Early blight resistance in tomato:
screening and genetic study

Promotor:

prof. dr. ir. P. Stam
Hoogleraar in de Plantenveredeling
Wageningen Universiteit

Co-promotor:

Dr. ir. R.E. Voorrips
Onderzoeker
Plant Research International

Promotiecommisie:

prof. dr. ir. P.J.G.M. de Wit, Wageningen Universiteit
prof. dr. R.F. Hoekstra, Wageningen Universiteit
Drs. J. J.M. Lambalk, ENZA ZADEN, Enkhuizen
prof. dr. ir. A.H.C. van Bruggen, Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoekschool Production Ecology and Resource Conservation

Early blight resistance in tomato:
screening and genetic study

Reni Chaerani

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op woensdag 8 februari 2006
des namiddags te vier uur in de Aula

CIP-DATA Koninklijke Bibliotheek, Den Haag

Chaerani R (2006)

Early blight resistance in tomato: screening and genetic study

Ph.D. thesis, Wageningen University, the Netherlands – with summaries in English, Dutch and Bahasa Indonesia

ISBN: 90-8504-355-7

Contents

Preface		7
Chapter 1	Tomato early blight (*Alternaria solani*): the pathogen, genetics and breeding for resistance	11
Chapter 2	Assessment of early blight (*Alternaria solani*) resistance in tomato using a droplet inoculation method in glasshouse	31
Chapter 3	QTL identification for early blight resistance (*Alternaria solani*) in a *Solanum lycopersicum* × *S. arcanum* cross	45
Chapter 4	General discussion	61
References		67
Summary		75
Samenvatting (*summary in Dutch***)**		77
Ringkasan (*summary in Bahasa Indonesia***)**		79
Acknowledgements		81
Curriculum vitae		83
PE&RC Ph.D. education statement		85

Preface

Tomato cultivation in Indonesia

Tomato (*Solanum lycopersicum* L. [syn. *Lycopersicon esculentum* Mill.]) was taken to South-East Asia in the 17th century from Europe (Opẽna and van der Vossen 1993). In Indonesia it is the fourth most important vegetable after hot pepper, onions and potato (Asandhi and Sastrosiswojo 1988). Tomato is mostly destined for the local market, used fresh as salad or processed in the ketchup industry. A minor proportion is exported to regional countries.

Tomato is cultivated in the open field in both lowland (<400 m altitude) and highland areas. The main production area is in highlands where optimum temperatures for growth and development (21-24^{o}C) can be achieved (Opẽna and van der Vossen 1993). The average productivity is only 12.7 t/ha, which is less than half the world average (27.2 t/ha, http://faostat.fao.org). The potential of growing tomatoes in Indonesia is great because it is labor intensive and thus generates rural employment; further it expands exports, improves nutrition of the people, and increases the income of growers (Villareal 1980). However, research aimed at the increasing tomato production, including research on resistance, is locally given low priority compared to rice.

In West Java many farmers use local cultivars (landraces) which are probably derived from an ancient imported variety “San Marzano”. This variety is known for its good taste and tolerance to late blight and other diseases (Opẽna and van der Vossen 1993). The recently introduced Taiwanese hybrids became popular because of their high yield capacity. The currently most popular Indonesian cultivars ‘Ratna’, ‘Intan’ and ‘Berlian’, which are based on Asian Vegetable Research and Development Center (AVRDC) lines, are adapted to growth at lower elevations and have bacterial wilt resistance (Opẽna and van der Vossen 1993). In the past few years commercial breeding of hybrid varieties has been initiated in Indonesia itself.

Early blight in Indonesia

It is not known with certainty when *Alternaria solani* (Ellis and Martin) Sorauer, the fungus causing early blight disease, was introduced in Indonesia. Already in the early 1900s it was reported to seriously damage potato plantations in the highlands of Western Java (Rant 1915). Later it spread to the highlands of North Sumatra (van Hall 1925). Potato plantations using healthy seeds showed normal green leaves at the early stage but prior to blooming brown, dry spots with distinct concentric circles appeared on the bottom leaves and later extend over the surface of the leaves (Rant 1915). The upper and lower

leaf sides of the lesions looked dark velvety and were clearly distinguished from those of late blight. Warm and rainy weather promoted the severity of the disease. In damp weather entire leaves were dying off and in only two weeks after the lower leaves were infected the plants usually died (Van Hall 1925). The Dutch name for early blight (EB) was “drogevlekken ziekte” (Rant 1915). The disease soon spread to potato fields at lower elevations causing considerable yield losses (van der Goot 1924). Other solanaceous species including tomato, *Datura* spp. (Jimson weed), *Solanum wendlandii* (giant potato creeper), *S. melongena* (eggplant), and *Cyphomandra betacea* (tree tomato) served as alternative hosts for the fungus (Rant 1915; Paravicini 1923).

At present EB is one of the major diseases of both potato and tomato in Indonesia (Semangun 1989). The expansion of tomato growing areas to lower altitudes, where late blight is less thriving, has increased the incidence of EB in recent years (Asandhi and Sastrosiswojo 1988; Semangun 1989; R. Rodenburg, pers. comm.). Control measures including rotation with non-host crops and sanitation are not entirely satisfactory since the fungus is primarily air-borne, has long survival ability in plant debris, and has a wide solanaceous host range (Semangun 1989). Fungicide treatments are the most effective way to control EB to a non-damaging level (Manohara 1977; Apandi 1979). Typically, fungicides are applied starting from two weeks after transplanting until two weeks before harvest at two- to three- week intervals, but in the wet season a fungicide treatment once or twice per week is necessary (Manohara 1977). Such heavy use of chemicals is not economically feasible for the generally resources-limited Indonesian growers. It also imposes health concerns for growers and consumers as well as environmental hazards. In the long run the intensive use of fungicides could stimulate the emergence of resistant variants of the fungus in Indonesia, in a similar way as has been reported recently in the U.S. (Pasche et al. 2004).

Early blight resistance breeding in Indonesia

Genetic resistance offers an attractive alternative to chemical control because it reduces both production costs and the negative impact of fungicides. Even partial resistance could be useful to reduce the frequency of fungicide applications. Unfortunately, the public tomato breeding program has very limited resources and is mainly devoted to selection and adaptation of introduced lines for high yield capacity. Breeding and research aimed at disease resistance have so far received little support.

The work reported in this thesis aims to stimulate tomato breeding for EB resistance in Indonesia. At the start of the research described here 40 isolates were collected in major tomato cultivation areas in West and East Java, from potato and hot pepper as well as from tomato (Suhardi, Kardin and Gunarto, unpubl. results). An improved resistance test method was applied to identify sources of EB resistance effective

towards Indonesian *A. solani* isolates (Chapter 2). Some materials described by other authors as resistant to *A. solani* was susceptible to an Indonesian isolate, which implies that working with local isolates is important when breeding for resistance effective in Indonesia.

The literature review in Chapter 1 of this thesis showed that EB resistance is expressed quantitatively, is influenced by environmental factors, and is controlled by several genes each with a limited effect. Therefore, classical breeding has so far achieved only limited success, and is also unlikely to result in the development of resistant varieties for the Indonesian market. For that reason, this study was aimed at the identification of genes effective in Indonesia, and to develop markers for these genes that can be used for marker-aided selection. The progress achieved in this direction is reported in Chapter 3.

Scope of this thesis

The research described in this thesis is one of the projects carried out under the auspices of the Biotechnology Research Indonesia–Netherlands (BIORIN) cooperative program. The project was aimed at supporting and improving Indonesian tomato breeding programs with respect to resistance to *A. solani*, the causal agent of EB.

Chapter 1 is a review of the extensive literature on EB and *A. solani* accumulated over the past 60 years. It was deemed useful to review the present knowledge about *A. solani*–tomato interaction to gain insight into the problems of breeding for EB resistance. The review covers all aspects pertaining to the *A. solani* biology, the resistance screening efforts, the characterization of resistance, and the genetic studies of resistance, either using the classical or QTL approaches.

Chapter 2 reports the work on an improved glasshouse test method and its use in identifying sources of EB resistance. Several EB resistance screening methods have been used in the past. For an objective assessment an inoculation technique originally developed in late 1940s was evaluated, improved and used to screen tomato material in glasshouse tests. From these glasshouse screenings, which were carried out with an Indonesian *A. solani* isolate, several strong source of EB resistance were identified, including one in *S. arcanum* (syn. *L. peruvianum*) accession LA2157.

Chapter 3 describes the results of a genetic study of the resistance present in this accession LA2157. A QTL mapping approach was used in an F2 population and the population of derived F3 lines. The results were compared with previous classical genetic and QTL analyses of different resistance sources.

The thesis concludes with a general discussion (Chapter 4). Here the results from the previous chapters are reviewed, and their implications for EB research and practical breeding for resistance to *A. solani* are discussed.

Chapter 1

Tomato early blight (*Alternaria solani*): the pathogen, genetics and breeding for resistance

R. Chaerani and R.E. Voorrips

Abstract

Alternaria solani causes symptoms on foliage (early blight), basal stem of seedlings (collar rot) and stem of adult plants (stem lesions), and on fruits (fruit rot) of tomato. Early blight is the most destructive of these symptoms and hence receives considerable attention in breeding. For over 60 years early blight resistance breeding has been practiced but the development of cultivars with high levels of resistance has been hampered by the lack of sources of strong resistance in the cultivated tomato, the quantitative expression and polygenic inheritance of the resistance. This literature review presents the current knowledge of the *A. solani*–tomato complex with respect to its biology, genetics and breeding.

Isolates of *A. solani* differ markedly with respect to morphological and physiological characteristics. However no conclusive evidence for physiological host specialization has been presented.

Several test methods have been used to assess resistance to *A. solani*, including tests of field of glasshouhouse-grown plants, tests with detached leaves or leaflets, and tests using toxins produced by *A. solani* rather than the fungus itself. Only tests using intact plants inoculated with fungal mycelium or conidia were shown to correlate well with resistance under normal growth conditions.

In some accessions of wild species high levels of resistance to one or more symptoms of *A. solani* have been found but breeding lines still show unfavorable horticultural traits from the donor parent. Recently, the first linkage maps with loci controlling early blight resistance have been developed based on interspecific crosses. These maps may facilitate marker-assisted selection.

(Submitted)

Introduction

Early blight (EB) is the major disease symptom caused by the fungus *Alternaria solani* (Ellis & Martin) Sorauer. This disease, which in severe cases can lead to complete defoliation, is most damaging on tomato (*Solanum lycopersicum* L. [Peralta et al. 2005, syn. *L. esculentum* Mill.]) in regions with heavy rainfall, high humidity and fairly high temperatures (24-29^{o}C). Epidemics can also occur in semiarid climates where frequent and prolonged nightly dews occur (Rotem and Reichert 1964). Apart from the leaf symptoms that are known as EB, *A. solani* causes other symptoms on tomato which are less economically important, including collar rot (basal stem lesions at the seedling stage), stem lesions in the adult plant stage and fruit rot (Walker 1952). Yield losses up to 79% due to EB damage were reported from Canada, India, USA, and Nigeria (Basu 1974b; Datar and Mayee 1981; Sherf and MacNab 1986; Gwary and Nahunnaro 1998). Collar rot can cause seedling losses in the field of 20 to 40% (Sherf and MacNab 1986).

The control measures include a 3- to 5-year crop rotation, routine applications of fungicides, and the use of disease-free transplants (Madden et al. 1978; Sherf and MacNab 1986). Fungicide treatments are generally the most effective control measures, but are not economically feasible in all areas of the world and may not be effective under weather conditions favorable for epidemics (Herriot et al. 1986). Resistant cultivars are potentially the most economical control measure as they can extend the fungicide spray intervals while maintaining control of the disease (Madden et al. 1978; Shtienberg et al. 1995; Keinath et al. 1996).

The progress in EB resistance breeding has been limited by the lack of effective resistance genes in cultivated tomato (Vakalounakis 1983; Poysa and Tu 1996; Banerjee et al. 1998; Vloutoglou 1999), quantitative expression and polygenic inheritance of the resistance (Barksdale and Stoner 1977; Maiero et al. 1989; Nash and Gardner 1988a; Maiero et al. 1990a; Thirthamalappa and Lohithaswa 2000). Sources for EB resistance have been identified in wild relatives of tomato. Some of these have been utilized through traditional breeding approaches but an increased level of resistance is negatively correlated to earliness (Nash and Gardner 1988a; Maiero 1989; Foolad and Lin 2001; Foolad et al. 2002a) and yield (Barrat and Richards 1944). The most resistant breeding lines and hybrid cultivars with acceptable horticultural characteristics that are currently available have moderate resistance to EB and are slightly later in maturity (Gardner 1988; Gardner and Shoemaker 1999; Gardner 2000). Therefore, resistant cultivars with better horticultural traits are still needed.

