other experiments (Straathof et al., 1993), although 'Snow Star' was less diseased than expected. Some deviations in resistance level of genotypes were found between the two tests. This could be due to differences in developmental stage or to the quality of the bulbs. Differences due to the developmental stage is unlikely as demonstrated by Straathof & Löffler (1994) in Asiatic hybrid lilies. The (inner) quality of the bulbs, however, may well be variable. Cultivars and especially species differ in their ability to produce scale bulblets. Furthermore, hardly any information about the temperature treatment for scale bulblet induction and the chilling temperature for

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shoot elongation of *Lilium* species is available. Because the most uniform scale bulblets were used for testing, the quality and number of the yearlings obtained after cultivation of the remainder scale bulblets was also limited.

Large differences in partial resistance were found between the lily cultivars and species. Amongst other bulb crops, variation in *Fusarium* resistance has been found in *Gladiolus* (Palmer & Pryor, 1958; Jones & Jenkins, 1975; Chandra et al., 1985) in *Tulipa* (Van Eijk et al., 1978) and in *Narcissus* (Tompsett, 1986; Linfield, 1992). In *Gladiolus* (Th.P. Straathof, *unpublished*) and *Narcissus* (Linfield, 1992), even absolute resistance was found. In the genus *Lilium*, absolute resistance has still not been detected.

Partial resistance has already been found in the Asiatic hybrid lilies (Straathof et al., 1993). Especially bulbs of 'Connecticut King' and its descendant 'Orlito' are only slightly affected after infection. Cultivars of the Asiatic hybrid lilies tested in this study came from different origins (e.g. U.S.A., Netherlands, U.K., Russia, Poland). Although highly resistant Asiatic hybrid lilies were again found, none of the cultivars tested was significantly better than 'Connecticut King'. The origin of resistance in the Asiatic hybrid lilies can probably be traced back to several of their ancestors like *L. dauricum*, *L. davidii* and *L. tigrinum.* The *Fusarium* problem in Asiatic hybrid lilies can probably be controlled at short notice by screening the present-day cultivars for resistance, followed by a specific choice by growers to cultivate only the most resistant ones. It is possible that a broader group of resistant cultivars can be developed in the near future with the knowledge obtained from the present study.

Within cultivars of *L. longiflorum*, variation in resistance was found, although the level of resistance was lower than in the most resistant Asiatic hybrid lilies. Because cultivars of *L. longiflorum* originated, as defined, from a single species, variation in resistance has to be found within this species. Introgression of resistant genes from e.g. *L. dauricum* or *L. regale* will lead to completely new lily types; otherwise long term back crossing programs have to be performed. Furthermore, hybridisation between *L. longiflorum* and *L. regale* appeared to be very difficult, although the two species belong to the same section. More resistance within *L. longiflorum* can possibly be traced in wild accessions, found in the Southern Islands of Japan.

Interspecific crosses between *L. longiflorum* and Asiatic hybrid lilies gave rise to the so called LA-hybrid lilies (Figure 4.1) (Van Tuyl et al., 1988) which have become more and more popular. *Fusarium* resistant LA-hybrid lilies can be produced if resistant Asiatic hybrid lilies and cultivars of *L. longiflorum* are crossed. Recent experiments with new LA-hybrid lilies showed that a resistance level of 'Connecticut King' can be achieved within the LA-hybrid lilies (data not shown).

The Oriental hybrid lilies (*Archelirion* section) showed little variation in *Fusarium* resistance. For short term solutions, it is important to test a wider range of Oriental hybrid lilies to detect whether any resistance is directly available or not. Two species

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tested within the *Archelirion* section (*L. speciosum* and *L. auratum*) were both susceptible. From *L. rubellum* (J.M. Van Tuyl, *unpublished*) and *L. japonicum* (Imle, 1942a; Imle, 1942b) it was already known that they were susceptible. Resistance can perhaps be introduced from resistant *L. henryi* accessions. Oriental-Henryi hybrid lilies tested so far were not significantly better than the most resistant Oriental hybrid lilies.

Within the section *Martagon*, a high level of resistance was found in the species *L. hansonii*. This was also reported by Imle (1942a; 1942b). This resistance might be used for introgression into *L. longiflorum*. The first successful cross between *L. martagon* of the *Martagon* section and *L. longiflorum* has already been reported (Van Creij et al., 1990).

Interspecific hybridisation can lead to sterile hybrids. Mitotic chromosome doubling can restore fertility (Van Tuyl, 1990). Furthermore, some interspecific hybrids produce unreduced gametes. Back crossing those hybrids can lead to triploid genotypes. The interspecific hybrid of *L. auratum* x *L. henryi* (Asano, 1980a) and Oriental hybrid lily 'Shikayama' x *L. henryi* (Asano, 1978) gave rise to only triploid progenies when crossed with diploid Oriental hybrid lilies (Van Tuyl, 1990). Those problems can be prevented by using a tetraploid genotype as back crossing parent. By mitotic and meiotic polyploidisation, tetraploid lily hybrids can be obtained. Comparisons of diploid and tetraploid cultivars show no influence of ploidy level on the level of *Fusarium* resistance (Straathof & Van Tuyl, 1990).

Of the ten species tested both by Imle (1942a; 1942b) and in these experiments, eight species reacted more or less the same, although variation within species can be expected as demonstrated in different accessions of *L. henryi*, *L. regale* and *L. longiflorum*. *L. callosum* and *L. canadense* were more susceptible in our studies than in the studies of Imle.

In conclusion, several sources of resistance are found in the genus *Lilium* and some can be used directly to produce new resistant cultivars. Resistance in unrelated species can only be used via more sophisticated breeding techniques. Based on existing interspecific hybridisation protocols and the new hybridisation techniques which are being developed, it can be expected that *Fusarium* resistance from a number of *Lilium* species can be transferred to economically important cultivar groups. After interspecific hybridisation, however, several back crossings will be necessary in most cases. Because of the large investments involved in interspecific hybridisation and back crossing programmes, it will be essential to estimate the risks of the appearance of physiological races of the pathogen against the most interesting *Fusarium* resistance donors. This will be the subject for future research.

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CHAPTER 5

Durability of resistance in lily to basal rot: evaluation of virulence among isolates of *Fusarium oxysporum* f.sp. *lilii*

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ABSTRACT

Prospects of durability of resistance in lily to basal rot have been evaluated by testing the virulence of 31 isolates of Fusarium oxysporum *f.sp.* lilii *towards a number of different resistance sources in* Lilium spp. *Isolates differed strongly in aggressiveness as did species and cultivars of* Lilium spp. *in resistance. Significant interactions were observed between isolates of the pathogen and genotypes of* Lilium spp., *but the magnitude was very small compared to the main effects. The interactions were mainly due to a small group of isolates with low aggressiveness. It is argued that the interactions might be based on minor genes. No major break down of the resistance was found. For practical purposes it will be sufficient to use highly aggressive isolates in screening tests.*

Keywords: bulb disease, basal rot, *Lilium spp.*, resistance breeding, scale test, races

INTRODUCTION

The soil-borne fungus *Fusarium oxysporum* is a pathogen which occurs worldwide and causes, in specialized form, serious diseases in many crops. In lily the forma specialis *lilii* causes bulb rot of lily, which threatens the bulb production worldwide. The predominant symptom of the disease is a brownish rot at the base of the bulb scales. This rot may spread over the whole bulb in a later stage of the disease, resulting in the total loss of the bulb (Imle 1942; Linderman, 1981; McRae, 1987). The disease can be controlled by disinfecting bulbs before planting in combination with soil fumigation. In the Netherlands, however, the application of chemical control will be reduced strongly in the near future in order to reduce environmental pollution. Control of the disease by cultivation of *Fusarium* resistant cultivars will be the first alternative.

High levels of partial resistance were found in some Asiatic hybrid lilies and some species. A moderate level of resistance was found in cultivars of *Lilium longiflorum* and almost none in the Oriental hybrid lilies (Straathof & Van Tuyl, 1994). The sources of resistance can be used in, interspecific, breeding programmes to develop new resistant lily cultivars.

It is not clear, however, what the durability of those resistances will be. Non-durable resistance evidently is of limited value in a breeding programme. In other crops, resistance to *Fusarium oxysporum* sometimes was found to be overcome by the pathogen, resulting in the emergence of different races of the pathogenic forma specialis (Armstrong & Armstrong, 1981; Roebroeck & Mes, 1992). The specificity of the interaction caused by races will be designated with the term 'virulence' throughout this paper, whereas the term 'aggressiveness' refers to the overall rate

of disease development (Van der Plank, 1984). Apart from a preliminary study in vegetative compatibility, aggressiveness and virulence in *F. oxysporum* f.sp. *lilii* (Löffler & Rumine, 1991), data on the existence of races within the f.sp. *lilii* are lacking. The present study assesses the prospects of durability of the resistance in lily to basal rot.

Durability is governed by two distinct factors. In the first place, genetic variation for virulence may exist already within the population of the pathogen. Cultivation of resistant cultivars might thus lead to selection of more virulent genotypes of the fungus. In the second place, the pathogen might be able to adapt easily to the resistance of the host plant. In that case a breaking down of the resistance would be due to changes in the genome of the pathogen. The present study reports on the first aspect. We tested the virulence of a large number of isolates from diverse geographic origin to a series of lily cultivars differing in resistance to basal rot. For comparison a number of isolates of other rot-inducing formae speciales were included. Such isolates may cross-react with lilies (Löffler & Mouris, 1993). For the screening a bulb scale assay was used (Smith & Maginnes, 1969; Maginnes & Smith, 1971; Löffler & Mouris, 1989). This assay is efficient in time and space and renders reliable data (Straathof & Löffler, 1994).

MATERIALS AND METHODS

Fungi

Isolates of *F. oxysporum* f.sp. *lilii* (Fol), *F. oxysporum* f.sp. *gladioli* (Fog; Foi) and *F. oxysporum* f.sp. *tulipae* (Fot) were kindly provided by E.J.A. Roebroeck (LBO: Bulb Research Centre, Lisse, The Netherlands), G. Bollen (LUW: Wageningen Agricultural University, The Netherlands), J. van Tuyl (CPRO-DLO: DLO Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands), P. Rumine (ISF: Istituto Sperimentale per la Floricoltura, Pescia, Italy), L.B. Orlikowski (RIPF: Research Institute of Pomology and Floriculture, Skierniwice, Poland), and P.E. Nelson (FRC: The Fusarium Research Centrum, State College (PA), USA). The origin of the isolates is summarized in Table 5.1. Single-spore isolates were made and screened for pathogenicity to a number of susceptible lily cultivars in a scale-assay (Smith & Maginnes, 1969; Maginnes & Smith, 1971; Löffler & Mouris, 1989). Non-pathogenic isolates were discarded; pathogenic ones were stored on PROTECT bacterial preserves (Technical Service Consultants) at -80 °C. Isolates selected for virulence tests were revitalized on OXOID Czapek Dox agar plates (CDA).

Table 5.1 Origin of isolates of *Fusarium oxysporum* f.p. *lilii* (Fol), f.sp. *gladioli* (Fog; Foi) and f.sp. *tulipae* (Fot) used in this study.

isolate	host	cultivar	source[z]	country
Fol-3	Asiatic hybrid lily	Enchantment	LBO	The Netherlands
Fol-4	Asiatic hybrid lily	Pirate	LBO	The Netherlands
Fol-5	Asiatic hybrid lily	Pirate	LBO	The Netherlands
Fol-7	*Lilium formosum*	-	LBO	The Netherlands
Fol-9	Asiatic hybrid lily	Amigo	LBO	The Netherlands
Fol-10	Asiatic hybrid lily	Krista	LUW	The Netherlands
Fol-11	Asiatic hybrid lily	Esther	LUW	The Netherlands
Fol-15	*Lilium concolor*	-	CPRO-DLO	The Netherlands
Fol-18	Lily	-	LBO	The Netherlands
Fol-19	Lily	-	LBO	The Netherlands
Fol-21	Asiatic hybrid lily	Casa Blanca	LBO	The Netherlands
Fol-28	Lily	-	ISF	Italy
Fol-30	Lily	-	ISF	Italy
Fol-33	Asiatic hybrid lily	Aleida	LBO	The Netherlands
Fol-35	Asiatic hybrid lily	Avignon	LBO	The Netherlands
Fol-36	Asiatic hybrid lily	Montreux	LBO	The Netherlands
Fol-38	Asiatic hybrid lily	Snow Queen	LBO	The Netherlands
Fol-40	Asiatic hybrid lily	Moulin Rouge	LBO	The Netherlands
Fol-42	Asiatic hybrid lily	Yellow Present	LBO	The Netherlands
Fol-43	Asiatic hybrid lily	Connecticut King	LBO	The Netherlands
Fol-63	Lily	-	FRC	USA
Fol-69	Asiatic hybrid lily	Escapade	ISF	Italy
Fol-71	*Lilium longiflorum*	Snow Queen	ISF	Italy
Fol-73	Asiatic hybrid lily	Enchantment	ISF	Italy
Fol-78	Lily	-	ISF	Italy
Fol-79	Asiatic hybrid lily	Boston	RIPF	Poland
Fol-80	Asiatic hybrid lily	Nellie White	RIPF	Poland
Fog-15	Gladiolus	-	LBO	The Netherlands
Foi-2	Iris	-	LBO	The Netherlands
Foi-7	Iris	-	LBO	The Netherlands
Fot-8	Tulip	-	LBO	The Netherlands

[z]See materials and methods for abbreviations.

Plant material

Lily genotypes known to be highly resistant to basal rot were used (Straathof et al., 1993; Straathof & Van Tuyl, 1994) along with moderately resistant and susceptible ones (Table 5.2). Bulbs were not disinfected after harvest but were stored as such at -0.5 °C until use. One day before planting the bulbs were brought at room temperature and scales were detached. Apparently healthy scales were disinfected for 10 minutes in 1 % hypochlorite with a drop of Tween 20, rinsed with tap water and spread on filter paper and dried overnight at room temperature.

Table 5.2 Resistance level and taxonomical position of cultivars and accessions of lilies (*Lilium* L.) used in experiment 1 and 2.

genotype	abbreviation	group[y]	resistance[z]	experiment
Aristo	AR	A	S	1,2
Gelria	GE	L	S	1
Star Gazer	SG	O	S	1
CPRO-12/1 (*L. henryi*)	HEN	S	M	2
CPRO-26/1	JSH	H	M	2
White Europe	WE	L	M	2
Banga	BA	A	R	2
Connecticut King	CK	A	R	1,2
CPRO-01/1 (*L. dauricum*)	DAU	S	R	2
CPRO-02/1 (*L. hansonii*)	HAN	S	R	2
CPRO-10/1 (*L. tigrinum*)	TIG	S	R	2
CPRO-22/2 (*L. davidii*)	DAV	S	R	2
Mont Blanc	MB	A	R	1
Napoli	NA	A	R	1
Orlito	OR	A	R	1
Prominence	PR	A	R	1
Vonnie	VO	A	R	2
Yellow Blaze	YB	A	R	1
Yellow Star	YS	A	R	2

[y]A: Asiatic hybrid lily; L: *Lilium longiflorum*; O: Oriental hybrid lily; S: botanical species; H: hybrid originating from Oriental hybrid lily x (Oriental hybrid lily x *L. henryi*).

[z]R: resistant; M: moderately resistant; S: susceptible (Straathof et al., 1993; Straathof & Van Tuyl, 1994).

Soil infestation

Inoculum was prepared by infecting 100 ml glass jars containing sterilized oatmeal-soil mixture (20 % w/w oatmeal) with five 5-mm agar plugs taken from CDA plates with the proper isolate and incubating the mixture for two weeks at 23 °C. Subsequently the mixture was ground by pressing it through a sieve (experiment 1) or by using a food processor (experiment 2). Ten litre of soil in 20-litre open trays was mixed with the ground mixture of the appropriate isolate (1 % w/w) and incubated for two weeks prior to use to allow the fungal population to stabilize (Löffler & Mouris, 1989).

Virulence screening

Nineteen genotypes of lily were screened in two experiments with 31 fungal isolates in a randomized block design of two blocks. Both blocks contained trays of all isolates and a single tray with uninfested soil. Scales were planted in rows of six according to genotype (nine in experiment 1 and twelve in experiment 2). Rows within trays and trays within blocks were fully randomized. In this way each combination of genotype and isolate was tested in 12-fold. After 8 weeks incubation

at 18 °C in the greenhouse the scales were harvested and rated for disease severity using the following scale: 1 (healthy), 2 (slightly rotten), 3 (moderately rotten), 4 (heavily rotten), 5 (very heavily rotten), and 6 (completely decayed).

Statistical analysis

The disease ratings were transformed from the non-linear categorical scale to a linear disease severity score (DSS) by applying a threshold model, according to Straathof et al. (1993). Conclusions concerning effects of blocks, genotypes, and isolates and the genotype x isolate interaction were based on deviance statistics (McCullagh & Nelder, 1989), which have to be compared with the chi-squared distribution.

The genotype x isolate interaction was further analyzed using two approaches. In the first one, standardized predictions of the interaction effect of each combination of genotype and isolate were used. Large prediction values (> 2.5 or < -2.5) were considered to be significant. In the second one, fungal isolates and lily genotypes were clustered according to Corsten & Denis (1990). This procedure identifies simultaneously groups of non-interacting genotypes and groups of non-interacting isolates in a two-way table of uncorrelated normally distributed observations with common variance. The interaction between genotypes and isolates is now concentrated in interactions between groups. Within groups no interaction is present. For the cluster analysis, DSS values were used as observations. Since, the threshold model does not provide a common estimate of the variance, no significance between groups could be presented.

RESULTS

Analysis of deviance showed that in both experiments the deviance was mainly due to the main effects (aggressiveness of *Fusarium* isolates and resistance of *Lilium* genotypes); block effects were nonsignificant. The isolate x genotype interaction was significant ($P < 0.001$) in both experiments but their contribution to the total deviance was relatively small (Table 5.3).

Isolates differed considerably in aggressiveness to *Lilium spp.*, as did the lily genotypes in resistance level to *Fusarium* (Table 5.4 and Table 5.5). Since most isolates were used in both experiments, their relative aggressiveness can be compared. In general the two data sets correlate fairly well ($r = 0.90$, $n = 24$). Isolates belonging to other formae speciales were the least aggressive ones, but they were able to infect the lily scales. All lily genotypes were affected by the fungus. The levels of resistance found in the experiments were in agreement with those mentioned in Table 5.2, except for Yellow Star which was more susceptible than expected. Two standard lily cultivars were included in both experiments. Cultivar

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Connecticut King was resistant in both experiments, while cultivar Aristo was, as expected, the most susceptible one.