Classical quantitative genetic analyses have provided estimates of the number of quantitative trait loci (QTLs) for EB resistance, average gene action and heritabilities which provided the prospects for progress in breeding programs based on phenotypic selection (Nash and Gardner 1988a; Maiero et al. 1990a; Maiero et al. 1990b). However, such studies are unable to determine the effects of individual genes and their locations on

the tomato genome. More recent genetic studies on EB resistance have been directed to the mapping and characterization of QTLs determining the resistance with the aid of molecular marker maps (Foolad et al. 2002b; Zhang et al. 2003; Chapter 3). Markers closely linked to QTLs can be used to select individual plants with the most desirable QTLs. By fine mapping it is also possible to resolve whether the unfavorable traits associated with EB resistance are due to pleiotropic effects of resistance genes or to closely linked genes. If they are linked marker-based selection might facilitate breaking the linkage.

In this chapter the literature pertaining to aspects of resistance to EB and to a lesser extent also collar rot and stem lesions, is reviewed. First we describe the biology of *A. solani* and the symptoms caused by the fungus, followed by methods for selecting resistance to EB and collar rot. The next section presents the known sources of resistance followed by classical genetic studies of EB, collar rot and stem lesion resistance, as well as their genetic interrelationship. Mapping of resistance genes is presented in the following section. Physiological aspects affecting EB resistance and characterization of EB resistance are discussed in the next two sections. The paper concludes with perspectives for EB resistance breeding.

The pathogen

Since the first description by Ellis and Martin in 1882 (cited in Sherf and MacNab 1986), *A. solani*, previously known as *A. porri* f. sp. *solani* (Neergaard 1945), has been the object of intensive studies (Strandberg 1992; Rotem 1994). *A. solani* belongs to the Fungi Imperfecti (Deuteromycotina) in the class Hyphomycetes and order Hyphales (Agrios 2005). An Ascomycete fungus, *Pleospora solani*, has been claimed by Esquivel (1984) as the teleomorphic stage of *A. solani*, but this has not been confirmed by others. *A. solani* belongs to the large-spored group within the genus *Alternaria*, which is characterized by separate conidia borne singly on simple conidiophores (Neergaard 1945). The conidia of *A. solani* are muriform and beaked (Neergaard 1945; Ellis and Gibson 1975). Like other members of the genus *Alternaria*, *A. solani* has transverse and longitudinal septate conidia, multinucleate cells, and dark-coloured (melanized) cells (Rotem 1994). Melanin gives protection against adverse environmental conditions including resistance to antagonistic microbes and their hydrolytic enzymes (Rotem 1994).

Disease cycle

Under free moisture or near saturated humidity conditions at a wide range of temperature (8 to 32^{o}C), conidia germinate to produce one or more germ tubes. These subsequently penetrate the host epidermal cells directly by means of penetrating hyphae or enter through stomata or wounds by hyphal growth (Sherf and MacNab 1986; Perez and

Martinez 1999; Agrios 2005; Figure 1). Penetration can occur at temperatures between 10 to 25°C (Sherf and MacNab 1986). Host colonization is facilitated by enzymes (cellulases, pectin methyl galacturonase) that degrade the host cell wall, and by alternaric acid, a toxin which kills host cells and enables the pathogen to derive nutrients from the dead cells (Langsdorf et al. 1991). Lesions become visible two to three days after infection and spore production occurs three to five days later (Sherf and MacNab 1986). This relatively short disease cycle allows a polycyclic infection (Sherf and MacNab 1986; van der Waals 2001). The fungus survives between crops as mycelia or conidia in soil, plant debris and seed (Sherf and MacNab 1986; Figure 1). Also chlamydospores can serve as survival structures (Basu 1974a; Patterson 1991). Therefore, the life cycle of *A. solani* includes soil-, seed- as well as air-borne stages which make the pathogen difficult to control by means of rotation and sanitation.

The main hosts of *A. solani* are solanaceous crops including tomato, potato, eggplant and pepper (Ellis and Gibson 1975; Neergaard 1945).

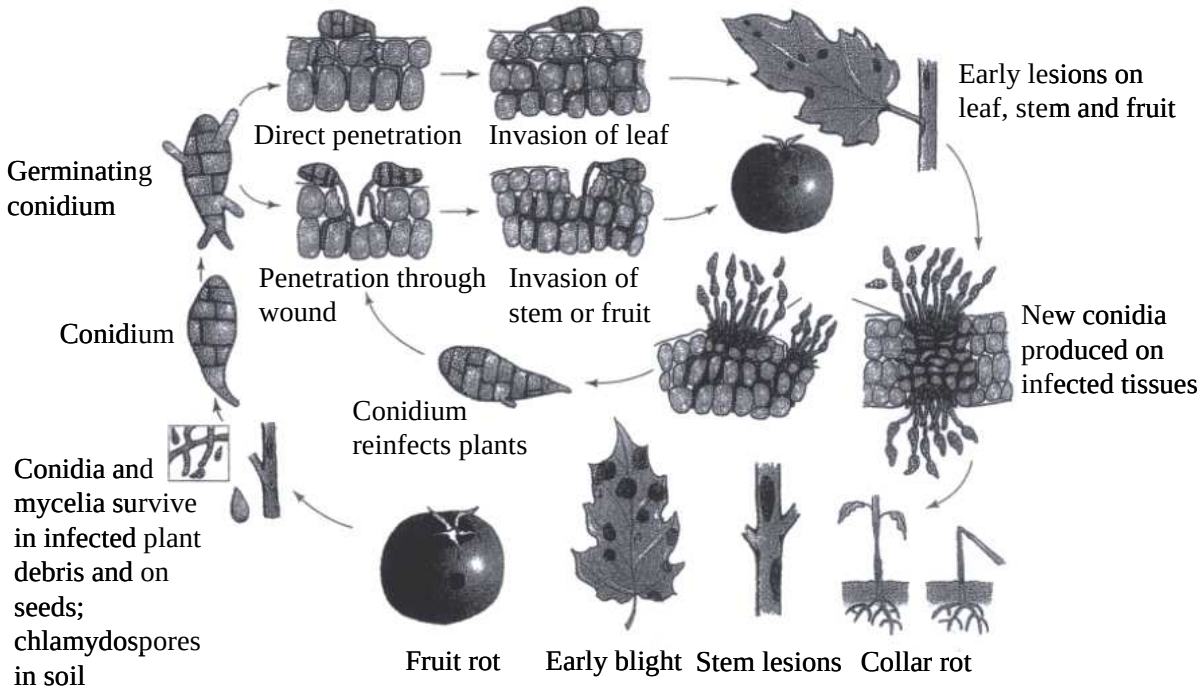

Figure 1 Infection process, development and symptoms of diseases caused by *Alternaria solani* (adapted from Agrios 2005)

Toxin production

Eleven toxins have been identified in culture filtrates of *A. solani* (Montemurro and Visconti 1992). Among these, alternaric acid, solanapyrone A, B, and C are able to induce necrotic symptoms similar to EB symptoms (Montemurro and Visconti 1992). Alternaric

acid is one of the major toxins found in the filtrates (Brian 1952) and is probably the main toxin for the development of necrotic and chlorotic symptoms (Pound and Stahmann 1951). Alternaric acid is already present in dormant spores and is produced and released by germinating spores (Langsdorf et al. 1990). Alternaric acid does not cause phytotoxicity when sprayed alone on tomato leaves, but it enhances the infection process and the development of necrotic symptoms when added to *A. solani* spore suspensions (Langsdorf et al. 1990). Another factor in *A. solani* spores was required for infection. This substance, referred to as S1, is non-toxic and is present in a water-soluble fraction from chloroform extracts of spore-germination fluid. This factor allowed the spores of a non-pathogenic strain of *Alternaria alternata* to cause necrotic symptoms on tomato and potato (Langsdorf et al. 1990).

Variability among isolates

Although *A. solani* appears to have only a non-sexual life cycle it exhibits a relatively large variation in morphology *in vivo* and *in vitro*, physiology, genetic makeup and pathogenicity among isolates (Bonde 1929; Wellman 1943; Neergaard 1945; Henning and Alexander 1959; Rotem 1966; Weir et al. 1998; Martinez et al. 2004; van der Waals et al. 2004). Bonde (1929) and Neergaard (1945) classified *A. solani* into conidial, mycelial and intermediate types of isolates. Pathogenic differences were found among isolates originating from different germ tube tips from the same conidium (Stall 1958).

A high genetic diversity was detected among the *A. solani* isolates originating from the U.S.A., South Africa, Cuba, Brazil, Turkey, Greece, Canada, China and Russia based on vegetative compatibility groups (VCG, van der Waals et al. 2004) and molecular markers (isozymes, random amplified polymorphic DNA [RAPDs], random amplified microsatellites [RAMs] and amplified fragment length polymorphism [AFLPs]; Petrunak and Christ 1992; Weir et al. 1998; Martinez et al. 2004; van der Waals et al. 2004). In studies where isolates from several countries were compared using VCG assays, RAMs (van der Waals et al. 2004) or RAPD markers (Weir et al. 1998), *A. solani* isolates cluster according to country, indicating some degree of genetic isolation. In contrast, isolates from the same country show no distinct separation based on geographical origin (Petrunak and Christ 1992; Weir et al. 1998; Martinez et al. 2004; van der Waals et al. 2004). This can be ascribed to short- or medium-distance dispersal of the air-borne spores and transport of plant material within the countries (Weir et al. 1998; van der Waals et al. 2004). In many cases isolates originating from tomato and potato clustered according to their hosts based on RAPD (Weir et al. 1998) and AFLP markers (Martinez et al. 2004), suggesting host specialization. Organ specificity was reported to occur among Bulgarian isolates by Stancheva (1990) but has not been described by other authors. Associations of

molecular markers with variability in physiology, morphology, and virulence are not known.

So far conclusive evidence for the existence of physiological races is lacking. Physiological races are defined based on differential host specificity (Mehrotra and Areja 1990; Schlegel 2003). Therefore, Bonde's (1929) report of the presence of *A. solani* physiological races is not correct according to the current definition since he described those races in terms of variability in physiological, morphological, and ecological characters in *in vitro* culture. Henning and Alexander (1959) characterized isolates on tomato and related species with quantitative variation in resistance. Some of these isolates, which showed cultural differences, appeared to be host-specific but the pattern of infection was not consistent between experiments. This was attributed to heterogeneity of the host lines and the unstable nature of the isolate cultures (Henning and Alexander 1959). Similarly Castro et al. (2000) could not demonstrate consistent host-specific reactions of isolates.

Heterokaryosis could be the driving force for genetic variation in *A. solani* (Stall 1958). Heterokaryosis is the occurrence of genetically different nuclei in the same cells. This can be the result of hyphal anastomosis, a process observed in *A. solani* (Stall and Alexander 1957; Stall 1958). After establishment of heterokaryosis, this state may be maintained or lost during further cell divisions. Also nuclear migration is possible through septal pores between cells of conidia, conidiophores, mycelia, and cells connecting these structures, allowing dissociation of unlike nuclei leading to homokaryosis and conversely, also the re-establishment of heterokaryosis (Stall 1958). Therefore, even isolates obtained from single conidia and hyphal tips are genetically unstable. In their studies Stall and Alexander (1957) observed frequent occurrence of anastomoses but failed to obtain heterokaryosis as indicated by the absence of segregation of cultural types.

The ability of *A. solani* to maintain a large genetic variability allows it to react quickly to changing environments. For example, a recent study demonstrated that isolates in the mid-western US have become less sensitive to a fungicide resulting in significant yield losses in glasshouse cultures (Pasche et al. 2004). The high genetic diversity and high degree of gene flow within countries could break down genetic resistance in the host; this has been advanced as one of the reasons for the absence of potato cultivars with complete resistance to *A. solani* in South Africa (van der Waals et al. 2004).

Disease symptoms

All above ground parts of plants can be infected by *A. solani* and various names are given for the different symptoms, which has often led to confusion (Sherf and MacNab 1986). In this paper we refer to the symptoms on foliage as early blight (EB), to symptoms on stems as collar rot when it affect seedlings and as stem lesions on adult plant stage, and to

symptoms on fruits as fruit rot (Walker 1952).

The first symptoms of EB are small, dark, necrotic lesions that usually appear on the older leaves and spread upward as the plants become older (Sherf and MacNab 1986). As lesions enlarge, they commonly show concentric rings giving a target board-like appearance and are often surrounded by a yellowing zone. In severe epidemics *A. solani* can cause premature defoliation, which weakens the plants and exposes the fruit to injury from sunscald (Sherf and MacNab 1986).

Large, dark and sunken lesions may appear on the stems of seedlings at the ground line, causing partial girdling known as collar rot (Sherf and MacNab 1986). Seedlings are weakened and can die when the stem is completely girdled by the lesion. On the main stem and side branches of adult plants, the fungus causes small, dark, slightly sunken areas that enlarge to form dark brown elongated spots, which occasionally show concentric rings like those on the leaves. These spots are scattered along the stem and branches (Walker 1952). Some authors make no distinction between collar rot and stem lesions (cf. Gardner 1990). In older literature collar rot and stem lesion are sometimes referred to as stem canker (Barksdale and Stoner 1977), a term which is currently reserved for the disease caused by *Alternaria alternata* (Sherf and MacNab 1986).