Combinations with a large interaction effect ($P < 0.001$), calculated by standardized predictions, are underlined in Table 5.4 and Table 5.5. In experiment 1, the overall susceptible cultivar 'Gelria' is less affected by the isolates Fol-69, Fol-30, and Fog-15 than most overall resistant cultivars (Table 5.4). The same holds for the isolates Foi-7, Fog-15, Fol-30, and Fol-69 on 'White Europe' in experiment 2 (Table 5.5).

Table 5.3 Analysis of deviance of disease ratings from experiment 1 and 2.

experiment	effect	d.f.	deviance	P-value
1	block	1	2	nonsignificant
1	isolate	30	2285	<0.001
1	genotype	8	2047	<0.001
1	isolate.genotype	278	776	<0.001
2	block	1	0	nonsignificant
2	isolate	23	2252	<0.001
2	genotype	11	1190	<0.001
2	isolate.genotype	287	1008	<0.001

In the cluster method, 'Gelria' in experiment 1 and 'White Europe' in experiment 2 are least clustered among the other genotypes. The isolates Fog-15, Fol-30, Fol-69, Fol-15, Foi-7, and Fol-7 are primarily responsible for the interaction in experiment 1 (Figure 5.1). Except for Fol-15, these isolates belong to the least aggressive ones. Fog-15, Foi-7, Fol-30, Fol-69, Fol-79, Fol-7, and Fot-8 are primarily responsible for the interaction accounted in experiment 2 (Figure 5.2), in which they were least aggressive.

DISCUSSION

In this study the existence of races of *Fusarium oxysporum* f.sp. *lilii* was investigated by studying the interaction between a number of fungal isolates with Fusarium susceptible and resistant lily genotypes. The level of interaction between isolates and lily genotypes was determined using a disease rating. Since disease rating data are qualitative and ordinal rather than quantitative and linear, care must be taken when applying statistics. Average disease ratings may not be appropriate since lilies rated 4 are not necessarily twice as diseased as lilies rated 2. Straathof et al. (1993) showed that such categorical data can be analyzed efficiently using a threshold model. With this model, disease severity scores (DSS) can be calculated by transforming the categorical data to an underlying linear scale.

Table 5.4 Disease severity score values of 9 lily genotypes inoculated with 31 isolates of *Fusarium oxysporum* in a scale-assay (n=12), sorted towards decreasing aggressiveness of the isolates (vertically) and decreasing resistance of the genotypes (horizontally). Data from experiment 1.

genotype isolate	PR[x]	YB	NA	MB	CK	OR	SG	GE	AR	aggressive-ness
Fol-11[y]	2.08	3.35	2.87	3.43	2.52	3.81	6.49	6.49	5.57	1.83
Fol-35	2.33	3.50	1.93	3.35	3.09	2.69	4.55	5.80	4.86	1.44
Fol-38	3.06	3.36	1.79	3.20	1.97	2.69	4.25	6.08	5.19	1.39
Fol-40	1.10	2.33	2.13	2.85	2.33	3.72	5.57	5.80	5.02	1.33
Fol-80	2.15	2.32	1.74	3.03	2.33	3.50	5.19	5.20	5.19	1.33
Fol-21	1.79	2.85	2.69	2.33	1.55	2.91	4.70	6.49	5.02	1.26
Fol-42	2.51	2.15	1.55	2.33	1.93	2.85	4.40	8.95	4.70	1.14
Fol-43	1.79	2.33	2.33	2.69	1.96	2.87	4.20	5.37	4.70	1.09
Fol-18	1.94	1.97	1.55	2.33	1.24	2.51	5.02	5.57	4.86	0.98
Fol-9	1.79	1.61	1.89	2.33	2.33	2.52	4.40	4.40	4.55	0.87
Fol-78	1.30	1.93	1.10	2.16	2.33	2.13	3.92	6.08	4.86	0.85
Fol-73	0.37	1.44	1.44	1.96	1.89	2.33	4.86	5.58	4.55	0.75
Fol-36	1.55	1.36	1.74	2.33	1.27	1.96	3.95	5.02	4.86	0.71
Fol-19	1.63	2.15	1.24	1.80	1.80	2.14	3.29	4.40	5.19	0.67
Fol-5	0.37	1.44	2.33	2.15	1.44	2.33	3.95	4.86	4.40	0.64
Fol-15	1.44	1.44	2.52	1.97	1.96	1.97	3.40	3.50	4.70	0.59
Fol-71	1.61	1.44	1.80	1.80	1.44	1.38	3.65	4.86	4.70	0.60
Fol-10	1.10	1.80	1.44	1.44	0.74	1.63	2.87	4.86	5.02	0.42
Fol-33	1.18	1.63	0.74	1.07	0.74	1.93	3.51	4.40	4.86	0.34
Fol-4	0.37	0.74	1.44	1.38	1.30	1.48	4.40	4.35	4.55	0.35
Fol-28	0.18	0.92	0.74	0.37	0.56	2.51	3.03	3.66	4.55	0.02
Fol-3	0.00	0.74	1.10	0.92	1.27	1.46	2.83	3.81	4.25	0.00
Fol-63	-1.51	0.17	0.56	-0.65	1.10	0.92	1.50	3.95	4.40	-0.53
Foi-2	-1.61	-1.32	-0.65	-0.79	1.36	0.55	3.81	5.38	2.91	-0.63
Fot-8	-0.51	-0.64	0.78	0.38	-0.36	0.78	-0.03	2.53	2.68	-0.98
Foi-7	-1.99	-0.65	-0.51	0.77	0.92	0.37	1.39	0.76	2.32	-1.19
Fol-69	-1.50	-0.51	0.18	-0.92	-0.02	-0.65	0.26	[z]-0.77	2.61	-1.59
Fol-30	-1.15	-0.19	0.95	-1.04	0.37	0.40	-0.28	-1.51	1.13	-1.63
Fol-7	-2.28	-0.65	-0.56	-2.45	-0.19	-0.02	1.35	0.73	2.15	-1.66
Fog-15	-2.41	-1.50	-0.77	-1.63	0.37	-1.62	1.50	-2.88	1.97	-2.11
Fol-79	-2.80	-1.64	-1.63	-1.07	-1.51	-1.3	-1.11	1.50	1.36	-2.23
resistance	-0.65	-0.11	-0.09	-0.01	0.00	0.34	1.60	2.16	2.34	

[x]See Table 5.1 for abbreviations of genotype names.
[y]See Table 5.2 for explanation of isolate names.
[z]Significant interactions are underlined.

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Table 5.5 Disease severity score values of 12 lily genotypes inoculated with 24 isolates of *Fusarium oxysporum* in a scale-assay (n=12) and sorted towards decreasing aggressiveness of the isolates (vertically) and decreasing resistance of the genotypes (horizontally). Data from experiment 2.

genotype isolate	CK[x]	VO	DAU	HA	TIG	BA	DAV	JSH	HE	WE	YS	AR	aggresive-ness
Fol-78[y]	0.68	2.72	3.52	4.05	2.93	3.77	4.38	3.84	2.88	4.26	4.72	5.09	1.21
Fol-18	0.90	1.82	1.95	3.99	3.86	3.77	3.69	4.16	3.96	4.16	3.96	4.62	1.11
Fol-43	1.05	1.58	2.84	3.65	3.65	3.68	3.96	2.92	3.67	4.78	3.69	4.72	1.00
Fol-21	0.28	2.44	1.90	3.88	2.49	3.04	3.59	3.51	3.14	3.94	4.38	8.46	0.90
Fol-42	0.42	2.04	2.00	4.29	1.39	3.30	3.90	3.51	3.16	5.34	3.57	5.34	0.88
Fol-40	0.22	1.83	3.34	3.48	3.12	2.47	2.87	3.33	3.04	4.05	3.50	4.89	0.72
Fol-35	0.42	1.92	1.52	3.17	2.30	2.92	2.87	3.14	3.24	4.89	4.14	5.34	0.71
Fol-33	0.00	1.16	1.49	3.32	2.60	3.13	2.73	3.06	3.41	3.79	3.94	4.67	0.55
Fol-71	-0.23	1.15	1.31	2.38	1.99	2.91	2.94	2.75	2.87	3.47	3.33	3.33	0.20
Fol-15	0.42	1.37	1.30	1.00	2.34	2.88	1.72	2.73	2.27	3.76	3.51	3.51	0.09
Fol-11	0.49	1.30	1.00	0.77	1.33	2.12	2.33	2.24	2.16	4.05	3.67	4.17	0.02
Fol-3	0.00	1.31	1.03	0.15	1.87	1.42	1.45	2.66	2.75	3.79	3.76	4.52	0.00
Fol-28	0.00	0.60	0.63	0.84	0.95	3.01	2.34	1.82	2.77	4.27	3.58	4.29	0.03
Fol-4	0.00	1.28	0.22	0.86	2.15	1.81	2.79	3.58	3.06	3.41	3.08	3.47	0.05
Foi-2	0.22	0.60	1.57	0.52	1.65	1.15	2.15	1.45	3.21	3.57	3.13	3.11	-0.23
Fol-5	-0.26	0.42	0.31	1.88	0.90	0.77	2.57	2.32	3.40	2.86	3.10	3.01	-0.25
Fol-63	-0.26	0.00	0.47	0.53	1.05	0.39	1.19	2.00	1.93	3.68	1.85	3.49	-0.60
Foi-7	-0.57	-1.40	-0.17	-0.57	-0.87	-0.88	-0.33	2.53	2.44	[z]-2.55	2.66	3.01	-1.37
Fog-15	0.00	-1.93	0.3	0.28	1.00	-0.26	0.95	1.16	1.71	-2.13	3.14	1.70	-1.38
Fol-7	-0.57	0.00	0.15	-0.57	0.28	-0.26	1.67	0.28	0.04	0.68	0.77	0.22	-1.57
Fol-30	-0.57	-0.26	0.31	-0.73	-0.57	-0.25	0.49	-0.77	0.22	-2.46	-0.26	2.04	-1.97
Fot-8	-0.57	-1.16	0.31	-1.4	-0.57	0.28	0.24	0.28	0.02	-0.83	0.00	0.14	-2.00
Fol-69	-0.57	-0.88	-0.57	-1.20	0.00	0.00	-0.30	-1.46	-0.57	-2.46	-0.57	0.85	-2.25
Fol-79	-1.83	-0.57	-1.73	-2.24	-0.57	-1.40	-1.05	-0.57	-0.57	-0.39	1.05	-1.53	-2.48
resistance	0.00	0.61	0.90	1.23	1.25	1.42	1.71	1.76	1.88	2.10	2.35	2.78	

[x]See Table 5.1 for abbreviations of genotype names.
[y]See Table 5.2 for explanation of isolate names.
[z]Significant interactions are underlined.

No major break-down of resistance was detected in this study. With all isolates, resistant genotypes were less affected than the susceptible controls. If the interaction would follow a gene-for-gene relationship in which major resistance genes and (a)virulence genes are involved, virulent isolates would assumedly incite disease levels in compatible 'resistant' genotypes to the same extent as in susceptible genotypes, which is clearly not the case in the present study. A gene-for-gene relationship, however, might also exist at minor gene level, as argued by Parlevliet & Zadoks (1977). Adaptation of the fungus to one or more minor resistance genes would result in strains differing slightly from one another in their virulence. The minor interactions found in both experiments point in this direction.
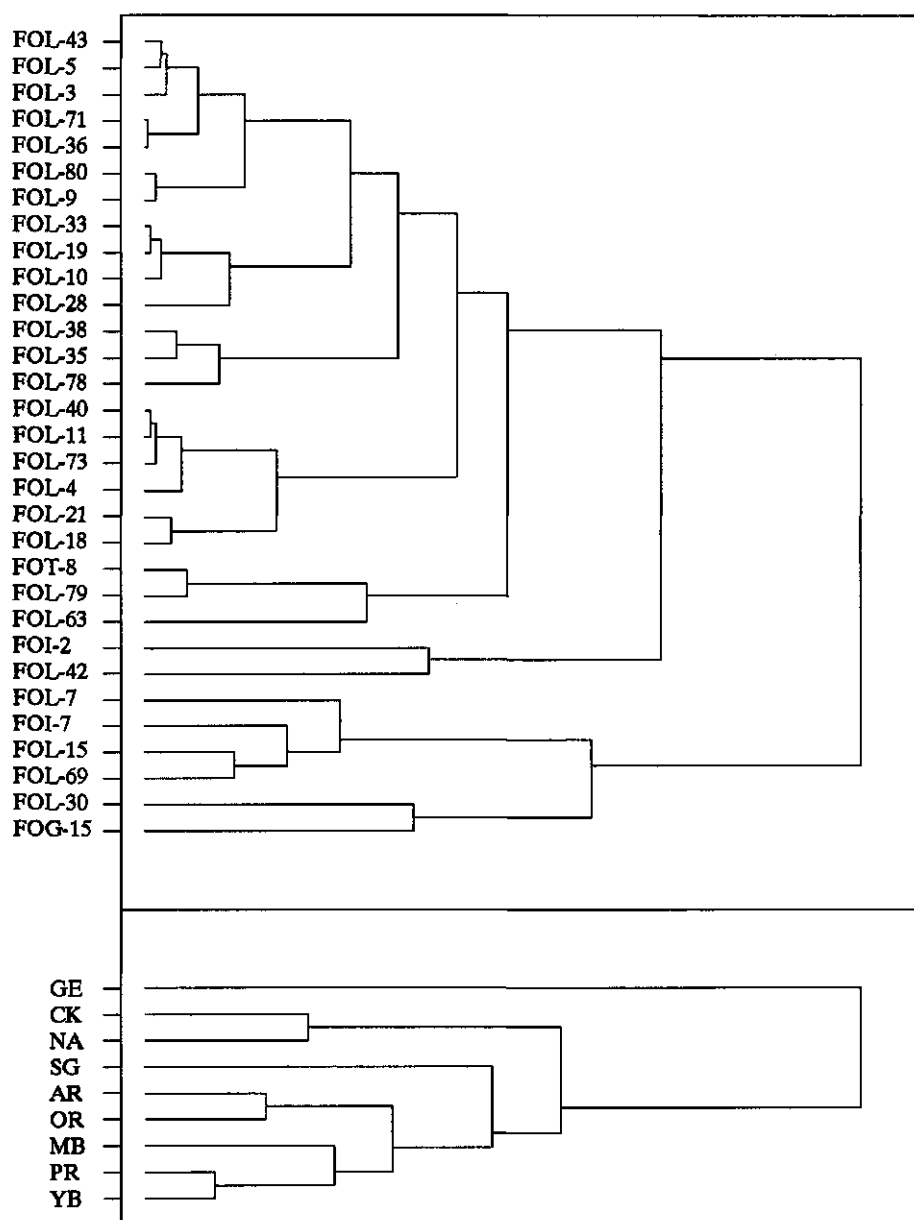
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Figure 5.1 Dendrogram produced by cluster analysis of fungal isolates and host cultivars according to Corsten & Denis (1990). The analysis was performed on disease severity score values from experiment 1. Isolates and cultivars are clustered successively according to similarity exclusively in terms of minimal contribution to variance for interaction. See Table 5.1 and Table 5.2 for abbreviations of *Fusarium*-isolate and lily-genotype names, respectively.
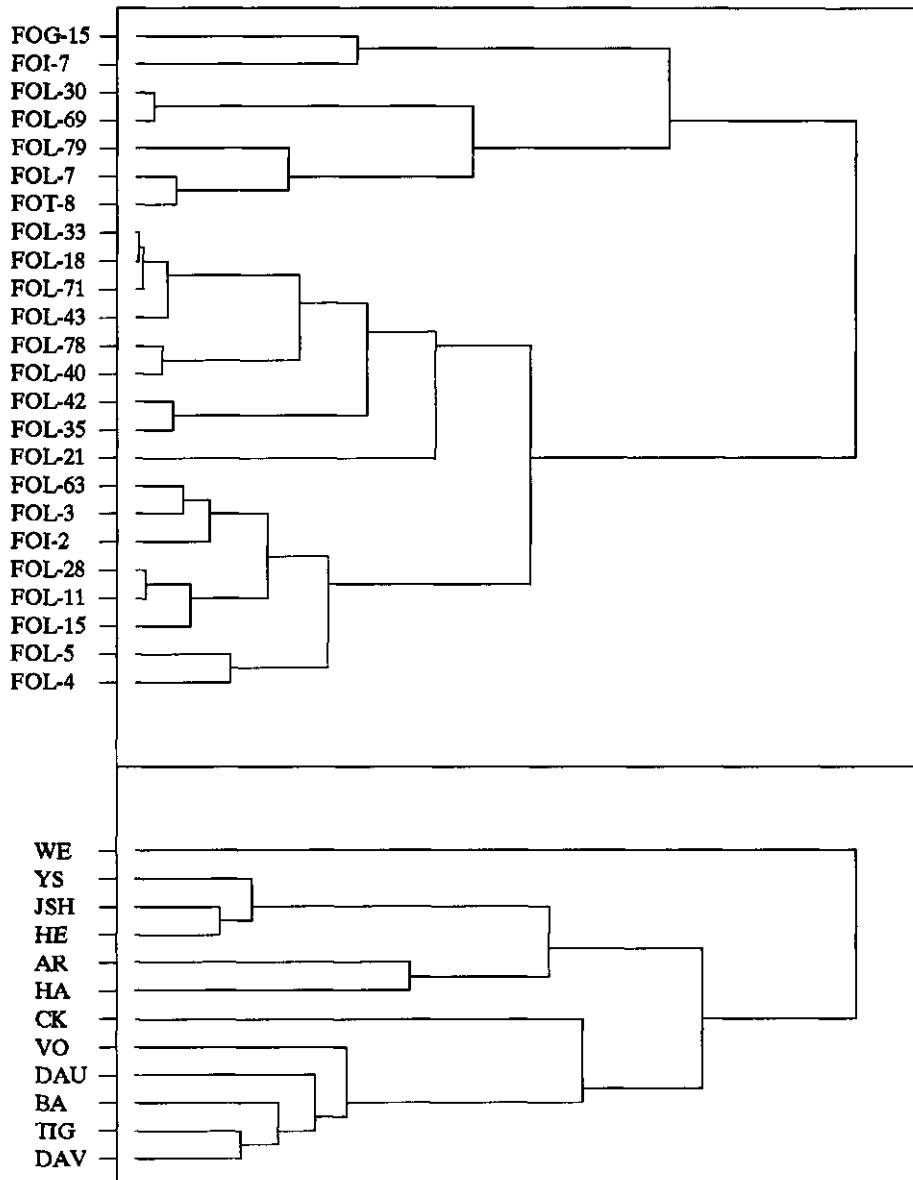
Figure 5.2 Dendrogram produced by cluster analysis of fungal isolates and host cultivars according to Corsten & Denis (1990). The analysis was performed on disease severity score values from experiment 2. Isolates and cultivars are clustered successively according to similarity exclusively in terms of minimal contribution to variance for interaction. See Table 5.1 and Table 5.2 for abbreviations of *Fusarium*-isolate and lily-genotype names, respectively.