On green or ripe fruits dark, velvety, sunken spots may occur at the stem end. These spots occasionally develop from mycelial extension from stem lesions, reach a considerable size and may show distinct concentric markings like those on the leaves (Sherf and MacNab 1986). Semi-ripe fruits are more susceptible than ripe ones (Mehta et al. 1975). Heavily infected fruits frequently drop before they mature. On susceptible genotypes the calyx and blossom may also become infected (Pandey et al. 2003).

Screening methods

Reliable and repeatable techniques for large-scale screening are necessary to identify host plant resistance. Techniques have been developed for EB and collar rot resistance screening under field, glasshouse, and laboratory conditions. In laboratory, both fungal inocula (spores and mycelia) and fungal toxins have been used in screening for resistance.

Inoculum production

A. solani can be artificially grown in various culture media but it does not readily sporulate *in vitro.* Spore production requires special conditions such as mycelial wounding or transferring pieces of the culture on minimal medium or filter paper followed by exposure to UV light, fluorescent light, direct sunlight or a combination of fluorescent light and partial desiccation (Charlton 1953; Lukens 1960; Barksdale 1969; Douglas and Pavek 1971; Padhi and Rath 1973; Shanin and Shepard 1979). Efficient sporulation can be

induced by exposing cultures under diurnal light in a partially opened culture dish, after removal of aerial mycelia (Barksdale 1969). For maintenance of wild type culture Barksdale (1969) suggested to mass transfer sections of culture which show 'normal appearing areas' since variants in culture are often obtained even though the culture is started from single spores. When *in vitro* culture-derived spores are difficult to obtain, mixed inocula of spores and mycelia obtained from dried (Thirthamalappa and Lohithaswa 2000) or freshly (Chapter 3) infected leaves are sometime used in field experiments.

Field screening

In field tests, large populations can be assessed under normal growth conditions during the whole life cycle of the plants. Artificial inoculation by (repeated) spraying of inoculum and/or the use of spreader rows is required to enhance natural infection and to obtain uniform disease pressure. Prior to inoculation it is often necessary to prevent or eradicate foliar diseases by scheduled fungicide sprays (Nash and Gardner 1988a).

EB severity in the field is assessed in terms of percent defoliation and the average fraction of necrotic leaf area on the plant (Horsfall and Barrat 1945). Symptoms on the upper leaves can be disregarded because the necrotic areas on these leaves are less than 2% of the total damage during the growing season (Basu 1974b). Therefore, counting the number of leaves having 75 to 100% necrotic area on lower half of plants (Basu 1974b), or estimating the percentage of necrotic area in the middle third of the plant canopy (Christ 1991) are reliable indicators for EB severity.

EB epidemics initially progress slowly but accelerate as plants mature, resulting in a typical sigmoidal disease progress curve (Nash and Gardner 1988b). Occasionally the disease curve is bimodal which could be due to the emergence of new healthy leaves after the first cycle of infection (Pandey et al. 2003). Therefore a once-only evaluation can underestimate or overestimate the actual level of resistance of a particular host, and field assessments must be based on several observations which are subsequently used to calculate the AUDPC. With the AUDPC the host, pathogen, and environmental effects occurring during the epidemic are integrated (Pandey et al. 2003).

In spite of their advantages, field tests also have their problems: they are slow, labor intensive, highly affected by the presence of other pathogens, not suitable for evaluation of single plants in a large-scale experiment, and they are sensitive to environmental conditions that are difficult to control.

Glasshouse screening

Glasshouse or controlled-environment chamber assays with seedlings or small plants provide uniform, favorable, repeatable environmental conditions and permit several cycles of screening per year, thus offering more reliable results. Glasshouse and field test results correspond well (Banerjee et al. 1998; Foolad et al. 2000). Glasshouse or controlled-environment chamber evaluations of young plants were mainly used for preliminary selection of *A. solani* resistance sources from large collections (Barksdale 1969; Vakalounakis 1983; Poysa and Tu 1996; Vloutoglou 1999) and to study collar rot inheritance. Glasshouse evaluation of EB resistance for genetic studies is for the first time described in Chapter 3.

The current glasshouse screening methods for *A. solani* resistance are based on the method established by Barksdale (1969). Generally, seedlings are spray inoculated with spores at an age of 4 to 6 weeks (Barksdale 1969; Marcinkowska 1982; Nash and Gardner 1988b; Banerjee et al. 1998; Vloutoglou 1999; Foolad et al. 2000). Leaves can be injured prior to spray by rubbing leaf surfaces between thumb and forefingers (Poysa and Tu 1996). Plants are incubated 24 hours under 100% relative humidity (RH) followed by 12-16 hours of 100% RH during the night period for 5-7 days in a mist chamber, mimicking repeated nightly dew in nature. During the day, plants are exposed to ambient RH to allow the development of disease symptoms. A leaf wetness period of at least 4 h after inoculation was required for infection (Moore 1942; Vloutoglou and Kalogerakis 2000). Increasing this period up to 24 h induced progressively higher EB severity, but more than 24 h humidity periods did not increase severity further (Vloutoglou 1999).

EB severity is usually determined at seven days after spray inoculation by estimating the percent necrotic area on leaves which were present at the time of inoculation (leaves emerging after inoculation are not affected, Barksdale 1969; Vloutoglou 1999). In the case of a low incidence of necrotic spots, EB severity is expressed as the number of lesions (Barksdale 1969).

Disease severity can be determined more precisely and objectively by measuring lesion sizes when the inoculum is applied as single drops on leaflets (Nash and Gardner 1988b; Chapter 2).

Glasshouse tests have also been used for assessing collar rot and stem lesion resistance (Gardner 1990; Maiero et al. 1990b). The basal stem of seedlings is sprayed with spores and covered with soil (Maiero et al. 1990b) or seedlings are placed in a humidity chamber (Gardner 1990). Collar rot is usually rated in three to five symptom grades (Reynard and Andrus 1945; Gardner 1990; Maeiro et al. 1990b). Screening for collar rot and stem lesions in the glasshouse is fast and can be used instead of field screening for EB resistance, provided that the resistance to these disease symptoms is closely associated in the materials used, such as in C1943 and derived lines (Gardner 1990).

Glasshouse tests have the advantage that conditions are more reproducible than in the field, that the duration of the test is shorter and that, especially after droplet inoculation, more objective and precise data can be obtained. Still, conditions in the glasshouse cannot be fully controlled, and some genotypes are not well adapted to glasshouse conditions.

Laboratory assays

Locke (1948) used detached leaflets assays for evaluation of EB resistance, as a means to circumvent the influence of growth habit, which may affect the reaction of plants in the field or glasshouse. The method involved the application of inoculum droplets on either punctured (Locke 1948) or non-punctured (Foolad et al. 2000) young, fully expanded leaflets. Locke (1948) claimed the method be reliable; Lynch et al. (1991) and Foolad et al. (2000) however, concluded that detached leaflet assays did not correlate well with field and glasshouse screenings. These results might imply that a whole plant is required for the expression of EB resistance, which is known to be influenced by the physiological state of plant such as maturity, determinism (Nash and Gardner 1988a; Maiero 1989; Foolad and Lin 2001; Foolad et al. 2002a), yielding ability (Barrat and Richards 1944) and also plant age and nutritional status (Rotem 1994).

To circumvent the problem of apparent resistance exhibited by late maturing cultivars, Bussey and Stevenson (1991) induced early senescence in juvenile potato leaf tissue by floating excised disks on a solution containing NAA or 2,4-D. A very late-maturing cultivar which was highly resistant in the field reacted more susceptible when tested using the leaf disk assay, suggesting that the assay may be less influenced by cultivar maturity than field test (Bussey and Stevenson 1991). The results of the other tested cultivars agreed with those obtained in the field (Bussey and Stevenson 1991).

Laboratory assays on detached leaflets therefore show promise for studying particular aspects of resistance and for eliminating confounding influences of whole-plant physiology. However, these methods need to be carefully tuned for the research question in hand, and cannot be relied on as a replacement for field or glasshouse tests.

Toxin assays

Several authors reported that *A. solani* culture filtrate could be used to distinguish EB resistant from susceptible genotypes, at least in progenies of some sources of resistance (Lodha 1977; Stancheva 1988; Maiero et al. 1991). Genotypes with collar rot resistance showed a higher tolerance to culture filtrate than those with only EB resistance (Maiero et al. 1991). In contrast, Lynch et al. (1991) found that the result of culture filtrate assays using detached leaflets did not correspond with the result of glasshouse or field tests.

Darakov (1995) proposed a new approach of selecting EB resistance by means of gametophytic selection in the presence of an unidentified toxin obtained from culture filtrate of *A. solani*. Pollen tube elongation correlated well with the level of EB resistance of the mother plant. Female gametophytic selection was done by treating styles of emasculated flowers with drops of toxin and after pollination, collecting seeds from plants which yielded most seeds. After two rounds of selection with toxin, selected plants with enhanced seed-bearing capacity were assessed in the field for EB resistance. Plant selections from toxin-treated plants showed enhanced EB resistance compared to those derived from plants selected with a water treatment.

Laboratory assays using alternaric acid can help to elucidate specific aspects of the pathogenesis process. However, alternaric acid only enhances the infection process and therefore cannot be the sole cause of differential interaction between isolates and host genotypes (Langsdorf et al. 1990). This is in contrast to toxins produced by formae speciales of *Alternaria alternata*, which do elicit most symptoms of the disease on susceptible plants and show the same differential host specificity as the fungal isolates, and which therefore can be used reliably for resistance screening (Gilchrist and Grogan 1975).

Sources of resistance

In the cultivated tomato high levels of resistance to EB are rare. Two old breeding lines, 71B2 and C1943, probably bred from *S. lycopersicum* sources, have been described as highly and moderately resistant to EB, respectively (Table 1). Some moderately resistant hybrids and breeding lines have been developed from these sources, such as ‘Plum Dandy’, NC EBR-5 and -6 (71B2), ‘Mountain Supreme’ and NC-EBR-2 (C1943). Poysa and Tu (1996) identified only eleven moderately resistant lines from more than 500 tomato cultivars and breeding lines after testing for EB resistance.

Some accessions of the wild species *S. habrochaites* (syn. *L. hirsutum*), *S. peruvianum* (syn. *L. peruvianum*) and *S. pimpinellifolium* (syn. *L. pimpinellifolium*) are resistant to EB (Table 1). Success to incorporate the resistance trait is limited as most breeding lines, e.g. NC EBR-1, NC EBR-2 (Gardner 1988), NC EBR-4 (Gardner and Shoemaker 1999), HRC90.303 and HRC91.341 (Poysa and Tu 1996) are still late maturing, indeterminate, and relatively low-yielding. These lines are derived from *L. hirsutum* accessions.

A high level of collar rot resistance has been found in the cultivated tomato such as in the old cultivar ‘Devon Surprize’ and breeding line C1943. Additionally, Stancheva et al. (1991a) reported sources of resistance to collar rot and stem lesions in several wild species (Table 1).

Table 1 Genetic sources of resistance to early blight, collar rot and stem lesion

Original source	Resistant line or variety	Test(s) used to confirm resistance	References
	Early blight resistance		
Solanum lycopersicum (syn. *Lycopersicon esculentum*)[a]			
Unknown source	C1943	Field	Barksdale 1971
68B134	71B2	Field	Barksdale 1969
Syn. *L. esculentum* f. sp. *cerasiforme*[b] PI 406758	-	Field	Martin and Hepperly 1987
C1943	NC EBR-2	Field, glasshouse	Gardner 1988
Unknown accessions	HRC90.145, HRC 90.158, HRC 90.159	Glasshouse	Poysa & Tu 1996
NC EBR-1	NC EBR-4	Field	Gardner and Shoemaker 1999
NC EBR-1	IHR1816	Field	Thirthamalappa and Lohithaswa 2000
NC EBR-1 and -2	NC EBR-3	Field	Gardner and Shoemaker 1999
NC EBR-3 and -4	'Mountain Supreme'	Field	Gardner and Shoemaker 1999
NC EBR-5 and -6	'Plum Dandy'	Field	Gardner 2000
71B2	NC EBR-5	Field	Gardner 2000
71B2	NC EBR-6	Field	Gardner 2000
S. habrochaites (syn. *L. hirsutum*)[a]			
PI 127827	-	Laboratory	Locke 1949
PI 390514, PI 390662	-	Field	Martin and Hepperly 1987
PI 126445	NC EBR-1	Field	Gardner 1988
PI 1390662	87B187	Field	Maiero et al. 1990a
B 6013	H-7, H-22, H-25	Field	Kallo and Banerjee 1993
Unknown accessions	HRC90.303, HRC 91.279, HRC 91.341	Glasshouse	Poysa and Tu 1996
LA2100, LA2124, LA2204	-	Glasshouse	Poysa and Tu 1996
PI 126445	NC39E	Field	Foolad et al. 2002a
S. peruvianum (*L. peruvianum*)[a]			
PE33	-	Glasshouse	Poysa and Tu 1996
S. pimpinellifolium (syn. *L. pimpinellifolium*)[a]			
PI 365912, PI 390519	-	Field	Martin and Hepperly 1987
A 1921	P-1	Field	Kallo and Banerjee 1993
L4394 (IHR1939)	-	Field	Thirthamalappa and Lohithaswa 2000
	Collar rot resistance		
Unknown source	'Devon Surprize'	Field	Reynard and Andrus 1945
Unknown source	C1943	Glasshouse	Maiero et al. 1990b
S. pimpinellifolium (syn. *L. racemigerum*)[b] 87610005	-	?	Stancheva et al. 1991a
S. lycopersicum (syn. *L. humboldtii*)[b] 87610003	-	?	Stancheva et al. 1991a
S. chilense (syn. *L. chilense*)[a] 87610011	-	?	Stancheva et al. 1991a
	Stem lesion resistance		
S. lycopersicum 83602029	-	?	Stancheva et al. 1991a
S. cheesmaniae (syn. *L. cheesmanii* f. *typicum*)[b] 15	-	?	Stancheva et al. 1991a
S. neorickii (syn. *L. minutum*)[b] 87610006	-	?	Stancheva et al. 1991a

[a]Peralta et al. (2005);

[b]I. Peralta, S. Knapp and D. Spooner (pers. comm.)

Classical studies of genetics of resistance

Most genetic studies on the inheritance of EB resistance using different sources of resistance (*S. lycopersicum*, *S. habrochaites* and *S. pimpinellifolium*) arrived at the same conclusions that the resistance is a quantitative trait that is controlled polygenically (Table 2). The estimated minimum number of controlling factors is two (Barksdale 1977) or three (Nash and Gardner 1988a). Analysis using quantitative genetic methods (generation mean analysis and scaling tests) and several sources of resistance (C1943, NC EBR-2, IHR 1939 and IHR 1816) revealed additive and dominant genetic control with the presence of epistatic effects (Maiero 1990a; Nash and Gardner 1988a; Thirthamalappa and Lohithaswa 2000).

The EB resistance genes in C1943 and 71B2 are recessive and not allelic (Barksdale and Stoner 1977; Maiero 1989). However, in crosses of these two resistance sources with another susceptible genotype, the F1 hybrids were intermediate, indicating additive genetic control or partial dominance (Maiero 1989). Recessive genes have been reported in *S. lycopersicum* 83602029 (Stancheva 1991), in IHR1939 and IHR1816 by Thirthamalappa and Lohithaswa (2000). Partially dominant inheritance has been found in *S. pimpinellifolium* and *S. habrochaites* (Martin and Hepperly 1987).

The line 87B187, derived from *S. habrochaites* PI 390662, shared common resistance genes with NC EBR-2, although this line was developed via C1943 from a *S. lycopersicum* source (Maiero 1990a). Also, Thirthamalappa and Lohithaswa (2000) reported independent genes in IHR 1939 (*S. pimpinellifolium* L4394) and those in IHR 1816 (derived from NC EBR-1, which was developed from *S. habrochaites* PI 126445).

In contrast to all studies described above, one study reported a monogenic, dominant inheritance in *S. habrochaites* PI 134417 (Datar and Lonkar 1985). Their conclusion is arguable since a highly resistant F1 does not necessarily indicate the complete dominance of EB resistance as was observed by Foolad and Lin (2001). The resistance phenotypes in F2 population derived from *S. habrochaites* PI 134417 were grouped into resistant, intermediate, and susceptible, and a 3 : 1 segregation was observed, leading to the conclusion of monogenic inheritance (Datar and Lonkar 1985). However, EB resistance is a quantitatively expressed character and the assignment of three phenotypic classes is therefore arbitrary and may have led accidentally to the 3 : 1 segregation (Foolad and Lin 2001).

There are only few genetic studies published on resistance to the other disease symptoms caused by *A. solani*, a fact that may be caused by the less damaging effect of these two disease symptoms (Table 2). One study on collar rot resistance reported a monogenic inheritance (Reynard and Andrus 1945) whereas a study by Maiero et al. (1990b) showed a quantitative expression of the resistance. Analysis by Maiero et al. (1990b) using a joint scaling test showed that both additive and dominance effects

controlled the collar rot resistance in C1943 and NC EBR-2 sources, although dominance effect of susceptibility appeared to be more important.

Only one study on stem lesion resistance has been published which reported that the resistance is a quantitative trait controlled by dominant genes in *S. lycopersicum* source (Stancheva 1991; Table 2). Both additive and dominant genetic components conferred resistance which was complicated by epistatic effects.

Fruit rot has escaped attention in genetic studies although it may cause substantial direct losses (Datar and Mayee 1981). Resistance to fruit rot may be controlled independently from EB resistance since fruit rot incidence is not necessarily associated with EB severity (Barksdale 1971).

Little is known about the genetic relationships among EB, collar rot and stem lesion resistance. Maiero et al. (1990b) postulated that the collar rot resistance gene in C1943 and its derived line, NC EBR-2, is one of the genes that confer EB resistance or is closely linked with EB resistance genes since these lines have both EB and collar rot resistance.

Also stem lesion resistance may be independent of EB resistance. Barksdale and Stoner (1973, 1977), based on field observations but unsupported by a genetic analysis, assumed that stem lesion resistance segregated independently from EB resistance. Recently, several QTLs which had effects on both EB severity and stem lesions have been reported (Chapter 3).

Heritability of EB resistance has been estimated in crosses involving *S. habrochaites* PI 126445 (Foolad and Lin 2001) and derived lines NC EBR-1 and NC39E (Nash and Gardner 1988a; Foolad et al. 2002a). Depending on the calculation method, heritability estimates were low to moderate in two crosses involving NC EBR-1 (Nash and Gardner 1988a). Based on parent–offspring (PO) regression narrow sense heritability (h^2) for AUDPC was estimated as 0.26 and 0.38 (Nash and Gardner 1988a). Higher h^2 estimates were obtained from a cross with *S. habrochaites* PI126445 (0.70, Foolad and Lin 2001) and from a cross with *S. lycopersicum* NC39E (0.65, Foolad et al. 2002a), also based on PO regression.

Those studies showed that additive genetic components play a small to moderate role in the quantitative expression of resistance. The low to moderate heritability estimates indicate that progress based on phenotypic evaluations only is likely to be slow. Further, these classical genetic studies give general indications on the likely progress in selection of resistant material but do not provide information on the effects of individual resistance genes and their location on the tomato genome.

Table 2 Classical genetic studies of early blight, collar rot and stem lesion resistance in tomato

Resistant parent[a]	Population type	Test[b]	Analysis method	Genetic control[c]	Reference
			Early blight resistance		
S. lycopersicum 71B2	F1	F	Diallel, midparent–hybrid comparison	Recessive polygenic	Maiero et al. (1989)
S. lycopersicum C1943	F1	F	Diallel, midparent–hybrid comparison	Recessive polygenic	Maiero et al. (1989)
S. lycopersicum C1943	F1, F2, BC1, BC2	F	Diallel, midparent–hybrid comparison, generation means, joint scaling tests	Recessive polygenic with additive and epistatic (dom × dom) effects	Maiero et al. (1990a)
S. lycopersicum NCEBR-1	F1, F2, BC1, BC2	F	Generation means, joint scaling tests	At least 3 genes with additive, dominance, and epistatic (add × add, add × dom, dom × dom) effects	Nash and Gardner (1988a)
S. lycopersicum NCEBR-2	F1	F	Diallel, midparent–hybrid comparison	Polygenic, partial dominant	Maiero et al. (1990a)
S. lycopersicum 87B187	F1	F	Diallel, midparent–hybrid comparison	Polygenic, partial dominant	Maiero et al. (1990)
S. lycopersicum 83602029	F1, F2, BC1, BC2	?	Diallel, generation means	Quantitative, dominant genes with additive, dominance, and epistatic effects	Stancheva (1991)
IHR 1816 (= *S. lycopersicum* NCEBR-1)	F1, F2, BC1, BC2	F	Joint scaling tests	Recessive polygenic with additive and epistatic (add × dom) effects at seedling stage; with additive, dominance and epistatic (add × add) effects at adult stage	Thirthama-lappa and Lohithaswa (2000)
IHR 1939 (= *S. pimpinellifolium* L4394)	F1, F2, BC1, BC2	F	Joint scaling tests	Recessive polygenic with additive and epistatic (add × dom) effects at seedling stage; with additive, dominance and epistatic (add × add) effects at adult stage	Thirthama-lappa and Lohithaswa (2000)
S. lycopersicum NC39E	F2, F3	F	Midparent-segregating population means comparison	Polygenic, partial dominant	Foolad et al. (2002a)
			Collar rot resistance		
S. lycopersicum C1943, NCEBR-2	F1, F2, BC1, BC2	GH	Diallel, midparent–hybrid comparison, generation means, joint scaling tests	Recessive polygenic with additive and dominant effects	Maiero et al. (1990b)
			Stem lesion resistance		
S. lycopersicum 83602029	F1, F2, BC1, BC2	?	Generation means	Recessive polygenic with additive, dominance, and epistatic effects	Stancheva (1991)

[a]new nomenclature based on Peralta et al. (2005); please refer to Table 1 for synonyms

[b]F = field, GH = glasshouse.

[c]add = additive, dom = dominance

Mapping resistance genes

Given the low to moderate heritability estimates, a marker-aided selection approach is potentially useful to accelerate the transfer of EB resistance genes into new tomato cultivars. Foolad et al. (2002b) were the first to map QTLs for EB resistance. They used backcross progenies of a cross between *S. habrochaites* PI 126445 and a susceptible tomato line. Mapping was done in the BC1 and validated in the BC1S generation. Fourteen QTLs were identified which together explained 57% of the total phenotypic variation. For all QTLs the positive allele originated from the resistant parent. In a subsequent study Zhang et al. (2003) used a selective genotyping approach on a different part of the same BC1 population. Seven QTLs were detected, including one previously mapped major and three minor QTLs. One of the QTLs in this study inherited the resistance allele from the susceptible parent.

Chaerani et al. (Chapter 3) identified six QTLs for EB resistance in F2 and F3 populations from a cross between the resistant *S. arcanum* LA2157 and a susceptible tomato. Different environments and phenotypic scoring methods were used in this study, in contrast to the previous mapping studies which used one type of environment and disease measure. In addition, resistance to stem lesions was also assessed in the F3 population. Interestingly, EB QTLs detected in the F2 population were not always detected in the F3 population, and vice versa. This indicates the presence of environment-specific or plant age-specific QTLs. Three QTL regions for stem lesion resistance coincided with EB resistance QTLs, which allows simultaneous selection for resistance to both types of disease symptoms. The explained phenotypic variation per EB resistance QTL, 7 to 16%, was in the same range as that of Foolad et al. (2002b). One QTL for stem lesion resistance, however, had a large effect, explaining 31% of the total variation. For two of the six QTLs, the susceptible parent contributed the resistance alleles. Several of the QTLs found in the cross of *S. habrochaites* PI 126445 (Foolad et al. 2002b; Zhang et al. 2003) overlapped with those found in the *S. arcanum* LA1257.

Although many EB resistance QTLs have been identified, many of them have relatively small effects. Not all QTLs need to be incorporated in order to achieve a significant increase in resistance. Foolad et al. (2002b) and Zhang et al. (2003) recommended combination of four to six QTLs, which explained more than 40% of total phenotypic variation for use in marker-assisted breeding, and Chaerani et al. (Chapter 3) suggested two QTL, which had prominent effects under different environments and gave both EB and stem lesion resistance. It still needs to be determined, however, whether the level of resistance contributed by these QTLs will be of sufficient practical importance. The EB mapping studies have not yet reached the stage where QTLs are mapped precisely enough to be included in a breeding program.

Association of early blight resistance with plant maturity, yielding ability and determinism

The strong correlation between EB resistance and late maturity, low yielding ability, and indeterminate plant type (Nash and Gardner 1988; Foolad 2001, Foolad et al. 2002a, b) has limited the development of lines or cultivars with a high level of resistance. The QTL study of Foolad et al. (2002b) described above aimed to identify QTLs for resistance without an effect on these agronomic traits. Therefore, they removed plants with poor characteristics from their population before attempting to map the QTLs (Foolad and Lin 2001; Foolad et al. 2002b). However, no one plant with resistance level equal to that of the donor parent or F1 hybrid was identified in subsequent generations (Foolad and Lin 2001; Foolad et al. 2002b).