In both experiments the interaction can mainly be ascribed to the least aggressive isolates. As argued by Straathof et al. (1993) the interaction might be partly due to a bias in the results, caused by the limitations of the ordinal observation scale. Weakly aggressive isolates can only affect any lily genotype slightly and thus will necessarily show less contrast between resistant and susceptible lily genotypes than highly aggressive isolates. This bias does not explain all interactions, however, since some inversions were found. The isolates Fol-30, Fol-69, Fog-15 and to some extent Foi-7 affected the generally susceptible cultivars 'Gelria' and 'White Europe' less than expected (Table 5.4; Table 5.5). Apparently these cultivars possess some specific resistance to these isolates. Similar results were obtained by Löffler & Rumine (1991) for the combination of 'Gelria' and Fol-30 (in earlier work designated as Fol-C). Since 'Gelria' and 'White Europe' are the only *L. longiflorum* cultivars included in the experiments, it is likely that *L. longiflorum*, which is quite distinct from the Oriental and Asiatic hybrid lilies, differs in specific resistance to these particular isolates. The observed interactions, however, were relatively small and only occurred in isolates with rather low aggressiveness. Therefore the biological significance of the phenomenon seems to be limited.

Cluster analyses as proposed by Corsten & Denis (1990) separated the *Fusarium* isolates and the lily genotypes into different groups (Figure 5.1; Figure 5.2). In experiment 1, the most deviating isolate group comprised Fog-15, Fol-30, Fol-69, Fol-15, Foi-7, and Fol-7, and the lily 'Gelria' deviated from the other lily genotypes. Three of these isolates (Fog-15, Fol-30, and Fol-69) showed inversions with 'Gelria' (Table 5.4). In experiment 2, isolates Fog-15, Foi-7, Fol-30, Fol-69, Fol-79, Fol-7, and Fot-8 were clustered separately whereas lily 'White Europe' differed from the remaining lily genotypes. Apart from Fol-79, Fol-7 and Fot-8, the other four isolates interact specifically with 'White Europe' (Table 5.5). In general, therefore, isolates exerting specific interactions with *L. longiflorum* cultivars tended to cluster. Except for Fol-15, all these isolates belong to the least aggressive ones. At present it can not be judged, however, whether groups of isolates, discriminated on base of their interaction patterns, are genetically distinct. Fog-15 and Foi-7 are related since they both belong to the same race in the formae specialis *'gladioli'* and share common RFLP-patterns (Roebroeck & Mes, 1991; Mes et al. 1994). Fol-30 and Fol-69 may be related to each other since they both originate from Italy. To elucidate whether these isolates might be related to those of f.sp. *gladioli*, further characterization of the isolates is necessary. Determination of vegetative compatibility among isolates (Aloi & Baayen, 1993; Roebroeck & Mes, 1992) and evaluation of DNA restriction fragment length polymorphisms (Manicom & Baayen, 1993; Mes et al., 1994) are suitable techniques for this purpose and will be carried out in the future.

None of the deviating isolates led to a higher disease incidence than observed with highly aggressive isolates in the same cultivar. Therefore lily genotypes, selected for

resistance to highly aggressive isolates, will not be affected by any of the deviating isolates. Thus for practical purposes it will be sufficient to screen genotypes against one well-characterized aggressive isolate.

As could be expected a large genotype effect was found. This is apparently due to the fact that in both experiments susceptible controls were included. Even the most resistant cultivars were affected by the fungus in this study. This is partly due to the use of bulb scales in the screening test. The wounded base of detached scales forms an easy entrance for the fungus and thus scales will be affected more than bulbs (Straathof & Löffler, 1994). Moreover, it confirms the partial character of the resistance. The level of resistance found in both experiments correlates well with the levels originally found and reported elsewhere (Straathof et al., 1993; Straathof & Van Tuyl, 1994).

A large variation in aggressiveness of the isolates was found. In general, the relative aggressiveness of the two data sets correlate fairly well (r = 0.90). Some discrepancies between the two data sets may be due to differences in the infection pressure (Straathof & Inggamer, 1992).

Isolates belonging to other formae speciales were able to infect lily as shown before (Löffler and Mouris, 1993). Some cross-infection thus does occur as previously reported by Valásková (1976) for freesia. Those findings indicate that the concept of formae speciales may have to be re-evaluated for the rot-producing forms. Phylogenetic analyses, for example based on DNA patterns, using appropriately conserved areas of the genome may be necessary to solve the question raised above.

In conclusion, specific interactions were found between lily genotypes and isolates of *F. oxysporum*, suggesting the existence of physiological races. On the other hand, isolates from lily may as well in fact belong to distinct formae speciales, in which case the previous conclusion is not (yet) justified. At any rate, differences among the isolates in virulence are relatively small and seem to be of limited biological significance. The use of a single highly aggressive isolate in screening tests should suffice in breeding for resistance in lily to basal rot.

ACKNOWLEDGMENT

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Screening for *Fusarium* resistance in seedling populations of Asiatic hybrid lily

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ABSTRACT

A test to select Fusarium *resistant seedlings of the Asiatic hybrid lily is described. Young seedlings of 28 populations, obtained from an incomplete diallel between eight parents with different levels of* Fusarium *resistance, were tested for resistance. Significant differences in* Fusarium *resistance between and within populations were detected. The average percentage of selected seedlings ranged from 34 % in resistant x resistant crosses to 2 % in susceptible x susceptible crosses. Although resistant descendants were obtained in susceptible x susceptible crosses, using at least one resistant parent produced higher percentages of resistant seedlings. The resistance level of the parents correlated highly with the general combining ability for* Fusarium *resistance based on the seedling test. For eight populations, seedlings selected for* Fusarium *resistance and non-tested (control) seedlings of the same cross were compared, after propagation, in a clonal test. Variation between and within populations, found at seedling level, was confirmed at clonal level. A positive selection response was found for all eight populations. In the seedling test, approximately 18 % of the seedlings were selected as resistant of which 15 % (2.7 % of seedlings tested) appeared to be susceptible escapes. Comparison between selection at seedling level and at clonal level indicated that approximately 25 % of the seedlings tested were missed (rejected resistant plants) in the seedling test. The practical use of a seedling test for* Fusarium *resistance in lily breeding programmes is discussed.*

Keywords: Diallel analysis, general combining ability, *Fusarium oxysporum* f.sp. *lilii, Lilium*, seedling test, selection response

INTRODUCTION

Lily (*Lilium* L.) is one of the most important flower bulb crops in the world and subject of many breeding programmes. During cultivation of seedlings, selections are carried out for bulb growth and morphological characteristics, e.g. flower colour and plant shape. The time period from seed to a commercial (adult) bulb takes at least two years. Selected plants are propagated vegetatively by scale bulblet induction either in vivo or in vitro and tested for additional traits at clonal level. It takes another two years to obtain a clone of commercial bulbs from scale bulblets. Resistance to diseases has become an important genetic trait, because chemical control of pathogens has to be reduced to limit environmental pollution. *Fusarium oxysporum* f.sp. *lilii* Imle, a soil-borne fungus, is one of the most serious pathogens of lily. Variation in *Fusarium* resistance is present in some cultivars and *Lilium* species (Imle, 1942a; Imle 1942b; Straathof & Van Tuyl, 1994). Screening tests

have been developed, but they require cloned material to estimate the resistance level of the cultivars (Straathof & Löffler, 1994). In the scale test (Smith & Maginnes, 1969; Löffler & Mouris, 1989; Straathof & Inggamer, 1992) and the scale bulblet test (Straathof et al., 1993) clones can consist of scales or scale bulblets obtained from a single commercial bulb. Commercial bulbs, however, are necessary and clonal tests can only be performed several years after the initial crossing.

Clones are very useful in a screening test because the resistance level can be determined more precisely when compared to an individual test. Selection of individual plants, however, would result in a more efficient breeding program. If young seedlings are used, tests can be performed in the first year and *Fusarium* susceptible plants can be discarded before the production of commercial bulbs. Two criteria have to be met for a useful seedling test. First, the *Fusarium* resistance in seedling stage and bulb stage must be expressed equally. Second, the seedling test at individual level must be accurate. Since environmental variation occurs (Straathof et al., 1993), it is expected that some resistant seedlings will be discarded (missings) and some susceptible plants selected (escapes). For an efficient selection process misclassifications of both types must be minimized.

Testing lilies at seedling level was performed by Imle (1942a; 1942b) who found that seedlings of all species investigated were highly susceptible. Seedling selection in flower bulbs for *Fusarium* resistance is carried out successfully in tulips (Van Eijk et al., 1979; Van Eijk & Eikelboom, 1983) and daffodils (Bowes et al., 1992).

If a seedling test is carried out with populations obtained from an (incomplete) diallel, the inheritance in terms of general and specific combining ability can be estimated. Those values can be used to predict the breeding value for *Fusarium* resistance of a parent and in some cases the number of genes involved can be estimated.

The objective of this research was to develop a seedling test for early selection of *Fusarium* resistance in lilies. The efficiency of the seedling test and its practical implications in breeding programmes were determined. The inheritance of the resistance was studied in terms of general and specific combining ability.

MATERIALS AND METHODS

Seedling test

In 1989, crossings were made between eight Asiatic hybrid lily cultivars (Table 6.1) with known *Fusarium* resistance level (Straathof et al., 1993). In 1990, seeds of 28 populations (Table 6.1), including 12 pairs of progenies from reciprocal crossings, were sown in flat trays with soil. For each population, 150 - 600 seeds were used. Trays were placed in a greenhouse at ± 17/15 °C (16 h day / 8 h night) with supplemental light. After 19 weeks, the young seedlings were placed at 5 °C in dark

for 9 weeks. Bulblets of eight populations from different resistance combinations were harvested and divided into two parts. They were either transferred to non-infested (control) or to *Fusarium* infested soil (tested). All bulblets of the remaining 20 populations were transplanted into infested soil.

Table 6.1 Crossing diagram of an incomplete diallel between eight Asiatic hybrid lily cultivars with different levels of *Fusarium* resistance.

cultivar	resistance level[z]	AR	CK	ES	HI	MB	OR	PI	PR
Aristo (AR)	susceptible	*	*	*		*			
Connecticut King (CK)	resistant	*		*		*	*	*	*
Esther (ES)	susceptible	*	*			*			
Hilde (HI)	moderately resistant					*			
Mont Blanc (MB)	resistant	*	*	*	*		*		*
Orlito (OR)	resistant		*			*		*	
Pirate (PI)	susceptible	*	*	*			*		
Prominence (PR)	resistant		*						

[z]Straathof et al. (1993).

Commercial bulbs of the eight parents were scaled. Scales were placed in perforated plastic bags with moist vermiculite at 25 °C for eight weeks to induce scale bulblets. This was followed by four weeks at 17 °C and eight weeks at 5 °C (Straathof et al., 1993). Scale bulblets, which were a little larger in size, and seedling bulblets, were planted simultaneously in *Fusarium* infested soil.

The seedling test was performed 28 weeks after sowing in a climate controlled greenhouse at 17 °C (day / night). Seedling bulblets and parental scale bulblets were planted in two large benches. Populations and parents were planted in rows apart. The control seedling bulblets of the eight populations were also planted in the two benches, but separated from the infested soil by a glass plate. Rows consisted of maximal 25 plants and were randomly dispersed over the two benches. The number of seedlings tested per population was dependent on the number of seedlings obtained (Table 6.2). A total of 3371 seedlings were tested. The control part of eight populations consisted of 50 (reciprocal present) or 100 (no reciprocal present) seedlings per population, 500 seedlings were used as controls. Scale bulblets of the eight parents were tested in 50-fold.

Seedlings and parental bulblets were harvested after five weeks to be able to readily determine resistant and susceptible individuals. After harvesting, the disease severity was measured using a disease rating scale. Decay of the infested plant material was rated visually according to an ordinal scale with five categories: 1 = healthy; 2 = slightly rotten; 3 = moderately rotten; 4 = heavily rotten; and 5 = completely decayed. The selected seedling bulblets with a disease rating 1 or 2 and all control seedlings were disinfected in captan (Captan Flow; 1.0 %) and prochloraz (Sportak;

0.2 %) and stored at 0-2 °C in plastic bags with moist peat.

Propagation and clonal test
In 1991 and 1992, all selected and control seedling bulblets were disinfected and planted separately in pots with soil and grown in a greenhouse for 8 and 5 months, respectively. The commercial bulbs obtained and commercial bulbs of the eight parents were scaled to induce scale bulblets as described above.

Scale bulblets were tested in *Fusarium* infested soil at clonal level in 1993 for nine weeks in a temperature controlled greenhouse at 18/14 °C (16 h day / 8 h night). In the clonal test, all selected and control seedlings and parental scale bulblets were tested in decuple. The ten scale bulblets of each genotype were placed in two pots with five plants each. A pot of each genotype was placed in a block. Disease severity was measured using the disease rating scale as described above.

Fungus
Two highly aggressive isolates of *Fusarium oxysporum* f.sp. *lilii* (CPRO-Fol4 and CPRO-Fol11) (Löffler & Mouris, 1989) were used in the seedling and clonal test. Inoculum was prepared and soil was infested as described by Straathof et al. (1993). The number of propagules was determined at planting time which was two weeks after infestation of the soil. The inoculum concentration was $\pm\ 7.10^4$ and $\pm\ 1.10^4$ propagules per gram of soil in the seedling (1990) and clonal (1993) test, respectively.

Statistical analysis
Disease ratings were analyzed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990), using a probit link function. For each population or clone within an experiment, a disease severity score (DSS) was calculated with the threshold model. This score may be considered as a transformed average value of the disease ratings of each population or clone on an underlying linear scale (Straathof et al., 1993). Conclusions concerning block and population effects were assessed by analysis of deviance (McCullagh & Nelder, 1989). Deviances were compared with the table of the chi-squared distribution. The computer package Genstat (Payne et al., 1987) was used to carry out calculations. General combining abilities (GCA's), specific combining abilities (SCA's) and reciprocal effects were calculated using the DSS values of the 28 populations in the seedling test. Conclusions concerning GCA, SCA and reciprocal effects were assessed by analysis of deviance.

For an accurate individual seedling test, the selection of susceptible plants (escapes) and the rejection of resistant plants (missings) must be minimal. The percentage of escapes and missings was calculated by the following formulas (symbols presented in Figure 6.1)

Escapes: %sel - %sr
Missings: %cr - %sr

were:

%sel = percentage plants selected as resistant at seedling level
%sr = percentage plants selected as resistant at seedling level and clonal level
(relative to the original number of seedlings)
%cr = percentage plants selected as resistant at clonal level

Because severely diseased lily seedlings (disease rating > 2) could not be kept alive, the percentage of missings could only be estimated in comparison with the control seedlings.
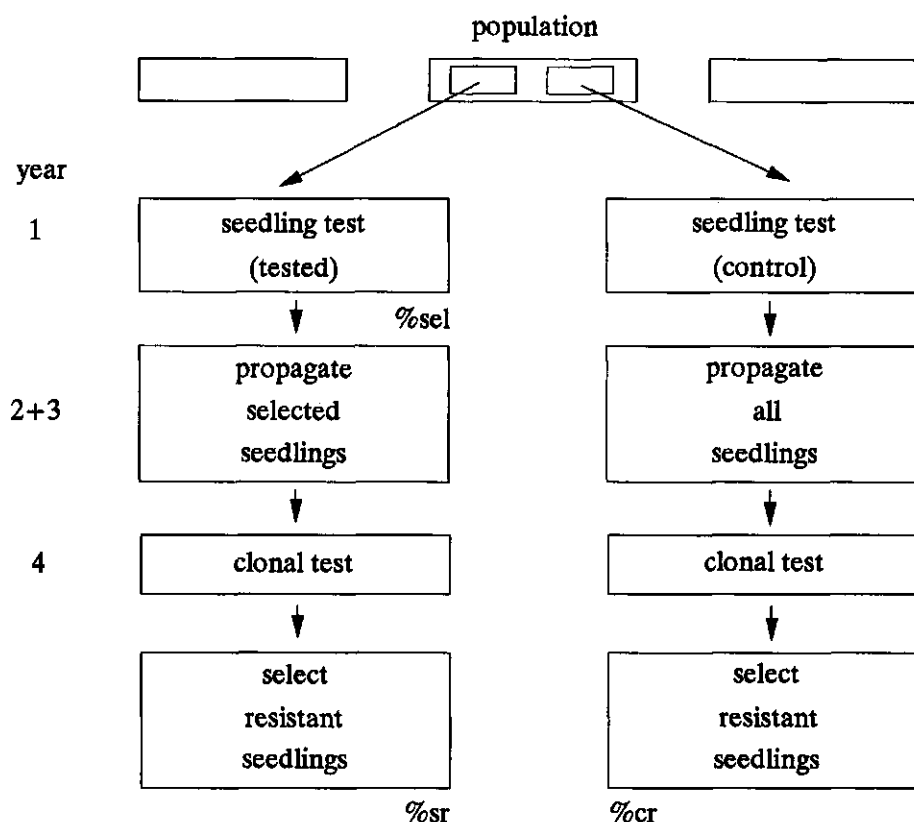

Figure 6.1 Experimental design of the *Fusarium* lily seedling test. See materials and methods for explanation of symbols.

RESULTS

The disease severity score (DSS) values per population of the tested seedlings is presented in Table 6.2. The analysis of deviance showed a clear population effect ($P < 0.001$). All populations obtained from two resistant parents were more resistant than populations obtained from one resistant and one susceptible parent, and the latter populations were more resistant than populations obtained from two susceptible parents. The control seedlings were all healthy (disease rating = 1) and no DSS values were calculated for those groups.

Table 6.2 Number of individual seedlings tested for *Fusarium* resistance at seedling stage, disease severity score values (DSS) and corresponding standard error of differences (s.e.d.; refer to differences with OR x CK) of 28 Asiatic hybrid lily populations originating from parents with various levels of *Fusarium* resistance.

♀	♂	cross[y]	no. of seedlings	DSS	s.e.d.
Connecticut King	Mont Blanc	RxR	110	1.27	0.13
Orlito	Connecticut King	RxR	171	1.38	-
Mont Blanc	Prominence	RxR	200	1.38	0.11
Prominence	Connecticut King	RxR	111	1.38	0.13
Mont Blanc	Orlito	RxR	97	1.41	0.13
Connecticut King	Orlito	RxR	150	1.50	0.12
Mont Blanc	Connecticut King	RxR	125	1.57	0.12
Mont Blanc	Hilde	RxM	100	1.60	0.13
Orlito	Mont Blanc	RxR	69	1.61	0.14
Hilde	Mont Blanc	MxR	119	1.74	0.13
Connecticut King	Prominence	RxR	108	1.95	0.13
Esther	Connecticut King	SxR	193	2.06	0.11
Pirate	Orlito	SxR	86	2.15	0.14
Aristo	Connecticut King	SxR	64	2.21	0.16
Orlito	Pirate	RxS	32	2.24	0.21
Pirate	Connecticut King	SxR	72	2.41	0.15
Connecticut King	Pirate	RxS	94	2.45	0.14
Aristo	Mont Blanc	SxR	13	2.45	0.31
Connecticut King	Esther	RxS	175	2.49	0.12
Mont Blanc	Esther	RxS	83	2.76	0.15
Esther	Mont Blanc	SxR	75	2.79	0.16
Mont Blanc	Aristo	RxS	309	2.80	0.10
Connecticut King	Aristo	RxS	119	2.86	0.13
Pirate	Aristo	SxS	210	3.15	0.12
Aristo	Esther	SxS	13	3.16	0.35
Esther	Aristo	SxS	213	3.18	0.12
Pirate	Esther	SxS	185	3.22	0.12
Aristo	Aristo	SxS	75	3.61	0.18

[y]Resistance levels: R = resistant, M = moderately resistant, and S = susceptible.

The distribution of the disease ratings for *Fusarium* resistance between and within four different resistance combinations is presented in Figure 6.2. In resistant x resistant as well as susceptible x susceptible combinations, seedlings were rated in all five classes. In the resistant x resistant combinations, an average of 34 % of the seedlings were selected (disease rating 1 + 2); while in resistant x moderate resistant combinations only 22 %. In resistant x susceptible combinations and in susceptible x susceptible combinations only 8 % and 2 % of the seedlings, respectively, were selected.
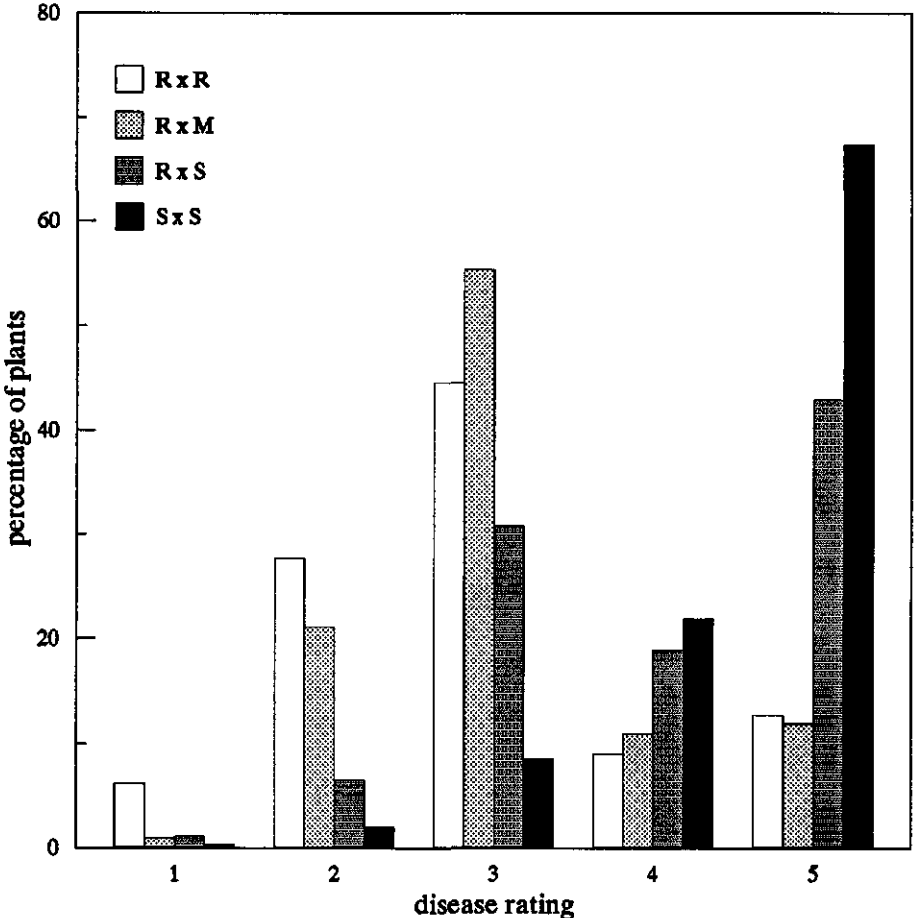

Figure 6.2   Average distribution of disease ratings of Asiatic hybrid lily seedlings from four different resistance combinations in the *Fusarium* seedling test. Resistance levels: R = resistant, M = moderately resistant, and S = susceptible.

The diallel analysis gave significant GCA (deviance = 204.7 with 7df; $P < 0.001$),

SCA (deviance $= 67.4$ with 8df; $P < 0.001$) and reciprocal effects (deviance $= 39.2$ with 12df; $P < 0.001$). The GCA value of each parent and the DSS values of the parents tested as scale bulblets in 1990 is presented in Table 6.3. Both were highly correlated ($r = 0.94$). The GCA values of 'Hilde' and 'Prominence' are only based on two, respectively, three cross combinations and are, therefore, less reliable. In Figure 6.3, the DSS values for the seedling populations was plotted against the DSS value per population calculated for GCA effects only. Vertical distances between each point and the line represent deviations from additivity. Although deviations from the additive model are highly significant, the additive model explains almost all variation between crosses.

Table 6.3   Disease severity score (DSS) and corresponding standard error of differences (s.e.d.; refer to differences with 'Connecticut King') of Asiatic hybrid lily cultivars tested for *Fusarium* resistance at scale bulblet level and corresponding general combining abilities (GCA) obtained from an incomplete diallel tested at seedling level.

cultivar	DSS	s.e.d.	GCA	s.e.d.
Mont Blanc	-0.23	0.23	0.05	0.10
Connecticut King	-0.16	-	-0.03	-
Orlito	0.27	0.23	0.00	0.10
Prominence	0.60	0.22	-0.01	0.10
Hilde	1.14	0.22	0.09	0.14
Esther	2.53	0.23	0.83	0.09
Pirate	2.84	0.23	0.74	0.11
Aristo	3.02	0.24	1.12	0.09
Grand mean			1.62	

Over all 28 populations, only 16 % (550/3371) or 18% (261/1446), if the same eight populations as in the control were considered, of the tested seedlings were selected as resistant plants. Those plants and all 500 control seedlings were cultivated for two years. The control seedlings produced in 59 % (294/500) commercial bulbs. The average percentage of commercial bulbs obtained from all the *Fusarium* resistant selected seedlings was 56 % (307/550) and 60% (157/261), if the same eight populations as in the control were considered. The fall out per population was independent from the resistance level of the population.

The DSS values of the eight populations with selected and control seedlings screened at clonal level is provided in Table 6.4. Significant population ($P < 0.001$), but no block effect, were detected. The DSS values of the selected populations was always lower than the corresponding DSS value of the control populations. This indicated a positive selection response for all eight populations. The DSS values of the eight control populations at clonal level corresponded with those found in the seedling test except for the progeny of the cross 'Mont Blanc' x 'Connecticut King', which was much more susceptible in the clonal test.
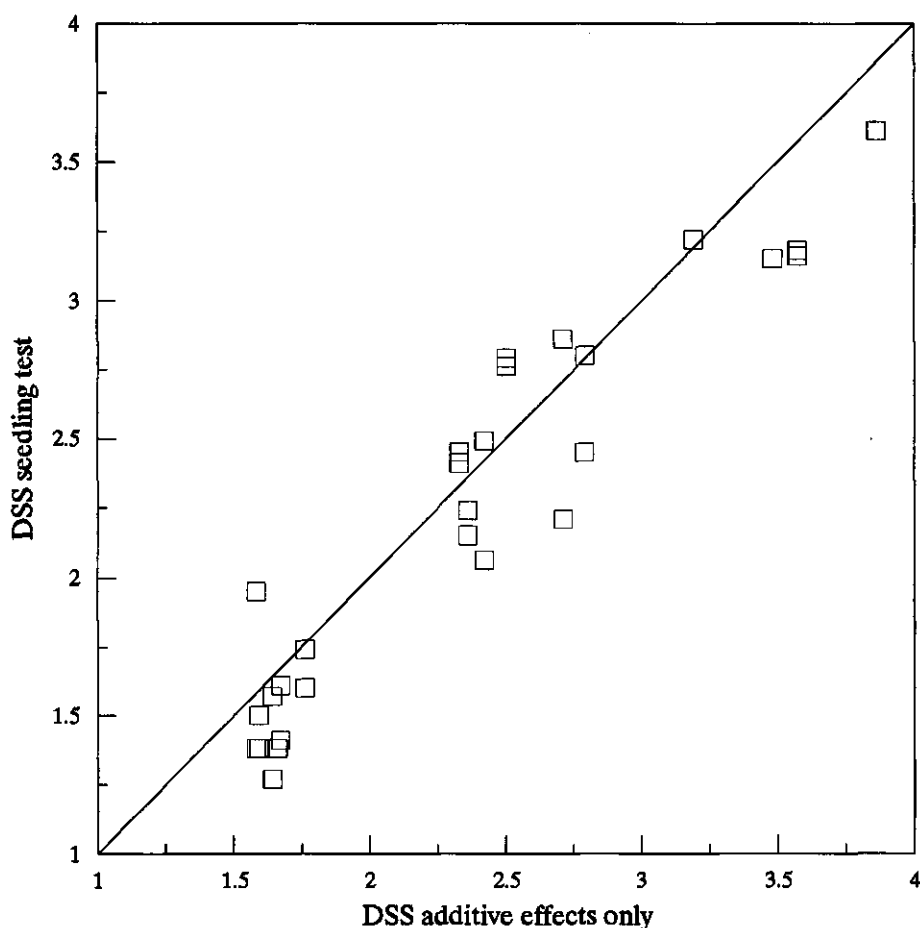
Figure 6.3 Relation between DSS values obtained after the *Fusarium* seedling test for 28 Asiatic hybrid lily populations and the DSS values calculated with only additive (GCA) effects.

The percentage of resistant plants at clonal level depended on the selection criterion applied. This selection criterion must be in agreement with practical standards. 'Hilde' is a moderately resistant genotype (Table 6.3; Straathof et al., 1993) and can be used for a minimum level of resistance. Genotypes were considered to be resistant if their DSS value at clonal level was lower or equal to the DSS value of 'Hilde'. The resulting percentage of resistant genotypes of the eight populations (Table 6.4) again showed a positive selection response between control and selected populations. The total percentage of resistant clones in eight control populations was 41 % (120/294), while in the selected group 85 % (133/157) were resistant and 76 % (233/307), if all 28 populations were considered (data not shown).

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Table 6.4 Disease severity score (DSS) and corresponding standard error of differences (s.e.d.; refer to differences with OR x CK) and percentage of resistant clones of eight Asiatic hybrid lily populations selected for *Fusarium* resistance at seedling level or as control tested at clonal level in scale bulblet stage.

♀	♂	cross[w]	control			selected		
			DSS	s.e.d.	%resistant[x]	DSS	s.e.d.	%resistant[x]
CK[y]	OR	RxR	-0.08	0.08	89	-0.35	0.08	98
OR	CK	RxR	0.19	-	71	0.05	0.08	88
CK	MB	RxR	0.53	0.09	40	0.03	0.09	79
CK	ES	RxS	0.56	0.08	37	0.24	0.19	75
ES	CK	SxR	0.64	0.08	33	-0.11	0.12	85
MB	AR	RxS	0.88	0.08	23	0.49	0.21	33
ES	AR	SxS	1.02	0.08	9	*[z]	*	*
MB	CK	RxR	1.06	0.08	23	0.18	0.10	61
(total					41			85)

[w]Resistance levels: R = resistant, M = moderately resistant, and S = susceptible.
[x]Disease severity score smaller or equal to DSS value of 'Hilde'.
[y]See Table 6.1 for abbreviations of cultivar names.
[z]All plants died during propagation.

Table 6.5 Percentages of plants selected for *Fusarium* resistance at seedling level and at clonal level and percentage escapes and missings of eight Asiatic hybrid lily populations.

♀	♂	cross[w]	%sel[x]	%sr[x]	%cr[x]	%escapes %sel-%sr[x]	%missings %cr-%sr[x]
CK[y]	OR	RxR	40.0	39.1	89.5	0.9	50.4
OR	CK	RxR	39.8	35.0	70.7	4.8	35.7
CK	MB	RxR	46.4	36.7	40.0	9.7	3.3
CK	ES	RxS	5.7	4.3	36.6	1.4	32.3
ES	CK	SxR	10.9	9.2	33.3	1.7	24.1
MB	CK	RxR	30.4	18.6	23.3	11.8	4.7
MB	AR	RxS	3.2	1.0	23.1	2.2	22.1
ES	AR	SxS	1.4	*[z]	9.1	*	*
(total			18.1	15.4	40.8	2.7	25.4)

[w]Resistance levels: R = resistant and S = susceptible.
[x]See materials and methods for abbreviations and explanation of formulas.
[y]See Table 6.1 for abbreviations of cultivar names.
[z]All plants died during propagation.

The DSS value of the selected populations was not constant and the percentage of resistant seedlings was not 100 % (Table 6.4), which suggested escapes (selected susceptible seedlings). The 41 % of resistant plants in eight control populations compared to 18 % of resistant plants selected at seedling level in the same eight populations suggested missings (rejected resistant seedlings). The percentages of escapes and missings of the eight populations calculated according to the formula's

in materials and methods are presented in Table 6.5. Over the eight populations, 2.7 % of the seedlings tested were abusively considered to be resistant and selected. Whereas, 25.4 % were abusively considered to be susceptible and discarded.

Evidently, the percentage of escapes and missings depends on the selection criteria applied. If a more resistant criterion (lower DSS value) is used, the percentage of escapes will be higher. Whereas, the number of missings will decrease. The percentage of escapes and missings, as average of eight populations, was calculated for a range of selection criteria (Figure 6.4).
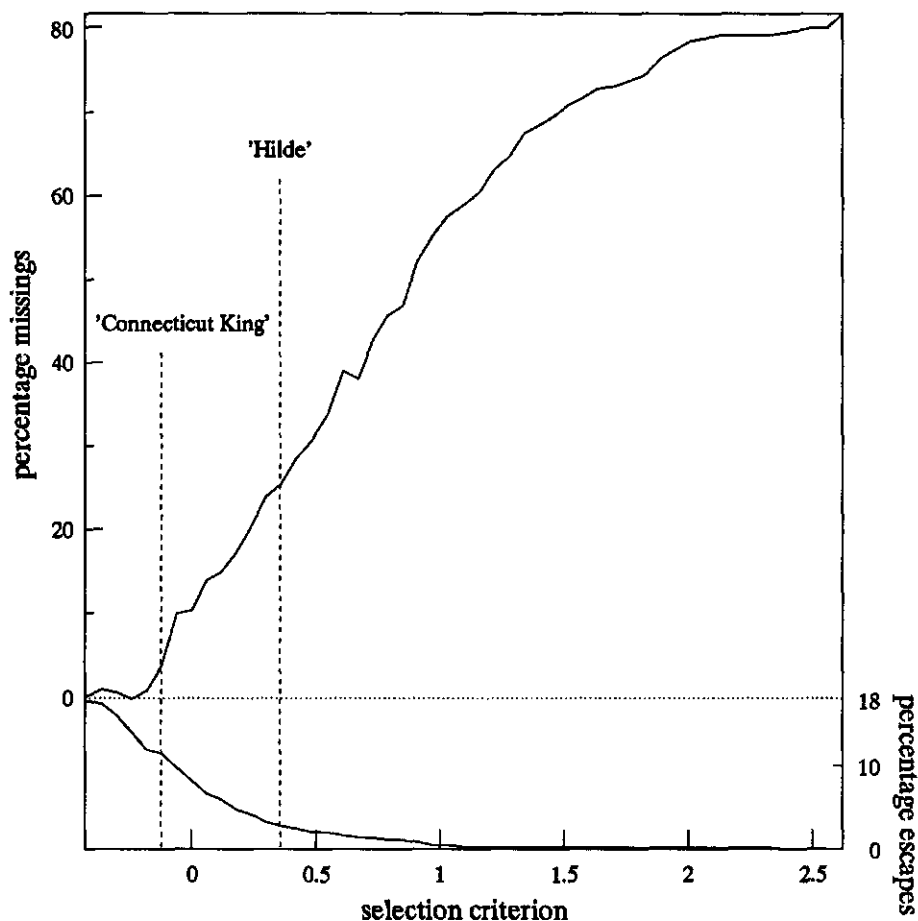
Figure 6.4 Relation between the percentage of escapes and missings, as average of eight Asiatic hybrid lily populations, and the selection criterion (DSS values). Dotted line represent the 18% seedlings selected as resistant (disease rating 1 + 2) in the seedling test. Percentage of escapes varied between 0 and 18 and percentage of missings between 18 and 82 of the number of seedlings tested at seedling level. Disease severity score values of 'Hilde' and 'Connecticut King' are presented as selection criteria.

DISCUSSION

Variation between and within populations was detected at seedling level (Figure 6.2) and confirmed at clonal level (Table 6.4). Most of the resistant descendants were obtained in resistant x resistant crosses. In the susceptible x susceptible crosses, however, some resistant descendants were found. Therefore, cross breeding provides possibilities for developing resistant cultivars. Although resistant descendants were obtained in susceptible x susceptible crosses, using at least one resistant parent resulted in higher percentages of resistant seedlings and is, therefore, recommended in Asiatic hybrid lily breeding strategies.

When selected and control seedlings of the same cross were compared in a clonal test (Table 6.4), a positive selection response was found in the DSS values of a population and in the percentage of resistant plants. *Fusarium* resistance of the populations was in agreement with the resistance level of corresponding parents (Table 6.2). This suggests an equal expression of *Fusarium* resistance in seedling and bulb stages. Selection for *Fusarium* resistance at seedling level appears, therefore, feasible. A part of the seedlings, however, were misclassified, i.e. escapes and missings (Table 6.5).

The ratio of escapes and missings depends on the selection criterion "resistant" in the seedling and clonal tests. In the seedling test all plants which survived after infestation (disease rating 1 and 2) were selected. Seedlings, which were selected with a disease rating 1, had similar resistance levels at clonal level as seedlings selected with disease rating 2 (data not shown). In the clonal test, the criterion for resistance is more complex. If the resistance of 'Connecticut King' is used as a selection criterion, fewer genotypes will be considered resistant than if the resistance level of 'Hilde' is used. Since the resistance of 'Hilde' meets the minimal requirements of practical cultivation, this level was chosen as criterion.