Substantial work on potato EB also documented the association of late maturity with EB resistance (e.g. Johanson and Thurston 1990). As in tomato, it is not yet clear whether this correlation is caused by closely linked genes or by pleiotropic effects of genes. A mapping study for EB and maturity in potato identified five EB resistance QTLs which explained 62% of the total phenotypic variation for resistance (Zhang 2004). Three of these five QTLs explained 98% of the total phenotypic variation for maturity. The other two EB resistance QTLs, which did not have an effect on (foliage) maturity, explained 33% of the total phenotypic variation for resistance (Zhang 2004). In potato therefore about half the genotypic variation for EB resistance is also linked to maturity; still this may be due to either close linkage or to pleiotropic effects. A very similar situation occurs in the potato–late blight (*Phytophthora infestans*) interaction (Visker et al. 2003).

Even on susceptible plants, the younger, topmost leaves are usually free of EB symptoms, while the older, lower leaves may be necrotized by the fungus (Johanson and Thurston 1990). Several studies attempted to clarify the physiological mechanisms for this apparent resistance in young tissues and plants. Low sugar content has been suggested as the cause of higher EB susceptibility in older or weakened leaves and plants (Rotem 1994): late in the season leaves of maturing plants might be susceptible due to translocation of sugars to the ripening fruit. An *in vitro* study by Sands and Lukens (1974) provided indirect evidence that abundant glucose in the medium inhibited the production of cell wall degrading enzymes by *A. solani*. The ‘low sugar content theory’ might explain the increase susceptibility of physiologically old plants or those which have a high fruit to foliage ratio (Barrat and Richards 1944). Another explanation of the relative resistance of young tissues is that the concentrations of three glycoalkaloids (solanine, chaconine and solanidine), which are capable of inhibiting growth of *A. solani in vitro*, are higher in young tomato leaves and steadily decline as leaves and plant matures (Sinden 1972).

The higher resistance of late maturing cultivars can similarly be explained in terms of sugar and alkaloid contents. Late maturing cultivars generally have an indeterminate, vine-type growth habit and continue producing new foliage (Johanson and Thurston

1990). In contrast, early maturing types have a determinate growth habit and do not continue producing new foliage throughout the season. Therefore, late maturing cultivars might appear resistant compared to the early maturing types just because fruit initiation is delayed and more young leaves are present throughout the season.

If the physiological mechanisms are the only cause of EB resistance then it might be impossible to find recombinants with a high resistance level and highly desirable horticultural characteristics in a segregating population. In that case, tomato breeders can only expect to obtain acceptable EB resistance level in varieties with mid- or late season maturity. However, in potato variation in resistance occurs between cultivars of the same maturity class, indicating that differences in resistance are not always and only an artifact of maturity effect (Holley et al. 1983; Christ 1991). So far EB resistance screening in tomato was studied without reference to maturity classes and yield while the latter traits are taken into consideration in EB potato research (Douglas and Pavek 1972).

Characterization of resistance

Several epidemiological parameters have been identified in *A. solani*-tomato and potato interactions, including infection efficiency (IE), lesion expansion rate (LER), latency period (LP), incubation period (IP), sporulation rate (SR) and sporulation capacity (SC). Tomato lines with a higher level of resistance typically showed a lower IE, slower LER, slower SR and lower SC but showed no significant difference in LP compared to susceptible lines (O'Leary and Shoemaker 1983). IP was most important in determining cultivar ranking in potato; resistant cultivars had a longer IP (Pelletier and Fry 1989). SC was found to correspond linearly with lesion size (Pelletier and Fry 1989; Johnson and Teng 1990).

Secondary plant metabolites correlated to EB resistance include the presence of a higher total phenolic content (tannins, flavonols, and phenols) in leaves and stems of EB resistant varieties (Bhatia et al. 1972). The tannin content in all varieties fluctuated as the plant matured but reached a maximum content by the 14^{th} week in leaves and by the 10^{th} week in stems. In addition, the fruit of resistant varieties contained a higher amount of phenol compound than that in susceptible one (Bhatia et al. 1972). The constitutive expression of phenols, which is thought to function as preformed inhibitors, has been associated with non-host resistance (Nicholson and Hammerschmidt 1992).

At the cellular level events during the *A. solani* infection involve general defense responses which are also found in other plant–pathogen interactions involving quantitative resistance. These responses are basically similar to those following hypersensitive responses in monogenic resistance, but they are expressed in more slowly and at a lower level (Agrios 2005). In EB resistant lines a higher and more rapid induction of the pathogenesis related (PR) proteins chitinase and β-1, 3-glucanase (Lawrence et al. 1996,

2000), peroxidase (PO, Fernandez et al. 1996), and phenyalalanine ammonium lyase (PAL; Solorzano et al. 1996) are observed during the early infection process compared to those in susceptible lines (Lawrence et al. 1996, 2000). Chitinases and glucanases probably slow the fungal ingress in the plant as indicated by their inhibitory effect on *A. solani* growth *in vitro* (Lawrence et al. 1996). Enzyme preparations from resistant lines also induced the release of hypersensitive response (HR) elicitors from *A. solani* cell walls *in vitro*, whereas enzymes from susceptible lines did not (Lawrence et al. 2000).

PO is involved in the production of reactive oxygen species, which are directly toxic to the pathogen or indirectly reduce the spread of the pathogen by increasing the crosslinking and lignification of the plant cell walls (Hammond-Kosack and Jones 1996). PAL is the key enzyme in the synthesis of the secondary, endogenous signaling molecule salicylic acid (SA) which in turn activates the expression of a variety of PR genes (Mauch-Mani and Slusarenko 1996).

Polyphenol oxidase (PPO) F is systemically up-regulated in response to *A. solani* infection and is detected in leaves of upper nodes but not in lower nodes (Thipyapong and Steffens 1997). This induction pattern matches with the observation of temporary resistance of young leaves to *A. solani* infection (Johanson and Thurston 1990). PPOs catalyze the oxidation of phenols to quinones, reactive molecules which induce cell death and barriers to secondary infection (Thipyapong and Steffens 1997). PPO F is induced within lesions but not around the lesions during early infection and necrotic lesion development. Other defense-related responses to infection with *A. solani* involve elevated expression of the PR-1B gene following exogenous application of SA on tomato roots (Spletzer and Enyedi 1999), PR-1 like protein after leaf treatment of tomato with arachidonic acid (Coquoz et al. 1995) and sequential expression of two ACC synthase genes (*ST-ACS4* and *ST-ACS5*) in potato (Schlagnhaufer et al. 1997).

The biological effects of the genes underlying the identified EB resistance QTLs remain unclear at this moment. A candidate gene approach, either using genes involved in the pathogen recognition process (resistance genes [*R* genes] or *R* gene analogs [RGAs], Foolad et al. 2002b) or those involved in the defense response process (defense response genes [DR genes], Faris et al. 1999) as molecular markers for QTL analysis, is potentially useful for the analysis of EB resistance. Since resistance to *A. solani* does not seem to be race-specific and is not mediated by genes with a major effect, *R*-genes are unlikely to be involved in this resistance. Therefore DR genes are more likely candidate genes for the QTLs involved in EB resistance. Faris et al. (1999) provided a convincing example. They mapped DR genes on a wheat linkage map where QTLs for several diseases had previously been identified. These DR genes were shown to have a more significant association with disease resistance and explained more of the phenotypic variation than the original markers used for QTL detection. Mapping at a higher resolution is also needed, however, before establishing any functional relationship.

Concluding remarks

A wealth of information on the tomato–*A. solani* interaction is available. However, some important aspects need further attention.

No conclusive evidence is available so far concerning the existence of physiological races. This should be studied using homozygous tester lines and isolates that are as homogenous as possible.

The strong association of negative horticultural traits with the expression of EB resistance seems to be a general rule, for which no conclusive genetic explanation has yet been offered. Meanwhile, breeders should be aware that selection for resistance will only produce useful results if the plant material is comparable in terms of earliness and yield.

QTLs for EB resistance have been identified in interspecific cross population. Before these can be used in marker-assisted breeding program, fine mapping is needed to avoid introgressing large parts of donor genome along with the resistance gene. Also, before QTLs are used in a breeding program, their pleiotropic effects on other traits should be investigated.

Acknowledgements

The comments of Dr. P. Lindhout and prof. dr. ir. P. Stam are gratefully acknowledged. Financial support was provided by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia–Netherlands.

Chapter 2

Assessment of early blight (*Alternaria solani*) resistance in tomato using a droplet inoculation method

R. Chaerani, R. Groenwold, P. Stam and R. E. Voorrips

Abstract

A droplet inoculation method was used for evaluation of tomato resistance to early blight, a destructive foliar disease of tomato caused by *Alternaria solani* (Ellis & Martin) Sorauer. In this test method, leaflets are inoculated with small droplets of a conidial suspension in water or 0.1% agar solution. Early blight resistance was evaluated based on lesion size. The droplet method gave a better discrimination of resistance level ($P<0.001$) at a range of conidial densities compared to the more commonly used spray inoculation method. Lesions generated by the droplet inoculation method at 7 days post inoculation ranged from small flecks to almost complete blight with an exponential-like distribution of lesion sizes. Significant correlations ($r = 0.52$, 0.58 and 0.63, $P<0.001$) were observed across three glasshouse tests of 54 accessions including wild species using the droplet test method. The most resistant accessions included wild species: one accession of *Solanum arcanum*, three accessions of *S. peruvianum*, one accession of *S. neorickii* and one of *S. chilense*. *S. pennellii* and *S. pimpinellifolium* accessions were susceptible, whereas *S. habrochaites* and *S. lycopersicum* accessions ranged from susceptible to moderately resistant. The droplet test method is simple to apply, offers a fine discrimination of early blight resistance levels and allows an objective evaluation.

(Submitted)

Introduction

Early blight (EB) of tomato leaves, caused by *Alternaria solani* (Ellis & Martin) Sorauer, is a serious disease in warm and humid regions (Sherf and MacNab 1986) and in semi arid areas where frequent and prolonged night dew occurs (Rotem and Reichert 1964). Early blight reduces the photosynthetic area and, in severe cases, can defoliate plants.

Cultivars highly resistant to EB are not known in cultivated tomato (*Solanum lycopersicum* [Peralta et al. 2005, syn. *Lycopersicon esculentum*]). All breeding lines and released cultivars are susceptible to moderately resistant (Vakalounakis 1983; Gardner 1988; Poysa and Tu 1996; Banerjee et al. 1998; Vloutoglou 1999; Gardner and Shoemaker 1999). Several wild species (*S. habrochaites* [syn. *L. hirsutum*], *S. pimpinellifolium* [syn. *L. pimpinellifolium*], *S. peruvianum* [syn. *L. peruvianum*], and *S. chilense* [syn. *L. chilense*]) have been identified as potential sources of resistance (Nash and Gardner 1988b; Kalloo and Banerjee 1993; Poysa and Tu 1996; Foolad et al. 2000; Thirthamalappa and Lohithaswa 2000). Some of these, primarily *S. habrochaites* accession PI 126445, have been used to develop moderately resistant breeding lines (Gardner 1988; Gardner and Shoemaker 1999). Identification of additional sources of resistance could facilitate the development of resistant cultivars.

Field evaluations can identify sources of resistance but the major drawbacks are the lengthy duration of the tests, uncontrollable environmental conditions necessary for infection and the presence of other foliar pathogens (Locke 1948; Foolad et al. 2000; Pandey et al. 2003). Glasshouse tests using spray inoculation of a conidial suspension on seedlings are widely used following the establishment of efficient screening and conidial inoculum production techniques by Barksdale (1969). The EB lesions resulting from spray inoculation are scattered on the leaves; this requires an observer to estimate the combined area of all lesions on all leaflets as a percentage of the total leaf area. Disease reading in this way, although rapid, is rather subjective. Another disadvantage of the spray inoculation method is that the inoculum may not be uniformly distributed on the leaves. Furthermore, the method is not sensitive enough to discriminate moderately resistant from susceptible plants (Gardner 1990).

An alternative method to obtain more precise and reliable disease readings is offered by a method in which individual droplets of fungal inoculum suspension are inoculated on leaflets. This method was first introduced by Locke (1948) to find sources of resistance to EB (Locke 1949). Detached leaflets were inoculated with mycelial suspension in a laboratory assay and the disease reaction was evaluated using a graded series of lesion diagrams with known diameters (Locke 1948; 1949). Henning and Alexander (1959) used the droplet method to investigate the existence of *A. solani* races by inoculating leaflets still attached to plants. Nash and Gardner (1988b) applied the method, which they called point inoculation, on a whole plant assay and measured the EB

lesion diameter. EB resistance of three parents and the F1's were tested in a glasshouse. Their results correlated well with field tests, but were based only on a few genotypes.

Screening of large numbers of accessions in the glasshouse has never been conducted using the droplet inoculation method. We describe here some improvements on the method, and its application to identify potential EB resistance sources in a collection of tomato accessions.