The low percentage of escapes (2.7 %) compared to the number of tested seedlings, demonstrated that the testing conditions at seedling level were strong enough to minimize the selection of susceptible plants. Compared to the number of selected plants, the percentage of escapes was 15. Therefore, retesting at clonal level is necessary. The high percentage of missings (25.4 %), demonstrated that the testing conditions at seedling level were too strong to minimize the rejection of resistant plants. For practical purposes, missings are of less importance in case sufficient resistance is available in a population. In populations with only a few resistant descendants, missings must be prevented. Since the selection criterion at seedling level can not be reduced, decrease of the percentage of missings can only be achieved by a seedling test with a lower disease pressure, e.g., lower inoculum concentrations, lower temperatures or shorter duration of the experiment (Straathof & Inggamer, 1992).

The conditions in the seedling test were comparable with those in the scale bulblet

test. Seedling bulblets, however, are perhaps more sensitive to *Fusarium* than the larger scale bulblets. This is sustained by earlier *Fusarium* experiments with different sized lily bulbs (Straathof & Löffler, 1994). In daffodils, Bowes et al. (1992) selected fewer *Fusarium* resistant seedlings in young populations than in older populations. As contrast, Van Eijk and Eikelboom (1983) found more resistant tulip seedlings in juvenile bulb stage than in the adult bulb stage. Imle (1942a; 1942b) found that all his lily seedlings were completely susceptible. This could be due, however, to the genotypes used, to the testing conditions and/or to a higher *Fusarium* sensitivity of his lily seedlings.

The control seedlings of the cross 'Mont Blanc' x 'Connecticut King' and reciprocal were more susceptible at clonal level than expected from the seedling test. The number of escapes was also high. This is an indication that seedlings of this population have a higher level of resistance than older plants (juvenile resistance).

During the two years of cultivation of the selected and the control seedlings almost 50 % of the plants died, with most in the first year. In normal lily bulb production from seedlings, only a few plants die during cultivation. The percentage of plants lost per population did not depend on the resistance level of the population. Furthermore, almost the same percentage of plants died in the control and selected seedlings. Therefore, it is not expected that the death of the selected plants is due to latent infections or that natural *Fusarium* selection occurred in the control populations. The loss could be due to the fact that the seedlings were transferred, after a low temperature treatment, to the soil and grown for only five weeks. In this period, the seedlings produce foliage and reserves are used from the bulblets (Blaney & Roberts, 1966). After harvest, the leaves and stems were removed and the bulblets were stored for a long period. The loss of selected and control seedlings is compensated in the calculation of the percentage of escapes and missings.

From the data of the seedling test the calculated GCA value of each parent was highly correlated with the DSS value of each parent tested as scale bulblets. This was also found in *Fusarium* seedling tests with tulips (Van Eijk et al., 1979) and daffodils (Bowes et al., 1992). The GCA effect explained a relatively large proportion of the observed deviance. SCA and reciprocal effects were, although significant, of less importance. A model with only GCA effects explained most of the variation. Therefore, the *Fusarium* resistance level of parents can be used to predict the *Fusarium* resistance at population level of the progeny. Some highly resistant seedlings were detected in the clonal test. Those seedlings will be propagated and used in future practical breeding programmes.

The number of genes involved in the *Fusarium* resistance in Asiatic hybrid lilies, which are heterozygous, can only be estimated if genotypic groups within populations can be established. Discontinuity is difficult to establish (Figure 6.2) but the clear GCA effects suggests that several genes are involved.

Screening for *Fusarium* resistance in an individual lily seedling test is effective. The

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test used, however, is very labour intensive and needs controlled conditions (e.g., temperatures and inoculum concentrations). Furthermore, many seedlings die during cultivation in the years after testing. Screening for resistance with a lower inoculum concentration and/or temperature (Straathof & Inggamer, 1992) can result in a longer testing period and, therefore, more surviving plants during cultivation in the years after testing. Asiatic hybrid lily populations with known resistance levels for *Fusarium* can be used as standard populations in new tests. For breeding purposes less labour intensive tests can be developed. Preliminary experiments with direct sowing in *Fusarium* infested soil with a low inoculum concentration, or pouring a spore suspension to germinated seedlings appears promising. An alternative is the use of one year old seedling bulblets in an individual screening test in the winter period. A more time consuming, but reliable alternative, is to grow commercial bulbs from seeds in two years and to test some of the scales during the winter period. Only resistant seedlings would be planted in the third year. The ideal seedling test, however, will be by indirect selection using molecular markers linked with *Fusarium* resistance genes (Beckmann & Soller, 1986; Gebhardt & Salamini, 1992). In that case, only a leaf or scale of a seedling would be required to test *Fusarium* resistance. Furthermore, several diseases could be tested simultaneously, if markers linked with different resistance genes are found. These possibilities are investigated in further studies.

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CHAPTER 7

Genetic analysis of inheritance of partial resistance to *Fusarium oxysporum* in Asiatic hybrid lily using RAPD markers

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ABSTRACT

Linkage of RAPD markers with loci involved in partial resistance to Fusarium *in Asiatic hybrid lilies was investigated. Variation in resistance was found in two greenhouse tests using scale bulblets of 150 descendants of a backcross population. The progeny did not show a clear Mendelian segregation in* Fusarium *resistance. Three out of 213 RAPD markers were significantly (P < 0.005)* linked to Fusarium *resistance explaining approximately 24 percent of the total variance of the resistance. The construction of a genetic map with all RAPD markers was hampered because of different segregation types due to the dominant marker system and the low number of descendants evaluated per RAPD marker. Genomic regions, where* Fusarium *resistance loci were calculated to be linked to markers, were constructed. The use of RAPD markers for selection of quantitative traits and construction of linkage maps is discussed.*

Keywords: Fusarium oxysporum, inheritance, *Lilium,* RAPD markers, partial resistance, quantitative trait loci.

INTRODUCTION

The lily (*Lilium* L.), a vegetatively propagated perennial crop, is one of the economically most important flower bulbs. The soil-borne pathogen *Fusarium oxysporum* f.sp. *lilii* Imle causes basal rot in lily and threatens bulb cultivation seriously. Resistant cultivars can play an important role in the prevention of damage and in the reduction of the application of fungicides to prevent further environmental pollution. Variation in partial resistance to *Fusarium* has been described for several cultivars and *Lilium* species, but so far absolute resistance has never been reported (Imle, 1942a; Imle, 1942b; Straathof & Van Tuyl, 1994). In order to select for new *Fusarium* resistant lily cultivars, screening tests at clonal level (Straathof & Löffler, 1994a) and seedling level (Straathof & Löffler, 1994b) have been developed. Screening tests at clonal level can only be performed several years after crossing. Seedlings can be tested in the first year after sowing. Selecting seedlings resulted in a positive selection response but because of environmental variation retesting at clonal level is necessary. The mode of inheritance of *Fusarium* resistance in Asiatic hybrids of lily has not been deciphered yet, but diallel analysis of seedlings suggests that several genes are involved (Straathof & Löffler, 1994b).

Molecular markers have been recognized as possible tools for indirect selection of traits, independently from environmental variation. They can be used to speed up selection (e.g. at seedling stage) and for genetic studies in crops with a long juvenile period and/or slow propagation rates (e.g. flower bulbs). For a review on potential

uses of molecular markers, see Gebhardt & Salamini (1992) or Tanksley (1994). In flower bulbs, no reports on the use of molecular markers for indirect selection have been reported so far.

In lily (2n = 2x = 24), the detection of conventional restriction fragment length polymorphisms (RFLPs) is not feasible (B. Dekker, *personal communication*), very likely due to the large genome size of lily. Sentry & Smyth (1989) estimate the genome size of *Lilium henryi* to be approximately 32 million kbp and Bennett & Smith (1976) indicate that genome sizes of other *Lilium* species are of the same magnitude. Because of this problem, we tried to use randomly amplified polymorphic DNAs (RAPDs) (Williams et al., 1990), a polymerase chain reaction (PCR) based molecular marker technique, in lily. RAPD markers are easy to obtain and have proven to be successful for linkage studies of qualitative traits (e.g. Michelmore et al., 1991, Klein Lankhorst et al., 1991), but to our knowledge, no reports have been published reporting linkage analysis of RAPD markers with quantitative traits.

In this paper, we describe the results of the linkage analysis of individual RAPD markers with loci involved in *Fusarium* resistance in Asiatic hybrid lilies. We also describe the current state of a *Lilium* RAPD map. The use of RAPD markers for selection of quantitative traits and construction of linkage maps is discussed.

MATERIALS AND METHODS

Plant material

The Asiatic hybrid lilies 'Connecticut King', partially resistant to *Fusarium*, and 'Pirate', highly susceptible to *Fusarium*, were crossed. One F_1 hybrid, the cultivar Orlito which is partially resistant to *Fusarium*, was used as father in a backcross with 'Connecticut King'. A population of 150 descendants was obtained and cultivated to yearling bulbs. To speed up the propagation, 97 descendants and the three (grand)parents were propagated in vitro on an artificial medium during 10 months. The propagation medium consisted of full strength MS macro and micro elements, 0.1 mg/l NAA and 5% (w/v) sucrose. After a cold period of 12 weeks, the in vitro obtained scale bulblets were cleaned, standardized by size (diameter) and used in a *Fusarium* screening test in 1992. Some scales of the yearling bulbs were used for DNA isolation. The remainder part of the original bulbs was cultivated in 1992 to obtain commercial bulbs.

In 1993, commercial bulbs of 144 descendants of the population and the three (grand)parents were scaled, and scale bulblets were induced in vivo as described by Straathof et al. (1993). The in vivo scale bulblets were standardized by size and tested for *Fusarium* resistance in 1993. Some of the remainder scale bulblets were used for DNA isolation. All bulb material which was not used in a *Fusarium* test or

for DNA isolation was grown under greenhouse conditions in 1993 and evaluated for three morphological traits (flower colour, flower spots, and male sterility).

Fusarium test

Two highly aggressive isolates of *Fusarium oxysporum* f.sp. *lilii* (CPRO-Fol4 and CPRO-Fol11) were used for soil infestation as described by Straathof et al. (1993). The inoculum concentration was determined at planting time and amounted to 30,000 and 15,000 propagules per gram of soil in the 1992 and 1993 test, respectively. Scale bulblets were planted in pots, filled with *Fusarium* infested soil, and placed in a temperature-controlled greenhouse at 18/14°C (16 h day / 8 h night). Each pot contained four (1992) or five (1993) bulblets of the same genotype. The experiment was arranged in ten (1992) or four (1993) blocks. The genotypes were randomly assigned to the pots. Observations were made six weeks after bulblets were planted. Disease severity was rated visually according to an ordinal scale with six categories: 1 = healthy; 2 = slightly rotten; 3 = moderately rotten; 4 = heavily rotten; 5 = very heavily rotten; and 6 = completely decayed (Straathof et al., 1993).

RAPD analysis

Scales and scale bulblets were used for total DNA isolation according to either a tomato leaf extraction procedure (Van der Beek et al., 1991) or a method based on the chemical disclosure of tissue with sodium ethyl-xanthogenate (Jhingan, 1992). DNA concentrations were determined using a fluorometric assay with Hoechst 33258 in a Hoefer TKO 100. DNA was diluted to 10 ng per μl in TE buffer, and stored in aliquots at -20 °C until use.

RAPD reactions were conducted in reaction mixtures containing 25-50 ng total DNA, 50 ng 10-mer primers (Operon technologies), 200 μM of each dNTP and 1 unit Amplitaq DNA polymerase or 2 units Amplitaq DNA polymerase Stoffelfragment (Perkin Elmer). RAPD reactions were performed in either a Perkin Elmer DNA thermal cycler 480 [40 cycles of denaturation at 92 °C (1 min), annealing at 35 °C (1 min) and extension at 72 °C (2 min)] or a Perkin Elmer GeneAmp PCR system 9600 [40 cycles 94.3 °C (24 sec), 35 °C (20 sec) and 72 °C (74 sec)]. In order to detect polymorphisms, the 10-mer primers were pre-tested on the three (grand)parents of the population.

RAPD products were stained with ethidium bromide after electrophoresis on 1.5% agarose gels in 1 x TBE according to Sambrook et al. (1989). Polymorphisms present in the resulting banding patterns were scored from photographs of the agarose gels, recorded in data matrices and used for statistical analysis.

Statistical analysis

Disease ratings were analyzed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990), using a probit link function. For each clone

within an experiment, a disease severity score (DSS) was calculated by the threshold model. This score may be considered as a transformed average value of the disease ratings of each clone on an underlying linear scale (Straathof et al., 1993). Conclusions concerning block and genotype effects were assessed by analysis of deviance (McCullagh & Nelder, 1989); deviances were compared with the table of chi-squared distribution.

The heritability of *Fusarium* resistance was calculated as $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e/n)$, with σ^2_g = expected genotypic variance, σ^2_e = expected residual variance, and n = the number of plants per genotype.

Association of an individual segregating RAPD marker with either the DSS values of 1992 or 1993 was assessed by applying Kruskal-Wallis rank-sum test (see e.g. Lehman, 1975), as implemented in the MapQTL computer program (J.W. Van Ooijen, *personal communication*). The Kruskal-Wallis test is the nonparametric equivalent of the analysis of variance and the Kruskal-Wallis test statistic has approximately a chi-squared distribution. Mapping of markers in linkage groups was performed with the JoinMap package (Stam, 1993).

Using MapQTL, markers from map segments where genetic linkage with *Fusarium* resistance was suspected were analyzed as a backcross with interval mapping (Lander & Botstein, 1989). This enabled the recovery of missing marker information with linked markers, and as such is more informative than one-way analysis of variance for a marker.

RESULTS

In the 1992 test 40 scale bulblets of 97 descendants and the three (grand)parents were tested for *Fusarium* resistance. Significant block effects and significant genotype effects were detected ($P < 0.001$). Block effects were due to three blocks, which were on one table. Those blocks had lower disease severity scores than the other seven blocks, probably because of stronger desiccation of the soil by mechanical ventilation. No correlation was found between the diameter of the scale bulblet planted and the disease rating score after harvest. The heritability of *Fusarium* resistance was estimated at 0.94 ($n = 40$). The cultivars Connecticut King (DSS = 1.86) and Pirate (DSS = 4.24, s.e.d. = 0.26 relative to 'Connecticut King') showed resistance and susceptibility, respectively as expected. The cultivar Orlito (DSS = 3.58, s.e.d. = 0.25), however, was found to be more susceptible than in earlier experiments (Straathof et al., 1993). The DSS values within the segregating population ranged from -0.34 to 4.54.

In 1993, 144 descendants and the three (grand)parents were tested. No block effects were found, the genotype effects were again highly significant ($P < 0.001$). No correlation between the size of the scale bulblets and the disease rating score was

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found. The heritability amounted 0.90 (n = 20). The cultivars Connecticut King and Orlito showed resistance (DSS = -0.19 and -0.18 (s.e.d. = 0.36), respectively), 'Pirate' showed susceptibility (DSS = 1.67, s.e.d. = 0.36). The DSS values within the population ranged from -1.21 to 1.78.

The correlation coefficient calculated between DSS values of the genotypes tested in 1992 and 1993 using data of 93 genotypes, including the (grand)parents was 0.61 (Figure 7.1). A distribution of the DSS values for the descendants tested in 1993 is presented in Figure 7.2.
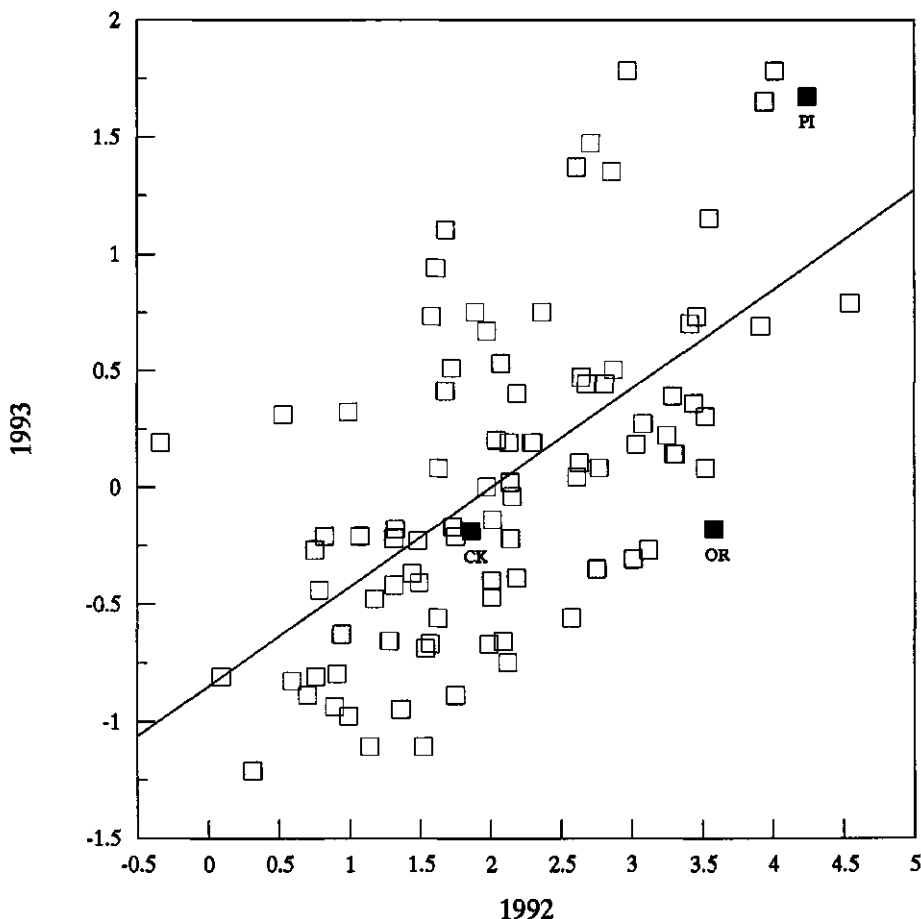

Figure 7.1 Correlation diagram between disease severity score values of the 1992 and 1993 *Fusarium* tests of 90 descendants of the lily cross 'Connecticut King' (CK) x 'Orlito' (OR), the two parents and the grandparent 'Pirate' (PI).

Three morphological markers were scored on 98 descendants of the population and corresponding parents during flowering in the greenhouse. Flower colours were scored as yellow or orange, and flower spots and male sterility were scored as present or absent. The female parent 'Connecticut King' was yellow, unspotted, and male fertile, the male parent 'Orlito' was orange, spotted, and of course male fertile. Flower colour (46 yellow and 52 orange flowering plants) and flower spots (54 spotted and 44 unspotted flowers) both segregated in a 1:1 ratio, whereas male sterility (78 male fertile and 20 male sterile plants) segregated in a 3:1 ratio (chi-squared test, $P > 0.05$).
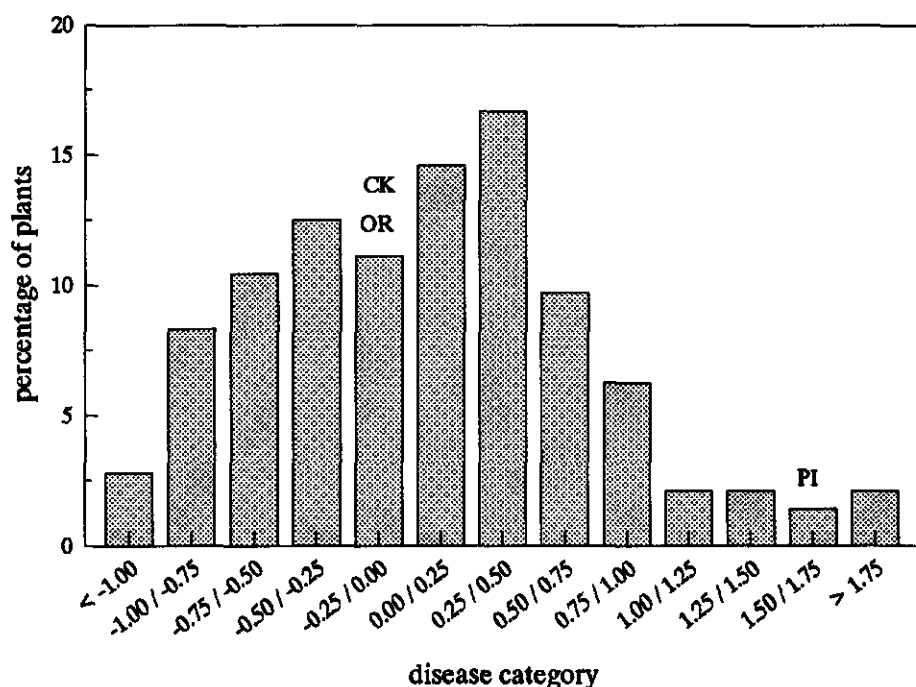

Figure 7.2 Distribution of disease severity score values in categories of 144 descendants of the lily cross 'Connecticut King' (CK) x 'Orlito' (OR) after screening for *Fusarium* resistance in 1993. The DSS values of the two parents and the grandparent 'Pirate' (PI) are indicated.

213 segregating RAPD markers were tested for association with loci, involved in *Fusarium* resistance in either 1992 or 1993 using the Kruskal-Wallis test. All 150 descendants of the segregating population were used in this test for linkage, but the number of descendants evaluated per RAPD marker varied between 29 and 96 descendants for 1992 (average number of genotype descendants per marker is 61) and between 29 and 128 for 1993 (average number of genotype descendants per

marker is 64). The Kruskal-Wallis test resulted in 24 significantly ($P < 0.1$) associated markers in 1992 and 36 markers in 1993, with 12 markers significant in both years (Table 7.1). Only three markers OPQ-08-05, OPS-13-03 and OPV-02-01 were significantly linked to the 1993 resistance data with $P < 0.005$.

The 213 RAPD markers, together with three morphological markers, were analyzed with JoinMap in order to create a genetic map of this *Lilium* cross. With a significance threshold for linkage of 3.0 LOD (^{10}log of odds) we were unable to separate the markers into a lower number of linkage groups. Even with a threshold of 2.5 LOD, more than 100 linkage groups were obtained.

Table 7.1 List of markers linked significantly to *Fusarium* data in either 1992 or 1993.

RAPD marker	1992	1993	RAPD marker	1992	1993	RAPD marker	1992	1993
EJO-03-01	.ᶻ	**	OPQ-08-08	-	*	OPV-16-01	-	*
EJO-08-01	-	**	OPQ-15-03	***	**	OPV-18-01	-	*
OPA-01-03	-	*	OPR-03-02	*	-	OPV-18-02	-	*
OPB-03-01	-	**	OPR-05-01	*	*	OPW-03-01	-	*
OPB-15-01	*	**	OPR-08-02	**	**	OPW-05-01	**	-
OPB-17-01	*	-	OPS-13-03	**	****	OPW-11-01	-	**
OPB-20-02	*	-	OPT-17-04	*	-	OPX-01-01	*	**
OPC-06-01	*	*	OPU-01-01	**	**	OPX-03-03	**	-
OPC-09-01	-	**	OPU-02-01	*	**	OPX-04-03	*	-
OPE-20-01	-	**	OPV-02-01	****	****	OPX-09-01	-	*
OPH-01-03	-	**	OPV-02-02	-	**	OPX-18-01	**	-
OPP-20-03	-	*	OPV-02-03	*	-	OPY-11-01	-	*
OPQ-08-02	*	*	OPV-08-01	-	***	OPZ-03-01	-	*
OPQ-08-03	*	-	OPV-10-01	**	-	OPZ-03-03	-	**
OPQ-08-05	*	****	OPV-12-02	-	**	OPAA-04-01	-	**
OPQ-08-06	**	-	OPV-15-01	-	*	OPAE-03-01	-	**

ᶻ-, *, **, ***, ****, Nonsignificant or significant at $P = 0.1$, 0,05, 0.01, or 0.005, respectively, by Kruskal-Wallis test. Marker codes are according to the original codes given by Operon technologies. The suffices give the order of segregating polymorphism scored with one primer according to fragment size. EJO-03 and EJO-08 are the primers 3 and 8 as described by Williams et al. (1990).

Linkage of the dominant RAPD markers is hampered since they may segregate in the gametes of one parent of the cross, in the other, or in both (Figure 7.3A-C, respectively). Markers segregating in one parent can be linked to each other. A marker segregating in one parent, however, cannot be linked directly to a marker segregating in the other parent, but only through markers segregating in both parents. Dominant RAPD markers segregating in both parents, however, provide less information than co-dominant markers.

With a significance threshold of 2.5 LOD, linkage analysis of separate data sets for the 62 markers segregating in 'Connecticut King' yielded 45 linkage groups and for the 60 markers segregating in 'Orlito' 50 linkage groups were obtained.
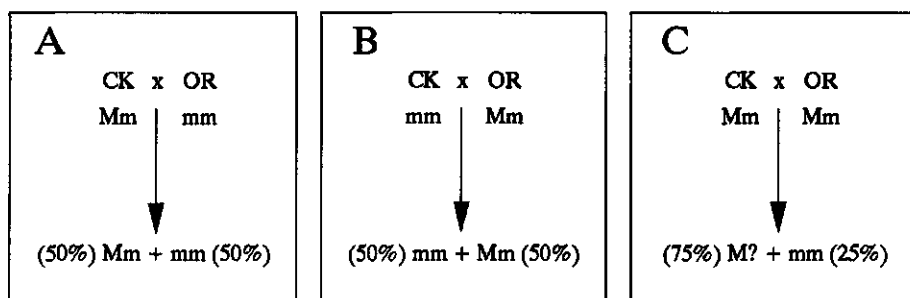
Figure 7.3 Three types of segregation of RAPD markers in the lily population 'Connecticut King' (CK) x 'Orlito' (OR). The M-allele corresponds with a band on the gel, the m-allele corresponds with the absence of a band. Marker segregates in the gametes of 'Connecticut King' (A), 'Orlito' (B) or both (C).

Flower colour was linked to two RAPD markers. Marker OPW-04-01 was located at 1.6 cM (3.0 LOD) and OPV-20-01 at 14 cM (4.6 LOD) from the colour locus. Both RAPD markers are of the Figure 7.3C type and, therefore, cannot be used efficiently as selection marker. Male sterility, also a Figure 7.3C type locus, was linked to two RAPD markers; OPAA-07-02 at 9 cM (5.8 LOD) and OPQ-08-06 at 19 cM (2.5 LOD). Flower spots was not linked (> 2.5 LOD) to any RAPD marker but was linked to male sterility (2 cM; 2.9 LOD). Only 4 out of 98 descendants (instead of approximately 12 if no linkage is present), were both sterile and had flower spots.

The linkage of the three RAPD markers which were highly associated with *Fusarium* resistance (OPQ-08-05, OPS-13-3, and OPV-02-01) with the other RAPD markers was studied in more detail. When using a significance threshold of 2.5 LOD, OPS-13-3 could not be linked to any other RAPD marker. This marker segregates in the gametes of both parents (segregation type Figure 7.3C), and the recessive allele is associated with a higher level of resistance. The markers OPQ-08-05 and OPV-02-01 formed a linkage group with four or three other markers, respectively (Figure 7.4). For both groups, Kruskal-Wallis test statistics are also presented in Figure 7.4. For OPQ-08-05 the recessive allele, whereas for OPV-02-01 the dominant allele is associated with a higher level of resistance. Both markers segregate in the gametes originating from 'Connecticut King' (segregation type Figure 7.3A).

Markers of both map segments with the same segregation type, were analyzed with interval mapping. The resulting estimates of phenotypic variance explained by the markers in the 1993 experiment were 7% for OPQ-08-05, and 8% for OPV-02-01. The estimation of the phenotypic variance explained by marker OPS-13-03 by one-way analysis of variance was 9%. This amounts to approximately 24% of the total

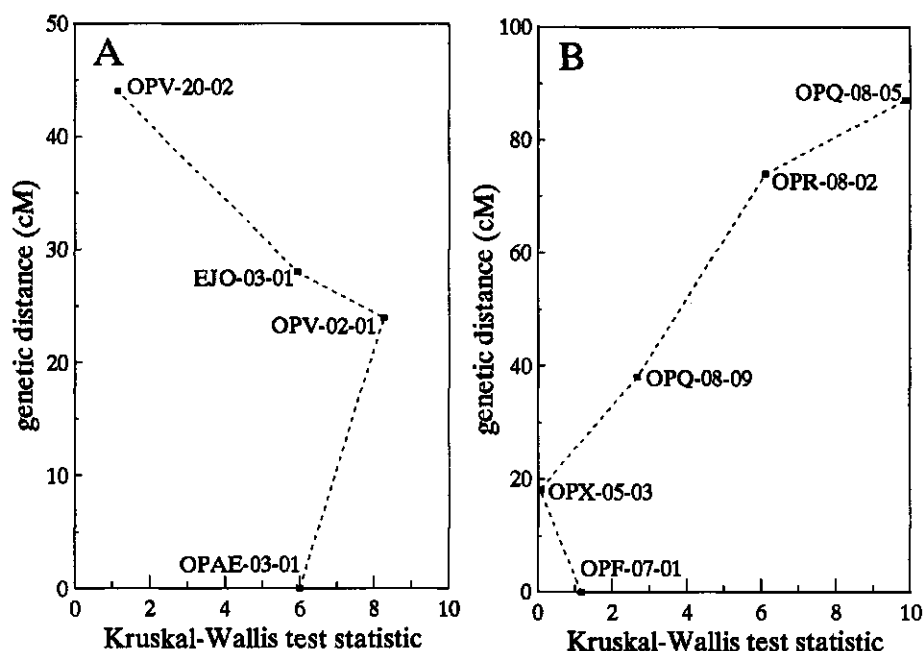phenotypic variance explained by the three markers together.

Figure 7.4 Maps of the two genomic regions, where *Fusarium* resistance loci were calculated to be linked to markers. A, the region including OPV-02-01 and B, the region including OPQ-08-05. The Kruskal-Wallis test statistic of 1993 of each marker is indicated right of the markers.

DISCUSSION

In this initial study to link *Fusarium* resistance in lily to RAPD markers, three significant ($P < 0.005$) markers are found in the 1993 test. Only one of these loci, however, was linked with the same significance with the results of the 1992 *Fusarium* test. Furthermore, both tests were not highly correlated ($r = 0.61$) and the resistant cultivar Orlito was scored susceptible in the 1992 test. Although clonal tests for *Fusarium* resistance in lily have proven to be reliable (Straathof et al., 1993; Straathof & Löffler, 1994a), in this experiment the repeatability was insufficient. For the first time, in vitro propagated scale bulblets were tested in large numbers (1992 test). Although preliminary experiments showed an acceptable correlation (data not shown) between *Fusarium* resistance of in vitro and in vivo propagated scale bulblets, several conditions in vitro could influence the *Fusarium* resistance. Since the initial number of scales was low, several propagation steps had

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to be made to obtain enough bulblets in vitro. This resulted in an tissue culture of 10 months with bulblets of different ages. Genotypes might obtain a different physiological status during this culture. It is known that the nitrogen supply during cultivation of lily bulbs influence the *Fusarium* sensitivity (Linderman, 1977). For all these reasons, the results of the 1993 test are expected to be more reliable. A *Fusarium* test in 1994 using in vivo obtained scale bulblets will be carried out to confirm this assumption.

Two hundred and thirteen RAPD markers were used to create a genetic map, which resulted in fragmentary maps only. These fragmentary maps could be due to the following four reasons. First, the dominant RAPD markers used in this study segregate in three different ways (Figure 7.3). Markers segregating in one parent can be linked to those segregating in the other parent through the markers segregating in both. The dominant markers segregating in both parents, however, provided so little linkage information that they were unable to perform this function. Second, markers were scored on subsets of all descendants only, resulting in a low numbers of plants in pairwise comparisons between markers. Third, the influence of the lily physical genome size on the genetical genome size. From mapping results in other crops it is known, however, that there is no direct relation between the physical and the genetical genome size (Nodari et al., 1993). Four, technical misscorings due to imperfect RAPD reactions and/or unclear band patterns. Repeatability of RAPD patterns in different labs and under different conditions is not always reliable (Penner et al., 1993). Furthermore, RAPD bands are sometimes difficult to score, when intensity varies due to experimental conditions or due to the presence of other bands that have almost the same size. For application of RAPD markers in plant breeding, highly linked markers have to be transformed to sequence characterized amplified regions (SCARs) (Paran & Michelmore, 1991).

Of the 213 RAPD markers tested, 34 (16 %) did not show a Mendelian segregation ($P < 0.01$; chi-squared test). Distorted segregation has been identified mainly in interspecific crosses in e.g., *Solanum* (Gebhardt et al., 1991), *Lycopersicon* (Miller and tanksley, 1990), and *Oryza* (Lin et al., 1992). The Asiatic hybrid lilies are obtained from interspecific hybridization of *Lilium* species within the section *Sinomartagon* (Van Creij et al., 1990).

Although no genetic map of lily is available, three markers highly significantly linked to *Fusarium* resistance were detected with the Kruskal-Wallis test. Therefore, this RAPD approach seems promising to obtain markers linked to *Fusarium* resistance loci. The use of a saturated linkage map of this population, however, is imperative to establish the number of loci involved in resistance and to ensure that markers identified to be linked with the Kruskal-Wallis analysis, but which are from different segregation types are not linked. To establish a saturated linkage map of this population, we are in the process of scoring more RAPD markers on all descendants and developing other PCR based marker types which inherit co-

dominantly. To obtain more markers linked to the three markers already identified as being linked to *Fusarium* resistance loci, we are currently performing the bulked segregant analysis (Michelmore et al., 1991).

Large variation in resistance was found between descendants of the population. This resulted in a high heritability value. The heritability in 1992 was higher than in 1993, because of the higher number of scale bulblets used. The inheritance of *Fusarium* resistance did not show a clear Mendelian segregation (Figure 7.2). This could be due to the ordinal measurement scale or the genetic background. Transformation to the quantitative disease severity score scale might reduce the measurement problem. This transformation, however, may work counteractive, when the underlying genetic distribution is not normally distributed. The limited number of descendants used in our experiments make conventional inheritance studies speculative, especially if several genes are involved as expected in this population (Straathof & Löffler, 1994b).

The distribution pattern of the resistance level of the descendants showed that most descendants were as resistant as or even more resistant than both parents (Figure 7.2). Resistance linked to the absence of marker OPS-13-03 may contribute to a level of resistance, which is higher than the level present in 'Connecticut King' or 'Orlito'. This result promises further possibilities for resistance breeding upon combining of alleles.

Three morphological markers were used in this study. Two traits (male sterility and flower spots) were found to be highly correlated. The inheritance of these traits is more complicated than shown in this study. Modifying genes are involved in flower colour (light/dark colour), spotting (few/many spots) and male sterility (functional male sterility).

The implications for practical breeding for *Fusarium* resistance in lily, when sufficient linkage is detected, are enormous. Estimates of how much can be gained with marker aided seedling selection depends on the number of loci and on how much these loci contribute to *Fusarium* resistance. This study does not reach definite conclusions about the number of loci involved in *Fusarium* resistance, but so far 24% of the resistance could be explained by the three markers. We speculate that more loci are involved in the coding for *Fusarium* resistance. The costs of testing seedlings on *Fusarium* resistance using linked markers, are strongly dependent upon the number of genes and consequently the number of PCR reactions which have to be performed. If many loci (e.g. ten) are involved in the coding for *Fusarium* resistance, costs for analysis may increase rapidly (e.g. 2-5 fold), depending on the number of SCAR reactions which can be combined in one PCR reaction.

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GENERAL DISCUSSION

INTRODUCTION

The investigation presented in this thesis was focused on the interaction between *Lilium* and *Fusarium oxysporum*. The objectives were to obtain knowledge on screening and breeding techniques and plant materials which can be used for the development of resistant lily cultivars. They are necessary for: (1) a more environmental friendly cultivation using less fungicides, (2) to ensure bulb quality at harvest time, and (3) to reduce production costs. The research consisted of three parts. To estimate different levels of *Fusarium* resistance in lily, screening methods were developed. They comprised the determination of resistance level (see Chapter 1), the comparison of resistance levels at different developmental stages (see Chapter 2), and study on the influence of the screening conditions (see Chapter 3). Since genetic variation is the basis of breeding, variation in *Fusarium* resistance in *Lilium* was investigated (see Chapter 4). To obtain knowledge about durability of the resistance, variation in virulence in the pathogen was studied (see Chapter 5). For an efficient selection of resistant genotypes from crossings, selection methods were developed. Selection was carried out at seedling level (see Chapter 6) and a method for indirect selection using molecular markers is described (see Chapter 7).

SCREENING METHODS

Before breeding can be conducted, the development of a screening method to determine the level of *Fusarium* resistance in lily was needed. A useful screening method must be repeatable, accurate, practicable and reliable.
Repeatability is defined as the agreement of screening results between experiments. All experiments which have been repeated showed significant correlations between the results of different years (see Chapter 1, 2, and 4). Some deviations were detected which could be due to the origin of the bulbs (e.g., cultivation locations, growers). The nitrogen supply during cultivation of the bulbs (Linderman, 1977), infection of other diseases (e.g., virus), and the use of fungicides after harvest could influence the *Fusarium* sensitivity of lily bulbs. The correlation of the results of two experiments to determine the level of resistance in the progeny of a 'Connecticut King' x 'Orlito' crossing was, although significant, much lower (see Chapter 7) than in the other experiments. This could be due to the in vitro propagation of the bulb material in one of the repeats. The influence of an in vitro period on the sensitivity of bulbs for *Fusarium* resistance, perhaps by a change in the physiological status of

these bulbs, is unknown. For the estimation of the *Fusarium* resistance in lily, the cultivation of bulbs under identical conditions in the year before testing is recommended.