Materials and methods

Plant material and culture conditions

Tomato seeds were germinated on moistened filter paper in 90 mm diameter Petri dishes for 5-7 days in darkness at 19^{o}C. Germinating seeds were planted to peat soil in boxes or plastic pots (see details in the following experiments). Plants were grown in a glasshouse in Wageningen, the Netherlands at day/night temperatures of 22/20^{o}C. Tomato accessions used in the screening experiments are listed in Table 3. They were propagated one generation before use; where possible inbred lines were obtained by selfing, but in the case of *S. peruvianum* half-sib families were harvested after intercrossing five plants per accession. In cases where clear morphological differences between the five plants of the original accession were observed, two lines or two half-sib families were included in the screening experiments.

Fungal culture and inoculum preparation

An *Alternaria solani* isolate obtained from infected tomato leaves in Sukabumi, West Java, Indonesia, was propagated on V8 juice agar in 90 mm diameter Petri dishes. The dishes were incubated at 21-22^{o}C in a 12-hr diurnal period of fluorescent light for 10-17 days. The cultures were induced to sporulate as described by Barksdale (1969). The number of conidia in the suspension was counted in five 10-µl samples. The yield per plate was about 0.7-13.0 x 10^5 conidia.

Conditions during infection

For the first 40 hr immediately after inoculation, plants were incubated on a glasshouse bench lined with a wet mat and covered with a transparent plastic tunnel. Periodic misting to maintain high humidity was supplied from a humidifier (Defensor®). After the initial incubation, each side of the tunnel was opened and the humidifier was turned off for 8 hr during the day to allow the plant surface to dry. Minimum light intensity in the plastic tunnel was approximately 14 µmol $m^{-2}s^{-1}$; when necessary, daylight was supplemented

with light from high-pressure sodium vapor lamps (17 µmol m^{-2} s^{-1}) for 16 hr day^{-1}. The temperature and relative humidity were recorded with a thermohygrograph.

Effect of conidial density on early blight (EB) severity with two inoculation methods

One moderately resistant (FT94-978; 99-213) and one susceptible (HRC90.145) *S. lycopersicum* line were grown for three weeks on peat soil in boxes of 34 cm x 29.5 cm x 4 cm. Two inoculation methods, the droplet and the standard spray inoculation method, were compared. Conidial density was varied (0, 1, 2, 4, 10, and 20 x 10^3 ml^{-1} water) to find the most discriminating level.

Plants inoculated using the droplet method were raised in boxes of 12 plants, with two rows of three plants of each genotype. Inoculation was done by applying a single drop of 10 µl of a conidial suspension on interveinal spaces of the upper surface of three apical leaflets. The two first expanded leaves were used. The six conidial densities were randomized over the six plants of each genotype in each box. The experiment was replicated over three boxes.

In the spray inoculation treatment boxes contained four rows of four plants, with the two genotypes in alternating rows. Each pair of rows was sprayed with one conidial density until run-off. The experiment was replicated four times (24 boxes).

The boxes were covered with a transparent lid and placed in the tunnel with intermittent misting for 15 min at 45-min intervals. After the first 24 hr the lids were removed. The temperatures during the day were 20 to 27°C and during the night 16 to 24°C. The relative humidity ranged from 40 to 72% during the day and 85 to 100% during the night. Symptom evaluations were done at seven days post inoculation (DPI). Length and width of lesions resulting from the droplet inoculation were measured. EB severity on each leaf of the sprayed plants was recorded on a scale of 0 to 5, where 0 = no visible lesions on leaf; 1 = up to 10% leaf area affected; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; and 5 = more than 75% leaf area affected or leaf abscised (Vakalounakis 1983). Leaves that were not completely unfurled during the inoculation were not assessed. The disease scales were converted into percentage of EB index (PEBI) for each plant using the following formula (Pandey et al. 2003):

$$\text{PEBI} = \frac{\text{sum of all ratings}}{\text{number of leaves sampled} \times \text{maximum disease scale}} \times 100$$

Resistance reaction of selected accessions with two inoculation methods in the glasshouse

The reproducibility of the droplet inoculation method in determining early blight resistance in a wider range of accessions was compared with the spray inoculation method. Nine accessions, including wild species, differing in mean EB lesion size were planted in pots and inoculated at six weeks after germination.

For spray inoculation, plants were sprayed with conidia in water until run-off. Droplet inoculation was performed by applying a single drop of 10 µl of 10^4 conidia ml^{-1} 0.1% agar solution on interveinal spaces of three apical leaflets of the four topmost expanded leaves. With agar the droplets were more likely to adhere to the leaves. A single concentration of 10^4 conidia ml^{-1} selected based on the most optimal inoculum level from the spray inoculation (see Results) was used for both inoculation methods. Two plants of each accession were tested in three replications for droplet inoculation and four replications for the spray inoculation method. Non-inoculated plants were used as controls for both inoculation methods. The plants were placed in the humidified tunnel directly after inoculation and received periodic misting for 45 s at 8-min intervals. Daytime temperatures ranged from 25 to 27^{o}C and nighttime temperatures from 20 to 22^{o}C. The relative humidity ranged from 59 to 69% during the day and was 98% during the night. Disease reactions were recorded at 7 DPI using the procedure for the respective inoculation methods as previously described.

Glasshouse screening of tomato accessions

1. Glasshouse screening 2001 (autumn)

Forty-one accessions including wild species were tested (Table 3; GH I and GH II). For eleven accessions two or three lines or half-sib families were tested because the original accession was not morphologically uniform. The plants were raised in boxes of 34 cm x 29.5 cm x 4 cm. Each box contained 12 plots of two plants, of 12 different accessions, which were randomized in the boxes. The plants were inoculated using the droplet method at 3 weeks after sowing, when most of them had two fully expanded leaves. Boxes were closed with transparent lids for 24 hr and placed in the tunnel. The misting period was 15 min per hour. The length and the perpendicular width of lesions were recorded at 7 DPI. The experiment was replicated five times at weekly intervals; each replicate was treated as a block in the statistical analysis.

In the first two replicates the three apical leaflets of two basal leaves of two plants of each accession were inoculated with 10 µl droplets of a suspension of $2x10^4$ conidia ml^{-1} water. However, the basal leaves of some wild species, both with lesion and without lesions were lost earlier (3 DPI) than those of the cultivated tomato possibly due to a

faster development and senescence. Early senescence and defoliation were accelerated by inoculation with the pathogen. On some susceptible accessions, lesions expanded rapidly and caused early development of blight. Because of these problems the droplet inoculation procedures were modified in the subsequent replicates. These first two replicates were treated as separate experiment, designated as "glasshouse test I" (GH I).

In the subsequent three replicates (GH II) the three apical leaflets of the four topmost leaves were inoculated to achieve a more uniform physiological age of leaves, and a lower inoculum density ($4x10^3$ conidia ml^{-1} water) was used to prevent too fast development of blight symptoms. Three replicates in time were performed. In the first replicate of GH II some accessions were represented by less than two plants due to poor germination.

Temperatures ranged from 20 to 23°C during the day and from 17 to 19°C during the night. Relative humidity ranged from 43 to 64% during the day and from 97 to 100% during the night.

2. Glasshouse screening 2002 (summer)

The same 41 accessions were re-tested together with 13 additional accessions in five replicated tests, performed at weekly intervals (Table 3; GH III). Plants were grown in 12-cm diameter pots (one seedling per pot) to facilitate inoculation and evaluation. Four weeks after sowing plants were inoculated at the three apical leaflets of the four topmost expanded leaves with a single drop of 10 µl of 10^4 conidia ml^{-1} in 0.1% agar solution. Each replicate included one plant of each accession. Also included in the tests were control plants, one plant of each species, which were inoculated with agar solution without conidia. Plants were placed in the tunnel and exposed to a fine mist for 45 s to 1 min at 6 to 8-min intervals. Five replicate tests were performed in the season at 1-week interval. The length and the perpendicular width of the lesions were measured at 7 DPI. Day temperatures ranged from 22 to 27°C, night temperatures from 20 to 22°C. Relative humidity ranged from 40 to 66% during the day and from 91 to 93% during the night.

Leaf staining

Leaflets that did not show appreciable EB lesions were sectioned around the inoculation site into 1x 1 cm^2 and immersed in fixative solution (glacial acetic acid : 96% ethanol [1 : 2, v/v], Pierre and Millar 1965). When they had been fully decolorized (approximately 48 h) leaf sections were rinsed three times with MQ water and stained with 0.05% toluidine blue in 0.025 M phosphate buffer (pH 7, w/v) using a modified protocol from Aveling et al. (1993). After 30 sec to 2 min of staining, samples were mounted in 50% glycerol and viewed under a bright-field microscope.

Experimental design and statistical analyses

The elementary data consisted of lesion size (length x width) for droplet inoculations, and PEBI per plant for spray inoculations. Heterogeneity of variances was observed in the data from both inoculation methods. Logarithmic and arcsine-square root transformation was applied before statistical analysis to the lesion size and PEBI data, respectively, to stabilize the variances.

Student's t-test was performed for the data of the conidial density experiment to compare means. All other experimental data were analyzed by ANOVA as a randomized complete block design. Mean separations were done by means of LSD tests ($P \leq 0.05$). GH II was analyzed using the unbalanced treatment structure procedure of ANOVA because of the unequal number of plants per block and per accession. All analyses were done using the GenStat 6 statistical package (Payne et al. 2002).

Results

The effect of conidial density on EB severity under two inoculation methods

EB lesions resulting from both the droplet and spray inoculation appeared within 3 DPI. Some droplet inoculations failed to develop into substantial lesions, but formed small spots (≤1 mm in diameter), or did not result in symptoms at all. This was observed on both accessions. The symptomless inoculations were scored as missing values.

With both inoculation methods, FT94-978; 99-213 (moderately resistant) showed significantly smaller lesion size or PEBI than HRC90.145 (susceptible) at all conidial densities at 7 DPI (Table 1). At all concentrations the droplet method gave a better discrimination of resistance level than the spray method as indicated by higher and more significant t-values ($P<0.001$). The most significant difference of PEBI between FT94-978; 99-213 and HRC90.145 was observed at a density of 1×10^4 conidia ml^{-1} while the differences were highly significant at all densities above 1×10^3 conidia ml^{-1} for the droplet inoculation method.

Resistance reaction of selected accessions with two inoculation methods in glasshouse tests

The comparison of the droplet and spray inoculation method was expanded to a set of nine accessions representing four tomato species, which from preliminary experiments were known to represent large differences in EB resistance. The wild accessions occasionally showed spontaneous blisters or necroses under glasshouse conditions. When EB developed on *S. habrochaites* leaves with necroses, the lesions would expand rapidly and result in severe blight symptom, complicating the measurement of lesion size. Leaflets

with severe blight symptoms where lesion size measurements were not possible were scored as missing values.

Table 1 Means of disease parameters obtained from droplet inoculation (lesion size) and from spray inoculation method (percentage of early blight index, PEBI) at different conidial densities

Conidial density	Lesion size mean (mm^2)[a]		t-value[b]	PEBI mean (%)[c]		t-value
($x10^3$ ml^{-1} water)	HRC90.145	FT94-978; 99-213		FT94-978; 99-213	HRC90.145	
1	29.92	10.50	2.16*	14.68	18.12	1.63
2	86.50	16.60	4.38****	19.23	28.93	2.61*
4	108.64	37.93	5.24****	27.64	44.88	2.53*
10	135.83	53.09	4.16****	53.31	74.91	4.16****
20	213.80	80.91	6.18****	75.21	61.62	2.98***

[a]Length x width measured at 7 DPI. Each value is an average of 3 replicates of 18 leaflets each (6 leaflets x 3 plants); data are back transformations of log (x).

[b]Asterisks indicate significance of t-value: *P<0.05, **P<0.01, ***P<0.005, and ****P<0.001.

[c]Based on individual leaf scores: 0 = no visible lesions on leaves; 1 = up to 10% leaf area affected; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; and 5 = more than 75% leaf area were affected or leaf shed. Each value is an average of 4 replicates of 4 plants each; data are back transformations of arcsine √(x/100).

The droplet method allowed a better separation of accessions than the spray inoculation method (Table 2) in accordance with the result of the conidial density experiment. Accession reactions under the two inoculation methods were not significantly correlated (r = 0.44, P>0.2). *S. habrochaites* PE36 showed inconsistent results between the two inoculation methods: it was ranked as susceptible under the droplet method but resistant under the spray method. Occasional spontaneous necroses on this accession inoculated with the droplet method exacerbated EB lesions. Excluding this accession from the analysis increased the correlation considerably (r = 0.66).

EB lesions on petioles were observed on sprayed plants. Large, sunken petiole lesions often caused loss of the leaf and thus raised the PEBI of accessions that showed small leaf lesions when inoculated by the droplet method. The petiole lesions were generated randomly as the spray inoculation was not purposely directed to petioles.