The accuracy determines which genotypic differences of resistance can be measured. Measurement of resistance to a bulb rot pathogen is difficult, because the major symptoms are not visible before harvest. In the *Fusarium*-lily interaction, wilting or inhibition of stem growth can be observed during the screening period. Those features, however, do not clearly indicate the progress of the disease, since stem roots can compensate for dying basal roots. In some of the tests, completely healthy stems were harvested, while no bulb scales and bulb roots were left. Since disease progress cannot be estimated from the above soil plant organs, precise harvest dates are difficult to determine. Interim harvest of some additional bulbs of genotypes with a well-known resistance ranging from susceptible via intermediate to resistant (e.g. 'Esther', 'Hilde', 'Connecticut King') can be helpful to determine the most suitable harvest date. Using these cultivars as standards in screening tests, comparison of different tests is possible.

After harvest, weight measurements can be used to estimate *Fusarium* resistance levels in lily. Resistant genotypes will thrive. Thus, they gain weight; whereas, susceptible genotypes either increase less in weight or, due to rot, even loose weight. Therefore, the relative weight change can be used as a measure of the resistance level of the genotype. An alternative is provided by qualitative non linear disease ratings. In this case, disease severity is rated visually according to an ordinal scale with several categories. Disease ratings can be analyzed using a threshold model, in which the disease ratings are transformed to a continuous linear scale resulting in disease severity score (DSS) values. Weight measurements analyzed by ANOVA and disease ratings analyzed with the threshold model were highly correlated and provided similar precision (see Chapter 1). Disease ratings, however, are often analyzed by ANOVA. In spite of the fact that disease ratings are not measured on a continuous scale, as required for an analysis of variance, similar results were obtained in comparison with the threshold model: i.e., standardised differences between genotypes were approximately equal. This can be explained by the fact that the number of categories used (six) was reasonably large and all categories were well occupied. For practical breeding, disease ratings can be averaged to estimate the resistance level. In research studies, disease rating can be transformed to DSS values by which also small differences or interactions can be determined more precisely.

Changing temperature, inoculum concentration, and the duration of the experiment did not change the ranking of the cultivars regarding the levels of resistance, but it effected the accuracy. Since all resistance found was of a partial level, cultivars can be classified as resistant or as susceptible depending on the testing conditions applied. Therefore, conditions stimulating disease development will eventually led

to diseased plants, regardless of their resistance level. This evidently will decrease accuracy. To obtain a high accuracy, testing under standardized conditions is preferred. If one of the conditions is not controlled, the other conditions can be changed to compensate for this. For example, a low inoculum concentration can be compensated by a higher temperature and/or a longer duration of the test (see Chapter 3).

The practical application depends on the amount of time, labour and resources (e.g., greenhouse capacity) needed for testing. The input of a screening method can be reduced by a simple disease measurement, such as qualitative disease rating (see Chapter 1), the use of small bulb material (see Chapter 2) or a short experiment by using a higher temperature and/or inoculum concentration (see Chapter 3).

As previously mentioned, weight measurements and disease ratings can be used to estimate *Fusarium* resistance levels in lily. Although the weight measurement is a quantitative trait and easy to analyze, the labour intensity and greenhouse capacity (equal number of infested and control pots) makes this method rather expensive. For disease ratings, only a small control group is necessary to detect if bulbs were already infected before planting.

Cultivation from scale bulblets to commercial lily bulbs normally takes two years, and different developmental stages can be distinguished. Disease resistance between developmental stages may differ. For practicable reasons, it is preferable to screen early for resistance. Therefore, the levels of resistance in different stages have been compared. The ranking of the genotypes according to their resistance was very similar in all stages. Smaller bulbs, however, generally are much more sensitive to the pathogen than larger bulbs. Infection of the basal plate in smaller bulbs directly lead to bulb disintegration. Testing scale bulblets reduces greenhouse capacity in area and duration. Further simplification of the test (e.g., in time, labour intensity, and greenhouse capacity) is possible using separated scales. Although some deviations in resistance levels were found for some genotypes, the scale test can be used to screen a large number of genotypes. Retesting of the selected genotypes at bulb level will reject the unjustly selected (susceptible) genotypes. Knowledge of the mechanism of resistance could be helpful in optimization and simplification of screening methods (Baayen, 1988).

Results of histological research in the *Fusarium*-lily interaction obtained so far (Baayen, 1992; Rijkenberg & Baayen, *in preparation*; R.P. Baayen, *personal communication*), showed that penetration of the fungus can occur in roots, the basal plate and scales. Roots are penetrated mostly via anticlinal walls of the epidermal layer immediately behind the root cap. In the zone of elongation, fungal germ tubes are probably attracted by leakage of nutrients. Suberization of the exodermis of roots can prevent leakage of nutrients and can be a barrier to penetration. The basal plate can be penetrated at the root rupture sites and scales via stomata. Wounds present in underground tissues can also be an entrance for the pathogen. After penetration

fungal growth was mainly intercellular. Intracellular growth was only observed in dead cells. The rot-causing f.sp. *lilii* does not enter the xylem, but colonizes only in cortical tissue. The pathogen probably utilises toxins and, certainly at a later stage in the pathogenesis, cell wall-degrading enzymes to kill and degrade the host cells. The host plant responds with strengthening of cell walls by infusion with phenolics, lignification, deposition of secondary wall layers and formation of so-called appositions. These responses prevent further penetration of cells and degradation of the cell walls by fungal enzymes, and thus retard the disease process. This defense reaction is stronger in resistant 'Connecticut King' than in susceptible 'Esther'. Besides a quicker and/or stronger defense reaction in the resistant cultivar, the resistance mechanism could also be due to detoxification of pathogenic products and/or a lower sensibility to the putative toxin, which is assumed to kill host cells in order to prevent defense responses.

Reliability is defined as the agreement between screening results and resistance under growers conditions. Field experiments could provide information comparable with growers conditions. Environmental variation, however, makes control of the progress of the disease difficult. The accuracy of the results of field experiments in comparison with testing under standardized conditions will be decreased, and screening will be more labour and time consuming. No field tests were performed in this study. Therefore, reliability is not known. As previously mentioned, changing temperature, inoculum concentration and the duration of the experiment did not change the ranking of the cultivars. Only a cultivar x time course interaction was found and this was probably due to the limited measurement scale (see Chapter 3). This indicates that the expression of resistance does not depend to a large extent on environmental conditions and, thus, will be exhibited similarly not only in greenhouse trials but also under growers conditions.

Comparison of resistance determined in greenhouse tests and field tests in the *Fusarium*-carnation interaction showed that only major effects (a high resistance level or a high susceptibility level) corresponded. The greenhouse test was unreliable as a predictor of the degree of resistance observed in the field (Ben-Yephet, 1993). In that study, greenhouse and field experiments were not completely standardized since not the same inoculum source (e.g. races) were used in both trials.

Recently, a survey of bulb growers was made (Van Keulen & Van Aartrijk, 1993) to obtain resistance data of cultivars under field conditions. In this survey, 157 cultivars were rated for their *Fusarium* resistance in lily, 21 of these were also used in tests described in this thesis. Comparison of both results, rated on an ordinal scale from 1 to 5, with 1 = resistant and 5 = completely susceptible, are presented in Figure 1. Cultivars are reported to be more resistant by the growers than they scored in the screening tests. In the survey only 9 % of the 157 cultivars was rated to be more than intermediate susceptible. This seems in contrast with the *Fusarium* problem in lily cultivation. Furthermore, susceptible cultivars which are rather new

and/or only grown on a small area could be considered resistant by growers in situations where fresh soil is used for cultivation. Also, the use of chemical disinfestation of bulbs and soil can lead to an improper resistance classification of a cultivar. Only in one case a cultivar (the Asiatic hybrid lily 'Prominence') was considered to be intermediate susceptible under field conditions, but appeared to be resistant in our tests.
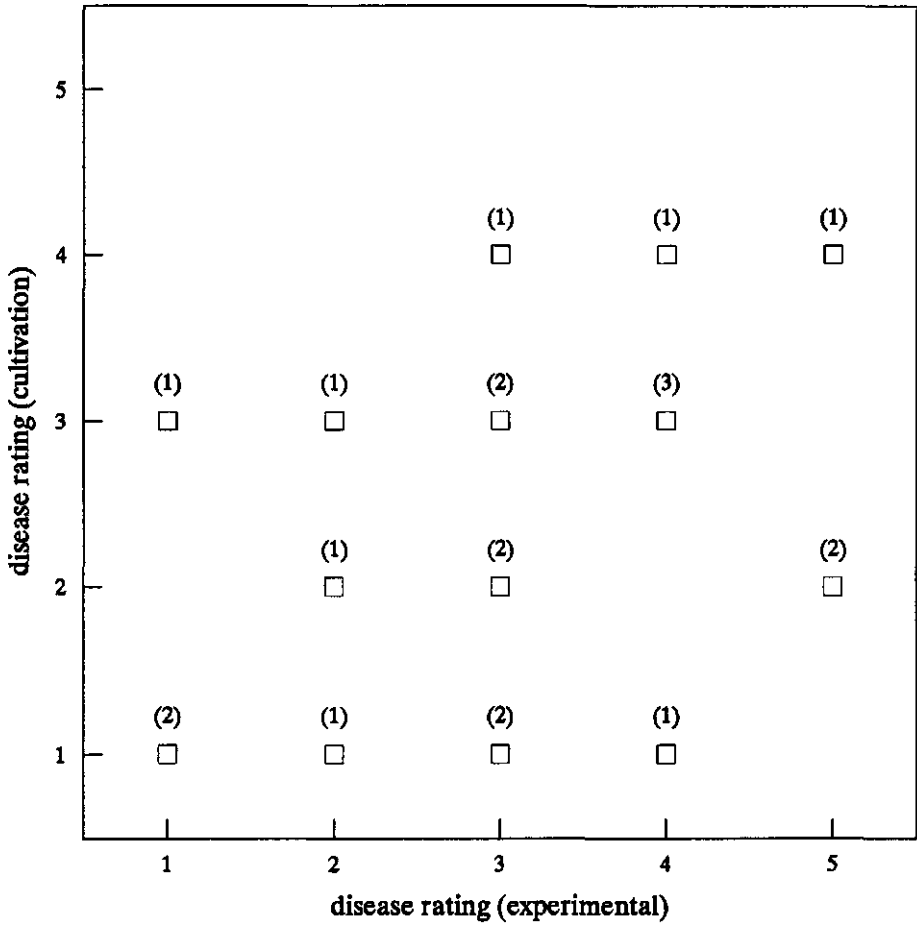

Figure 1. Comparison of *Fusarium* resistance levels of 21 lily cultivars after testing under standardized conditions and from an inquiry under bulb growers. Resistance was rated from 1 to 5, with 1 = resistant and 5 = completely susceptible. Between brackets the number of cultivars are given.

VARIATION

If screening methods are available, variation within cultivar groups can be determined. This is necessary for resistance breeding programmes. Variation in *Fusarium* resistance in lily cultivars was mentioned by Imle (1942a; 1942b) and Smith & Maginnes (1969; 1971). In this study (see Chapter 1 and 4), cultivars of the three most important lily groups were tested for *Fusarium* resistance. In the Asiatic hybrids, cultivars with a reasonably high resistance level were found, which can be used in breeding programmes. In the Oriental hybrids, no resistance was found. Additional cultivars, however, have to be screened in order to obtain a representative overview of the resistance within this group. In *L. longiflorum*, intermediate levels of *Fusarium* resistance was found, but the level of resistance was lower than in the most resistant Asiatic hybrids. Strong selection can probably lead to more *L. longiflorum* cultivars with a higher level of resistance. All currently grown lily cultivars have to be screened for *Fusarium* resistance followed by the decision of the growers to cultivate only the most resistant ones.

Variation in *Fusarium* resistance in *Lilium* species can be introduced by interspecific hybridization using in vivo and in vitro pollination and embryo-rescue techniques. In the genus *Lilium*, interspecific hybridization has been widely studied (Van Creij et al., 1990; Van Tuyl et al., 1991) and many hybrids have been obtained. Furthermore, protoplast isolation and fusion in lily to obtain more interspecific hybrids is being investigated (Mii & Yuzawa, 1991; Sugiura, 1993; I. Famelaer, *personal communication*). Variation in *Fusarium* resistance in *Lilium* species was only investigated by Imle (1942a; 1942b). In this study (see Chapter 4), accessions of species of five different *Lilium* sections were tested for *Fusarium* resistance. In the *Sinomartagon* section, species with high levels of partial resistance were found. Resistance genes within these species (e.g., *L. dauricum, L. tigrinum* and *L. davidii*) are probably the donors of the resistance present in Asiatic hybrid lilies. Within the *Archelirion* section most species are susceptible (e.g., *L. speciosum, L. auratum*). Within the related species *L. henryi*, variation in *Fusarium* resistance has been found. Using the most resistant *L. henryi* genotypes in interspecific hybridization, the resistance of the Oriental hybrid lilies should be increased. Interspecific hybridization between the Oriental hybrid lilies and the Asiatic hybrid lilies is under investigation in the Netherlands and Japan. If this succeeds, it will take several generations, however, before the Asiatic hybrid lily resistance genes are introduced into Oriental hybrid lilies. Introduction of new resistance genes into *L. longiflorum* by interspecific hybridization also requires a time-consuming back crossing programme. Hybridization of *L. longiflorum* with the Asiatic hybrid lilies has been accomplished, resulting in the LA-hybrids. Using resistant Asiatic hybrid lily cultivars, high resistance levels can be obtained within the LA-hybrids.

It is not known whether introgression of the *Fusarium* resistance genes will always

take place in interspecific hybridization and back crossing. Within the hybrid cultivar groups obtained so far, no disturbance of segregation has been found. In crosses using more unrelated species, sterility of the hybrid can occur. By polyploidisation, this sterility can be overcome, and back crossing becomes possible (Van Tuyl, 1990). No data are available as to whether genomes within those hybrids are sorted out or whether recombination occurs.

If no or only little variation in *Fusarium* resistance is available within a specific cultivar group (e.g., the Oriental hybrids) or the genetic variation of resistance is small (e.g., *L. longiflorum*) and interspecific hybridization will not result in the recombinants desired variation can be introduced by other techniques. Using somaclonal variation, in combination with in vitro selection, new resistant genotypes might be obtained (Van den Bulk, 1991). The availability of a tissue culture procedure and of a specific selection agent are a prerequisite of such a system.

Another technique is based on the introduction of resistance in plants by transformation. The lily, a monocot, is difficult to transform, but first results of external DNA transfer using particle gun bombardments of lily pollen and successive pollination showed that transformants were obtained (Bino et al., 1990; Van der Leede-Plegt et al., 1992; L.M. Van der Leede-Plegt, *personal communication*). Besides a transformation technique, *Fusarium* resistance genes are required. With the isolation of genes coding for enzymes detoxifying fusaric acid (Ouchi et al., 1989) and Pathogen-Related (PR) proteins (Sela-Buurlage et al., 1993; Logemann et al., 1994) the first steps have been made in that direction.

Besides variation within the host, variation in the pathogen can occur. Knowledge of this variation is important for estimation of the durability of the *Fusarium* resistance. If this variation concerns the virulence (sensu Van der Plank) of the pathogen, by definition physiological races are present that are adapted to some resistant genotypes. Genetic variation for virulence either might already be present within the population of the pathogen or it might be introduced by an adaptation to the resistance of the host. In studying pre-existing variation in the pathogen (see Chapter 5), only significant interactions were found when low aggressive *Fusarium* isolates were tested on *L. longiflorum*. This species probably has additional gene(s) for resistance against virulence genes in a distinct group of isolates. For lily breeding purposes, it is sufficient to test with the most aggressive isolates. Löffler et al. (*in preparation*) found no adaptation of the pathogen to resistance in the host. Based on these results, durability of *Fusarium* resistance in lily is expected.

Remarkably, even *Fusarium* isolates of other crops like gladiolus and tulip, which were determined as other formae speciales, were able to infect lily. This makes the use of crop rotation with various bulb crops questionable.

SELECTION METHODS

After recombination of genomes by crossing, plants with the characteristics important for lily cultivation and flower production have to be selected. *Fusarium* resistance can be selected according to the screening methods described in Chapters 1, 2, and 3. This requires, however, clones which can only be obtained after several years of cultivation. A scale test can be applied in the winter period two years after sowing. Although clone testing is accurate, selection in an earlier stage would speed up the selection procedure and reduce costs. In Chapter 6, the development of a seedling test is described and it resulted in a positive selection response. The test described is similar to bulb testing as described in Chapter 1 and 2, since young seedling bulbs are transplanted after a low temperature treatment to infested soil. This cold period replaces the storage of bulbs during the winter period. In fact, two cultivation seasons are replaced by one. The testing period, however, was very short. Since the seedlings were still very small, this exhausted the bulbs resulting in fall out of the selected seedlings in further cultivation. Testing one year old seedling bulbs can prevent this reduction and is less labour intensive than the transplant technique as used in this study. Such a test may be performed in the winter period, thus, saving time. Alternatively, testing in the first year can be performed by sowing in infested soil. On top of the infested soil two centimetre of fresh soil is placed in which seeds can be sown. Seeds are able to germinate in a pathogen-free surrounding, and come in contact with the fungus by root growth. A preliminary test showed promising results. Since the young seedlings are very sensitive to *Fusarium*, the inoculum concentration, temperature, and water supply must be precisely controlled.

Large variation in resistance within and between populations was found. Using resistant genotypes as parents gave the highest chance of a resistant progeny. Variation within populations makes selection possible.

In the seedling test described (see Chapter 6) escapes (seedlings unjustly scored resistant) and missings (seedlings unjustly scored susceptible) were found. Escapes and missings were expected since variation in clones was found. The percentage of escapes was rather low compared to the number of seedlings tested, while the percentage of missings was rather high. This is probably due to the rather high disease pressure applied. No clear evidence was found that at the seedling level different resistance mechanisms and genes are involved than in older bulbs.

The inheritance of the *Fusarium* resistance in lily could not be studied in detail. Therefore, large number of descendants of related populations (e.g., back crossings and selfings) are necessary. Analysis of the seedling test results leads to the conclusion that several genes are involved. This is based on the fact that the general combining ability (GCA) was the most important effect (see Chapter 6).

Molecular markers can be helpful in genetic studies and can be used for indirect

selection when a close linkage between the marker and resistance gene is obtained. Such markers have several advantages in comparison to traditional screening tests. They can be used at an early stage and require only a little plant material. Furthermore, they are not influenced by environmental changes. The lily genome is very large, which prevents the detection of bands in restriction fragment lengths polymorphisms (RFLP) studies. An alternative would be the use of markers multiplied by the polymerase chain reaction (PCR). Randomly amplified polymorphic DNA (RAPD) markers are easy to obtain. They are, however, difficult to use in genetic studies because of their dominant behaviour. So far, three RAPD markers which are linked with *Fusarium* resistance are detected in lily (see Chapter 7). With these markers, however, approximately a quarter of the variation found in screening tests was explained. Furthermore, using the three markers as selection criterion, susceptible seedlings can still be selected. The linkage map of lily is yet very fragmentary because of the low number of plants investigated per RAPD marker.

PRACTICAL PURPOSES AND FURTHER RESEARCH

In the Netherlands, the use of chemical control of pathogens in lily cultivation must be reduced to prevent further environmental pollution. Specific soil disinfection chemicals will be eliminated or only allowed on a limited basis. Soil pathogens, like *Fusarium*, can become a greater threat for bulb production. Cultivation methods, bulb disinfection, and biological control (Duijff, 1994) in combination with highly resistant cultivars can reduce damage and ensure economic profitability of lily cultivation. The variation in resistance found in this study and the testing and selection techniques described make it possible to obtain new lily cultivars with high levels of *Fusarium* resistance. The resistance appears to be durable. So far, breeding for *Fusarium* resistance in the flower bulb industry is only carried out in gladiolus. *Fusarium* resistant gladiolus cultivars can be expected at short notice. In the other flower bulb crops (i.e., lily, tulip and narcissus), however, breeding for *Fusarium* resistance is only carried out at experimental stations and is mainly focused on research. To prevent *Fusarium* affection in bulb cultivation, resistant cultivars have to be developed by the breeding industry. The long time between crossing and introduction of a new flower bulb cultivar requires a direct action of the bulb breeders.

The reliability of the standardized screening method depends on the importance of the genetic component compared to environmental cultivation conditions. Reduction in plant vigour by other pathogens could influence the *Fusarium* resistance. Thus, testing virus-infected and virus-free lots of a cultivar for *Fusarium* resistance can give an estimation of such an influence. To determine if resistance levels found

under greenhouse conditions are reliable and if they are adequate when less fungicides are used, field experiments have to be performed. These trials can be carried out on experimental stations using integrated plant protection management (Stokkers, 1992). The comparison of bulb lots of a cultivar which have been grown at different locations and screened for *Fusarium* resistance in a standardized test will give an idea of the relation between genetic component and its bulb history.

Reducing chemical disinfection could stimulate the importance of other (soil) pathogens. Therefore, resistance breeding against other lily pathogens must have a high priority. The effect of *Fusarium* resistance against the other bulb rot pathogen *Cylindrocarpon* has to be studied.

Genetic maps are important especially in crops with a long juvenile phase and slow propagation rate. More markers and plants have to be scored to saturate the lily map and to find a better linkage with *Fusarium* loci. Consequently, a better view of the inheritance will be obtained. Co-dominant marker systems have to be developed for less complicated linkage studies using PCR.

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The soil-borne fungus *Fusarium oxysporum* f.sp. *lilii* Imle causes bulb and scale rot of lilies (*Lilium* L.), annually resulting in a considerable economical damage in bulb and flower cultivation. Presently, the prevention of *Fusarium* damage depends on the application of a combination of fungicides and cultivation practices, such as crop rotation and the use of healthy starting material, however, this is not satisfactory. In the Netherlands the application of soil-disinfestation chemicals must be significantly reduced in the future. An environmental friendly and durable solution is the cultivation of (partial) resistant cultivars. These are, however, not widely available in the current assortment and, therefore, must be developed. The objectives of the experiments described in this thesis were: (1) the development of screening methods, (2) the evaluation of genetic variation in lily and *Fusarium*, and (3) the development of selection methods, by which the breeding companies will be able to develop *Fusarium* resistant lily cultivars (see General Introduction).

A standardized screening method for an accurate determination of partial resistance to *Fusarium* in lily clones is described. The level of resistance can be measured by weight changes of the plant caused by *Fusarium* affection, as well as by a classification of the affected bulbs in ordinal disease ratings (on a scale of 1 to 6, with 1 = not affected and 6 = completely decayed). The data of the weight measurements were analyzed statistically using an analysis of variance. The disease ratings were analyzed using a threshold model. This model calculates a disease severity score (DSS) using an underlying continuous and linear scale. Both measurements were repeatable, accurate, and significantly correlated. The use of disease ratings is, despite a more complicated analysis, less laborious and can suffice with a smaller control group (see Chapter 1).

Vegetative propagation of lily has several developmental stages. It was possible to test reproducibly lily plant material in four different stages (separate scales, scale bulblets, yearling bulbs, and commercial bulbs). The sensitivity for *Fusarium* decreased with an increasing bulb age. A significant correlation was found between the resistance in the four stages mentioned, which allows testing in one stage only. In the scale test some deviating results were obtained, possibly because of the presence of a large wound. Since the scale test is more efficient than the bulb tests, this test is preferable for determination of *Fusarium* resistance in large numbers of genotypes. Selected material has to be retested, preferably using the scale bulblet test which is second in efficiency (see Chapter 2).

Standardisation of the tests is not always possible. A high temperature, a high inoculum concentration, and a longer duration of the experiment raised the level of affection. The ranking of the cultivars was not changed; only the accuracy was

reduced using more extreme testing conditions (see Chapter 3). Since no genotype x environmental interactions were found, it is expected that cultivars which are most resistant in the standardized tests, will also be most resistant under field conditions (see General Discussion).

Sources of resistance are necessary for breeding purposes. Within the Asiatic hybrid lilies, which belong to the *Sinomartagon* section, a high level of *Fusarium* resistance has been found. Also, in species within this section which are related to the Asiatic hybrid lilies, such as *L.dauricum*, *L.davidii*, and *L tigrinum*, high levels of resistance were detected. Absolute resistance was not found. In the Oriental hybrid lilies (*Archelirion* section), a low level of resistance was found. In the Oriental hybrid lily related species of the *Archelirion* section no resistance was obtained. In *L. henryi*, however, resistance was observed. This species can be crossed with the Oriental hybrid lilies. Since only a few Oriental hybrid lilies were tested, it is recommended that a large collection will be screened for *Fusarium* resistance. In cultivars of *L. longiflorum* a moderate level of resistance was found. The level of resistance of cultivars within this species can probably be raised by specific cross combinations and selection. More variation may be detected in wild accessions from Japan. Interspecific hybridisation research showed that crossings between genotypes from different sections is possible. This can result in the introduction of *Fusarium* resistance in the overall cultivar assortment (see Chapter 4).

Research on the variation of the pathogen, testing different *Fusarium*-isolates against the most resistant lily genotypes, showed that a sustainable durability of the resistance can be expected. A deviating pattern of virulence was only found in some low aggressive isolates. These isolates appeared to affect *L. longiflorum* less than expected on the basis of their aggressiveness. The affection of these deviating isolates was, however, lower than using aggressive isolates. Some *Fusarium*-isolates of other formae speciales (f.sp. *gladioli* and f.sp. *tulipae*) could also affect lily at a low level. This could have negative consequences for the crop rotation schedules used for bulb crops (see Chapter 5).

Selection at seedling level can substantially reduce costs and time. A seedling test for selection for *Fusarium* resistance in lily is described. Variation within and between populations was demonstrated. The seedling test resulted in a clear selection response, and this makes testing at a seedling level practical. The efficiency of the seedling test was determined on the basis of the percentage of escapes (selected susceptible plants) and missings (rejected resistant plants). The percentage of escapes was low compared to the number of seedlings tested, the percentage of missings on the other hand was rather high. This could be due to a high disease pressure. The percentage of escapes compared to the number of selected seedlings was such that retesting at clonal level is necessary. Genetic analysis of the results of the seedling test showed that the general combining ability (GCA) was the most important genetic component. The GCA-value of a cultivar was in agreement with the level of

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resistance at clonal level (see Chapter 6).

Indirect selection using molecular markers is independent of environmental conditions and can be carried out with a small amount of plant tissue. Using the Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD), in descendants of the cross 'Connecticut King' x 'Orlito' three markers were traced, which were highly linked to *Fusarium* resistance. These markers, however, only explained a part (24 %) of the resistance. Linkage of markers to each other to obtain a genetic map of lily was hampered due to the marker type and the low number of the same plants used per marker (see Chapter 7).

The methods described in this thesis can be of a great importance for advanced breeding programmes of lilies. The importance of partial *Fusarium*-resistance under field conditions in an integrated disease control programme, in combination with a reduction of chemical disinfection and the influence of other pathogens, requires further attention (see General Discussion).

De bodemschimmel *Fusarium oxysporum* f.sp. *lilii* Imle veroorzaakt bol- en schubrot bij lelie (*Lilium* L.) hetgeen jaarlijks leidt tot een aanzienlijke economische schade bij bollen- en bloementeelt. De huidige bestrijding van *Fusarium* bestaat uit toepassing van een combinatie van gewasbeschermingsmiddelen en diverse teelttechnische maatregelen, zoals vruchtwisseling en het gebruik van gezond uitgangsmateriaal, maar dit is niet afdoende. In Nederland dient het gebruik van met name bodemontsmettingsmiddelen de komende jaren sterk te worden verminderd. Een milieu-vriendelijke en duurzame oplossing is het telen van (partieel) resistente cultivars. Deze zijn echter nog onvoldoende aanwezig in het huidige cultuursortiment en moeten daarom ontwikkeld worden. De in dit proefschrift beschreven experimenten hadden tot doel: (1) het ontwikkelen van toetsmethoden, (2) het opsporen van genetische variatie in lelie en *Fusarium*, en (3) het ontwikkelen van selectiemethoden waarmee de veredelingsbedrijven in staat moeten zijn om *Fusarium*-resistente leliecultivars te ontwikkelen (zie Algemene Inleiding).

Een gestandaardiseerde toetsmethode voor een nauwkeurige bepaling van partiële *Fusarium*-resistentie in lelieklonen is beschreven. De mate van resistentie is zowel met behulp van gewichtsverandering van de plant ten gevolge van *Fusarium* aantasting als met behulp van een indeling van aangetaste bollen in ordinale ziekteklassen (op een schaal van 1 tot 6, met 1 = geen aantasting en 6 = volledig verrot) bepaald. De gegevens van de gewichtsmetingen werden statistisch verwerkt met behulp van een variantie-analyse. De ziekteklassen werden geanalyseerd met een drempelmodel. Dit model berekent een 'disease severity score' (DSS) op een onderliggende continue en lineaire schaal. Beide waarnemingen waren herhaalbaar en nauwkeurig, en bleken significant gecorreleerd. Het gebruik van ziekteklassen is, ondanks een moeilijkere analyse, minder bewerkelijk en er kan worden volstaan met een kleinere controle-groep (zie Hoofdstuk 1).

De vegetatieve vermeerdering van lelie verloopt via een aantal ontwikkelingsstadia. Het bleek mogelijk om plantmateriaal van lelie in vier verschillende stadia (losse schubben, schubbolletjes, plantgoed en leverbare bollen) reproduceerbaar te toetsen. De gevoeligheid voor *Fusarium* nam af naarmate de bol ouder werd. Er werd een significante correlatie gevonden tussen de resistentie in de vier genoemde stadia hetgeen toetsing in slechts één stadium mogelijk maakt. In de schubtoets werden echter enkele afwijkende resultaten verkregen, mogelijk door de aanwezigheid van een groot wondoppervlak. Aangezien de schubtoets efficiënter uitvoerbaar is dan de boltoetsen, is deze toets voor het bepalen van *Fusarium*-resistentie in grote aantallen genotypen toch te prefereren. Geselecteerd materiaal dient dan op bolniveau, waarbij de schubboltoets het meest efficiënt is, te worden nagetoetst (zie Hoofdstuk 2).

Standaardisatie van toetsen is niet altijd mogelijk. Een hogere temperatuur, een hogere inoculumconcentratie en langere duur van het experiment verhoogde de mate van aantasting. De resistentievolgorde van de cultivars veranderde echter niet, wel was de nauwkeurigheid lager bij extremere toetsomstandigheden (zie Hoofdstuk 3). Aangezien er geen genotype x milieu-interacties zijn gevonden, is de verwachting gerechtvaardigd dat de cultivars die in gestandaardiseerde toetsen het meest resistent bleken, dit tevens onder veldomstandigheden zullen zijn (zie Algemene Discussie). Resistentiebronnen zijn noodzakelijk in de resistentieveredeling. Binnen de Aziatische leliehybriden die behoren tot de *Sinomartagon*-sectie, werd een hoog niveau van *Fusarium*-resistentie gevonden. Ook in soorten binnen deze sectie die verwant zijn aan de Aziatische leliehybriden, zoals *L. dauricum*, *L. davidii*, en *L. tigrinum*, werden hoge niveau's van resistentie aangetoond. Absolute resistentie werd niet gevonden. In de Oriental leliehybriden (*Archelirion*-sectie) werd slechts een laag niveau van resistentie gevonden. Ook in de aan de Oriental leliehybriden verwante soorten van de *Archelirion*-sectie werd geen resistentie opgespoord. In *L. henryi* werd wel resistentie aangetoond. Deze soort is kruisbaar met de Oriental leliehybriden. Aangezien echter nog maar weinig Oriental leliehybriden zijn getoetst, verdient het aanbeveling op korte termijn een groot sortiment op *Fusarium* resistentie te onderzoeken. In *L. longiflorum*-cultivars (sectie *Leucolirion*) werd een redelijk niveau van resistentie gevonden. Het resistentieniveau van cultivars binnen deze soort kan mogelijk worden verhoogd door gerichte kruisingen en selectie. Meer variatie kan wellicht worden gevonden in wilde herkomsten in Japan. Uit soortkruisingsonderzoek is gebleken dat ook kruisingen tussen genotypen uit verschillende secties, en daarmee de introductie van *Fusarium*-resistentie in het gehele cultivarsortiment, tot de mogelijkheden behoort (zie Hoofdstuk 4).

Uit onderzoek naar variatie in het pathogeen, waarbij verschillende *Fusarium*-isolaten op de meest resistente leliegenotypen werden getoetst, bleek dat er geen duidelijke bedreiging voor de duurzaamheid van de resistenties verwacht wordt. Een afwijkend virulentiepatroon werd bij enkele weinig agressieve isolaten gevonden. Deze isolaten bleken *L. longiflorum* iets minder aan te tasten dan verwacht op basis van hun agressiviteit. De aantasting van deze afwijkende isolaten was echter lager dan bij het gebruik van agressieve isolaten. Enkele isolaten van andere formae speciales (f.sp. *gladioli* en f.sp. *tulipae*) bleken in staat lelie in lichte mate aan te tasten. Dit kan nadelige gevolgen hebben voor de huidige vruchtwisselingschema's in de bolgewassen (zie Hoofdstuk 5).

Selectie op zaailingniveau kan een aanzienlijke besparing in kosten en tijd opleveren. Een zaailingtoets voor selectie op *Fusarium*-resistentie bij lelie is beschreven. Variatie tussen en binnen populaties werd aangetoond. Bij de zaailingtoets werd een duidelijke selectierespons gevonden, hetgeen selectie op zaailingniveau zinvol maakt. De efficiëntie van de zaailingtoets werd bepaald aan de hand van het percentage ontsnappers (geselecteerde vatbare planten) en missers (niet-geselecteerde resistente

planten). Het percentage ontsnappers was laag ten opzichte van het aantal getoetste zaailingen, het percentage missers daarentegen was behoorlijk hoog. Dit werd mogelijk veroorzaakt door een hoge ziektedruk. Het percentage ontsnappers ten opzichte van het aantal geselecteerde zaailingen was dusdanig dat natoetsing op kloonniveau noodzakelijk is. Uit de genetische analyse van de resultaten van de zaailingtoets bleek dat de algemene combinatie geschiktheid (ACG) de belangrijkste genetische component was. De ACG-waarde van een cultivar kwam goed overeen met de mate van resistentie bepaald op kloonniveau (zie Hoofdstuk 6).

Indirecte selectie, gebruik makend van moleculaire merkers is onafhankelijk van milieuomstandigheden en kan aan weinig plantmateriaal worden uitgevoerd. Gebruik makend van de Polymerase Chain Reaction (PCR) en Random Amplified Polymorphic DNA (RAPD) werden in nakomelingen van de kruising 'Connecticut King' x 'Orlito' drie zeer significante merkers gekoppeld met *Fusarium*-resistentie opgespoord. Deze merkers verklaarden echter slechts een deel (24 %) van de resistentie. Koppeling van merkers onderling ter verkrijging van een genetische kaart van lelie werd bemoeilijkt door het gebruikte merkertype en het geringe aantal dezelfde onderzochte planten per merker (zie Hoofdstuk 7).

De beschreven methoden in dit proefschrift kunnen een belangrijke bijdrage leveren aan de huidige veredelingsprogramma's bij lelie. Het belang van partiële *Fusarium*-resistentie onder veldomstandigheden in een geïntegreerd bestrijdingsysteem, in combinatie met een reductie van bestrijdingsmiddelen en de invloed van andere pathogenen, verdient nadere aandacht (zie Algemene Discussie).

RELATED PUBLICATIONS

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CURRICULUM VITAE

Theodorus Paulus (Dolf) Straathof werd op 26 maart 1962 geboren te Lisse. Afkomstig uit een bloembollenfamilie was hij van jongs af aan betrokken bij de teelt, broei en export van bloembolgewassen. In 1980 behaalde hij het Atheneum B diploma aan het Fioretticollege te Lisse. In september van dat jaar begon hij aan zijn studie Plantenveredeling aan de Landbouwuniversiteit te Wageningen. De praktijktijd werd in 1985 doorgebracht bij het Department of Horticultural Science van de North Carolina State University. Het doctoraalexamen met verzwaard hoofdvak Plantenveredeling en de bijvakken Tuinbouwplantenteelt, Agrarische Bedrijfseconomie en Erfelijkheidsleer werd in september 1987 behaald. Vanaf 1 oktober 1987 tot 1 februari 1989 heeft hij gewerkt aan celbiologisch onderzoek bij vlas bij de Stichting voor Plantenveredeling in dienst van de Stichting Biogenes. Vanaf 1 februari 1989 tot 1 februari 1994 heeft hij gewerkt op het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek aan het project Resistentieveredeling tegen *Fusarium oxysporum* bij lelie en gladiool in het kader van het door de overheid en bloembollenbedrijfsleven gefinancierde Urgentieprogramma Bollenziekte- en veredelingsonderzoek, hetgeen leidde tot dit proefschrift.