Lesion size distribution

Inoculations using the droplet method did not always develop into a noticeable lesion, irrespective of the level of resistance of a plant. We first assumed that the conidia dried out before successfully penetrating the host tissue during the initial hours of incubation in the tunnel. However, incorporation of 0.1% agar solution into the conidial suspension, which apart from immobilizing the droplets also decreased evaporation, did not influence the probability of lesion formation.

Table 2 Means of disease parameters obtained from droplet inoculation (lesion size) and from spray inoculation method (percentage of early blight index, PEBI) of nine selected accessions

Genotype	Lesion size (mm^2)[a]	PEBI[b]
S. peruvianum PE44	1.19 a	67.43 a-c
S. peruvianum PE33	6.67 b	59.03 a
S. lycopersicum HRC90.158	7.73 bc	66.28 a-c
S. pimpinellifolium G1.1554	12.59 cd	86.69 cd
S. lycopersicum NC EBR-4	15.07 d	62.43 ab
S. habrochaites G1.1561	15.21 d	84.98 b-d
S. habrochaites 864086-2; PI272745	29.72 e	97.71 d
S. habrochaites PE36	40.18 e	60.40 ab
S. lycopersicum FT97-515; 99-214	45.92 e	90.86 d

[a]Length x width measured at 7 DPI. Each value is an average of 3 replicates of 24 leaflets (3 leaflets x 4 leaves x 2 plants); data are back transformations of log(x). Values within a column followed by the same letters are not significantly different at $P = 0.05$.

[b]Based on individual leaf scores: 0 = no visible lesions on leaves; 1 = up to 10% leaf area affected; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; and 5 = more than 75% leaf area were affected or leaf shed. Each value is an average of 4 replicates of 2 plants; data are back transformation of arcsine $\sqrt{(x/100)}$.

When the lesion sizes of the nine accessions, after grouping them into resistant, moderately resistant and susceptible genotypes according to their average lesion sizes, were plotted, an exponential-like distribution was observed (Figure 1). When fitting an exponential distribution, the parameter λ in the probability density function ($f(x) = \lambda \cdot e^{-\lambda x}$) was estimated as 0.0406, 0.0173 and 0.0109 for the resistant, intermediate and susceptible classes, respectively. The more resistant accessions showed a higher frequency of small lesions, a lower frequency of larger lesions and a lower mean lesion size. Symptomless leaflets were observed with an average of 0.9%, 7% and 18.2% for susceptible, intermediate and resistant accessions respectively; for flecks (≤ 1 mm^2) these frequencies were 2%, 9% and 17%. Microscopic evaluation of leaflets with flecks after staining procedure showed that infection had occurred as indicated by the presence of germ tube penetration, but mycelial proliferation was absent. An exponential-like distribution of lesion size was also observed on sprayed inoculation plants (data not shown).

Glasshouse screenings

Lesions near leaf veins of the susceptible *S. lycopersicum* accessions were often accompanied by smaller lesions of angular shape without concentric rings along the vein and at the distal ends of the vein. These smaller lesions rapidly expanded and eventually merged with the primary lesion, resulting in almost completely blighted leaves before lesion measurement at 7 DPI. Flecks, which did not further expand, and symptomless

inoculations were again observed on all tested accessions. Leaflets showing severe blight symptoms and those without appreciable lesions were scored as missing values.

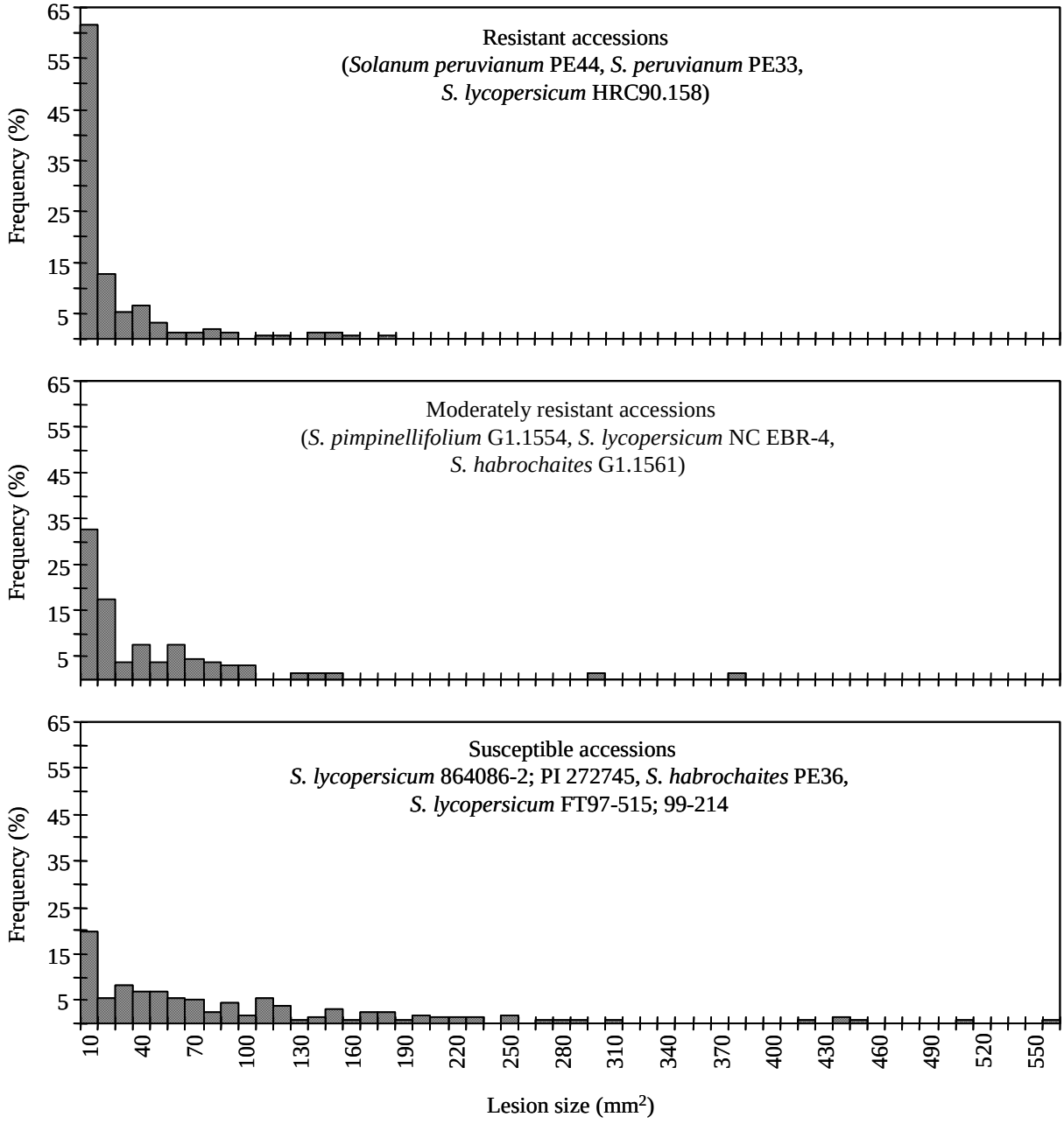

Figure 1 Distribution of lesion sizes on droplet inoculated plants of nine accession, grouped based on resistance level

Table 3 Early blight lesion sizes[a] of *Lycopersicon* accessions in glasshouse tests after droplet inoculation

Species	Accessions	Sources[b]	GH I[c,d]	GH II[e]	GH III[f]	Weighted average[g]
S. arcanum	LA2157	1	NT[h]	NT	1.40 a	1.40
S. peruvianum	PE44	4	NT	NT	1.46 ab	1.46
S. peruvianum	PI 390665	4	NT	NT	4.07 c-h	4.07
S. peruvianum	PE33	4	11.38 a	1.54 a	3.30 c-f	6.23
S. neorickii	G1.1601	2	NT	NT	6.68 g-m	6.68
S. chilense	G1.1556	2	NT	NT	6.68 g-m	6.68
S. lycopersicum	NC EBR-6	3	16.90 ab	2.59 a-d	5.02 d-k	9.56
S. lycopersicum	NC EBR-6	3	17.10 ab	4.56 a-i	2.61 bc	10.49
S. lycopersicum	HRC86.320	4	24.27 a-d	1.57 a	4.32 c-l	10.61
S. lycopersicum	cv. Santacruz	6	NT	NT	11.67 m-p	11.67
S. habrochaites	LA2650	4	18.58 ab	3.60 a-g	16.79 p-u	12.24
S. lycopersicum	HRC90.159	4	25.82 a-d	2.92 a-e	3.16 c-f	12.56
S. lycopersicum	HRC86.320	4	32.36 a-e	2.06 ab	2.93 cd	13.29
S. habrochaites	PE36	4	31.48 a-e	2.34 a-c	10.45 m-p	15.00
S. peruvianum	PI390665	4	16.94 ab	11.00 f-k	16.52 p-u	15.19
S. lycopersicum	HRC90.159	4	30.55 a-e	3.90 a-i	4.84 d-j	15.65
S. lycopersicum	HRC91.341	4	30.97 a-e	4.62 a-j	4.56 c-l	16.40
S. lycopersicum	NC EBR-1	3	28.91 a-e	4.73 a-j	10.69 m-p	16.94
S. lycopersicum	NC EBR-3	3	22.49 a-d	9.59 e-k	7.59 i-n	16.97
S. lycopersicum	HRC90.190	4	32.58 a-e	4.98 a-j	4.82 d-j	17.36
S. peruvianum	PI270435	4	NT	NT	17.50 p-u	17.50
S. lycopersicum	cv. Sufan n.1	6	NT	NT	17.54 p-u	17.54
S. habrochaites	LA1777	2	NT	NT	17.54 p-u	17.54
S. lycopersicum	NC EBR-2	3	21.43 a-c	15.10 jk	10.40 l-p	18.46
S. lycopersicum	NC EBR-5	3	42.46 b-e	3.10 a-f	5.73 f-l	18.67
S. lycopersicum	HRC86.329	4	36.39 a-e	3.71 a-h	12.94 n-r	18.88
S. lycopersicum	HRC86.329	4	32.66 a-e	5.16 a-j	14.16 o-s	19.23
S. lycopersicum	HRC90.157	4	30.76 a-e	8.24 c-k	3.83 c-g	19.28
S. lycopersicum	HRC86.321	4	41.88 b-e	4.00 a-i	5.33 e-k	19.60
S. lycopersicum	HRC90.159	4	39.08 a-e	5.93 b-k	3.12 c-e	19.72
S. lycopersicum	NC EBR-3	3	33.50 a-e	6.04 b-k	NT	20.34
S. lycopersicum	HRC90.157	4	41.40 b-e	4.33 a-i	8.36 j-o	20.60
S. lycopersicum	FT94-978; 99-213	5	39.72 a-e	4.60 a-j	9.73 l-p	20.62
S. lycopersicum	NC EBR-4	3	36.90 a-e	6.50 b-k	15.63 p-t	22.50
S. lycopersicum	FT94-968; 99-212	5	33.11 a-e	10.80 f-k	11.35 m-p	22.86
S. habrochaites	G1.1561	2	42.95 b-e	6.67 b-k	7.62 i-n	23.33
S. lycopersicum	HRC91.341	4	34.28 a-e	12.80 g-k	7.76 i-n	23.63
S. lycopersicum	FT97-515; 99-214	5	40.18 b-e	6.38 b-k	17.50 p-u	23.69
S. lycopersicum	FT94-978; 99-213	5	42.07 b-e	7.80 c-k	9.04 k-p	24.27
S. lycopersicum	cv. Allround	1	NT	NT	24.32 s-u	24.32
S. pimpinellifolium	G1.1554	1	47.53 b-e	8.59 d-k	6.75 g-m	24.70
S. lycopersicum	HRC90.158	4	49.66 b-e	7.24 b-k	4.60 c-j	25.13
S. lycopersicum	NC EBR-2	3	39.36 a-e	13.20 h-k	10.21 l-p	26.73
S. lycopersicum	PI79532	6	NT	NT	26.98 t-v	26.98
S. lycopersicum	FT94-968; 99-212	4	48.19 b-e	9.57 e-k	9.77 l-p	28.15
S. lycopersicum	cv. Vogliotti	6	NT	NT	29.17 uv	29.17
S. lycopersicum	HRC89.302	4	44.26 b-e	12.20 g-k	21.04 q-u	30.15
S. lycopersicum	864084-2; PI 273048	5	68.39 c-e	12.10 g-k	7.05 h-m	36.63
S. lycopersicum	HRC89.302	4	53.33 b-e	16.40 jk	21.09 r-u	36.74
S. lycopersicum	cv. Moneymaker	1	75.34 de	12.40 g-k	17.62 p-u	42.52
S. lycopersicum	HRC86.327	4	72.11 c-e	13.70 l-k	45.08 v	45.88
S. lycopersicum	HRC90.145	4	71.45 c-e	20.50 k	28.84 uv	48.49
S. lycopersicum	864086-2; PI 272745	5	94.84 e	18.80 k	45.08 v	59.49
S. pennellii	LA716	1	NT	NT	107.65 w	107.65

[a]Length x width (mm^2) measured at 7 DPI. Data are back transformations of log(x).

(continued)
[b]1 = Plant Research International, Wageningen, The Netherlands; 2 = Dr. P. Lindhout, Laboratory of Plant Breeding, Wageningen University, The Netherlands; 3 = Dr. R. G. Gardner, North Carolina Agricultural Research Institute, North Carolina State University, Raleigh, North Carolina, USA; 4 = Dr. V. Poysa, Agriculture and Agri-Food Canada, Harrow Research Center, Harrow, Ontario, Canada; 5 = Prof. M. Mutschler, Department of Plant Breeding, Cornell University, Ithaca, New York, USA; 6 = Nunhems Zaden BV, Haelen, the Netherlands.
[c]Values followed by the same letters within a column are not significantly different at $P = 0.05$.
[d]Inoculated with 20,000 conidia ml^{-1} water. Each value is the average of two replicates of 12 leaflets (3 leaflets x 2 leaves x 2 plants).
[e]Inoculated with 4,000 conidia ml^{-1} water. Each value is the average of three replicates of 12 or 24 leaflets (3 leaflets x 4 leaves x 1 plants or 3 leaflets x 4 leaves x 2 plants).
[f]Inoculated with 10,000 conidia ml^{-1} 0.1% agar. Each value is an average of five replicates of 12 leaflets (3 leaflets x 4 leaves x 1 plant).
[g]$\Sigma(Y_i/s^2_i)/\Sigma(1/s^2_i)$
[h]NT = not tested.

The means of the lesion sizes from each glasshouse test were weighted with the reciprocals of the variances to obtain overall accession mean values. This adjustment was necessary since variances of the means among the three tests were unequal. After calculating the weighted average, we observed a continuous range of resistance levels, from highly susceptible (*S. pennellii* LA716, average lesion size 107.65 mm^2) to highly resistant (*S. arcanum* LA2157, 1.40 mm^2) (Table 3). However, no complete resistance was found. Among the glasshouse tests significant correlation were observed ($P<0.001$), with correlation coefficients of 0.58 (between GH I and II), 0.52 (GH I and III), and 0.63 (GH II and III). Most of the resistant accessions belonged to wild species (*S. arcanum*, *S. peruvianum*, *S. neorickii* and *S. chilense*). However, both susceptible and moderately resistant *S. habrochaites* accessions were found, and other wild accessions belonging to *S. pimpinellifolium* and especially *S. pennellii* were susceptible. Among different lines of previously reported moderately resistant HRC91.341 (Poysa and Tu 1996) there were significant differences in EB lesion sizes. This accession was susceptible in GH II but resistant or moderately resistant in GH I and GH III, while the other was resistant in all three tests. Lines derived from NC EBR-2 and NC EBR-3 also showed a different reaction in GH II compared with GH I and GH III. Single lesions resulting from the droplet inoculation method permitted detailed observation on lesion phenotypes. Necrotic lesions on *S. neorickii* (syn. *L. parviflorum*), some accessions of *S. habrochaites* and *S. peruvianum*, and *S. lycopersicum* NC EBR-6 were surrounded by narrow chlorotic halos, whereas those on *S. pennellii*, *S. chilense*, and other accessions of *S. habrochaites* and *S. peruvianum* were not accompanied by chlorotic halos. *S. pimpinellifolium* and other *S. lycopersicum* genotypes showed a range of intermediate to wide halos. The extent of the halos did not seem to correlate with the necrotic lesion sizes (data not shown).

Discussion

The droplet inoculation method offers better discrimination between accessions than the spray method. This was observed in the conidial density experiment and in the 9-accession experiment. Variance of lesion size increased with increasing means in both experiments. Distribution of lesion sizes seemed to be exponential, with many small lesions and few large ones. This trend was observed at all levels of resistance.

The discrepancy between the results of the droplet and spray methods can partly be explained by the following two factors. Firstly, some wild species develop severe necroses in the glasshouse experiment even without inoculation. After spraying inoculation these necroses are often indistinguishable from EB lesions, whereas after droplet inoculation they are simply recognized and treated as missing values. Secondly, spray inoculation may lead to randomly occurring leaf shedding due to petiole lesions and therefore erratic high symptom scores. The droplet method offers the possibility to test the effect of petiole lesion in a controlled way.

Across the three glasshouse screenings significant correlations were observed. GH II and III yielded a better separation than GH I. Also the correlation between GH II and III was higher than that between GH I and the other two tests. This may be due to 1) the lower number of observations in GH I, 2) the higher inoculum density in GH I, and 3) the difference in the selection of leaves between GH I and the other two tests. We used a lower inoculum concentration (40 to 200 conidia per droplet) in the glasshouse screenings but observed overall larger lesion sizes than Nash and Gardner (1988b) who applied more inoculum (500 to 750 conidia per droplet) on basal leaves. This indicates that our tests were performed in near optimal conditions for infection and disease development. Another difference between our work and the study of Nash and Gardner (1988b) was that they selected one lesion per plant to be measured whereas we measured all inoculations without selection. As a result we observed a large range of lesion type from small lesions ($\leq$1 mm^2 in diameter) to almost blighted leaflets and also symptomless leaflets within the same accession. Small lesions ($\leq$1 mm^2 in diameter) occurred on all genotypes but their frequency corresponded with the resistance level. This was indicated by a high correlation between lesion size and the percentage of small lesions data, most notably in GH II and III where a lower conidial density was applied: r = -0.73 (GH II, P<0.001) and r = -0.77 (GH III, P<0.001).

Variability in pathogenicity of *A. solani* isolates has been widely described (e.g. Bonde 1929; Henning and Alexander 1959; Rotem 1966) but no evidence has been presented for the existence of pathological races. The results of our study, in which we used a single highly pathogenic Indonesian isolate, can therefore be expected to be representative for other Indonesian isolates as well.

The droplet inoculation method is simple to apply and allows an objective evaluation of EB severity. The method has been used to evaluate EB resistance

components (O'Leary and Shoemaker 1983). Single lesions created by the droplet method allow detailed observation on lesion phenotype such as differential extent of halos among genotypes. The importance of the chlorotic halo as an indicator of resistance has not been studied so far. Improvements of the method have been made by incorporating agar solution in the conidial suspension and the use of upper leaves as opposed to the bottom leaves.

The considerable amount of the time required to measure the lesion sizes may make this method less attractive for large-scale screenings, but it can be circumvented by determining the percentage of small lesions. The described advantages of the droplet inoculation method make this the method of choice where a fine discrimination of resistance level and accurate quantitative data are required, for example in QTL studies of resistance or in assessing breeding material during advanced backcross programs.

Acknowledgements

We thank Dr. Suhardi (Indonesian Ornamental Plants Research Institute, IOPRI) and Dr. Eri Sofiari (Indonesian Vegetables Research Institute, IVEGRI) for providing the *A. solani* isolate, and D. Geurtsen and A. Hermsen for their assistance in the glasshouse. Financial support was provided by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia–Netherlands.

Chapter 3

QTL identification for early blight resistance (*Alternaria solani*) in a *Solanum lycopersicum* × *S. arcanum* cross

R. Chaerani, M.J.M. Smulders, P. Stam, R. E. Voorrips

Abstract

Alternaria solani (Ellis & Martin) Sorauer, the causal agent of early blight (EB) disease, infects aerial parts of tomato at both seedling and adult plant stages. Resistant cultivars would facilitate a sustainable early blight (EB) management. EB resistance is a quantitatively expressed character, a fact that has hampered effective breeding. In order to identify and estimate the effect of genes conditioning resistance to EB, a QTL mapping study was performed in F2 and F3 populations derived from the cross between the susceptible *Solanum lycopersicum* (syn. *Lycopersicon esculentum*) cv. 'Solentos' and the resistant *S. arcanum* LA2157. Two evaluation criteria of resistance were used: measurements of EB lesion growth on the F2 plants in glasshouse tests and visual ratings of EB severity on foliage in a field test on the F3 lines. A total of six QTL regions were mapped on chromosomes 1, 2, 5, 6, 7, and 9 with LOD scores ranging from 3.4 to 16.4. Three EB QTLs also confer resistance to stem lesions in the field, which has not been reported before. All QTLs displayed significant additive gene action; in some cases a dominance effect was found. Additive × additive epistatic interactions were detected between one pair of QTLs. For two QTLs, the susceptible parent contributed resistance alleles to both EB and stem lesion resistance. Three of the QTLs showed an effect in all tests despite methodological and environmental differences.

(Submitted)

Introduction

Early blight (EB), incited by *Alternaria solani* (Ellis & Martin) Sorauer, is one of the most damaging diseases in many tomato production areas worldwide (Sherf and MacNab 1986). Other disease symptoms caused by *A. solani* include collar rot on seedlings, stem lesions and fruit rot. The disease is characterized by formation of dark, necrotic lesions with concentric rings giving a target-like appearance. EB is the most devastating of these symptoms. EB lesions first appear on the oldest leaves and spread upwards as the plants grow. Lesions enlarge and merge, resulting in early senescence and gradual defoliation. Complete defoliation may occur and leave fruits exposed to sun-scalding.

EB is prevalent in Indonesia and can cause yield losses as high as 23% (Bos and Kartapradja 1977; Manohara 1977). Frequent applications of fungicides are necessary to control the disease; however, the incidence and severity of EB remain high due to heavy and frequent rainfall in the region. Even partial resistance would be an important improvement, because in combination with fungicides it could extend the intervals of fungicidal spray and therefore increase the net return of the growers.

A strong source of resistance to an Indonesian isolate of *A. solani* was identified in *Solanum arcanum* LA2157 (syn. *Lycopersicon peruvianum* LA2157) (Chapter 2). In glasshouse tests, the average lesion size was only 1.4 mm^2 compared to 23.0 to 108.0 mm^2 on susceptible tomato accessions. *S. arcanum* LA2157 is known as resistance source to other pathogens, including bacterial canker (Sandbrink et al. 1995; van Heusden et al. 1999) and root knot nematode (Veremis et al. 1999). The cross with *S. lycopersicum* (syn. *L. esculentum*) is difficult but possible through *in vitro* embryo rescue (Brüggemann et al. 1996).

Resistance may be difficult to transfer from wild species to cultivated tomato since it is accompanied by unacceptable horticultural traits including inferior fruit quality, late maturity, low yield and indeterminate growth habit. Moreover, the quantitative expression and polygenic inheritance of EB resistance has limited the development of EB resistant cultivars using traditional breeding approaches (Foolad et al. 2002a, b; Zhang et al. 2003).

Classical genetic studies revealed at least two genes with additive and dominance effects and epistatic interactions that confer resistance to EB symptoms (Barksdale and Stoner 1977; Nash and Gardner 1988a; Maiero et al. 1990a; Thirthamalappa and Lohithaswa 2000). According to Stancheva (1991) resistance to stem lesions was a quantitative trait conferred by additive and dominant genes with epistatic effects but the correlation with EB resistance was not investigated.

The identification of markers closely linked to resistance genes is of great benefit for breeding for two reasons. First, these markers allow selection based on marker genotype rather than resistant phenotype and secondly they enable minimizing unfavorable linkage drag. With the aid of a genetic linkage map, Foolad and co-workers (Foolad et al. 2002b; Zhang et al. 2003) have identified and estimated the magnitude of

quantitative trait loci (QTLs) effects in a *S. habrochaites* (syn. *L. hirsutum*) resistance source using backcross populations. Using interval mapping and selective genotyping approaches, they identified fourteen QTLs dispersed over 11 tomato chromosomes. Four QTLs were potentially useful in marker assisted-breeding programs since they were stable across environments. It should be realized that such genes may not be effective in other regions of the world, where different *A. solani* populations may occur and other growth conditions prevail.

The current study is aimed at identification of QTLs for EB resistance effective in Indonesia. Using F2 and F3 populations derived from a cross with *S. arcanum* LA2157 as the donor parent we have located EB resistance QTLs, some of which also confer resistance to stem lesions. To our knowledge this is the first report of QTLs for stem lesion resistance.

Materials and methods

Plant material

The mapping population was composed of 176 F2 individuals obtained from one embryo-rescued F1 plant of a cross between EB susceptible *S. lycopersicum* cv. 'Solentos' (De Ruiter Seeds) and an EB resistant *S. arcanum* LA2157 (Brüggemann et al. 1996). To allow replicated tests the F2 individuals were clonally propagated *in vitro*. Seeds were germinated on MS medium containing 1.0% sucrose and 0.8% agar (Murashige and Skoog 1962) at 25^{o}C. After two to three weeks shoots were cut and transferred to MS medium supplemented with 2.0% sucrose and 0.4% agar. Clones were multiplied by transferring nodes to a fresh medium and cultured for three to four weeks. Prior to transfer to the glasshouse, shoots with two leaves were cut and root formation was induced on MS medium containing 1.5% sucrose, 0.8% agar, and 0.25 mg/l filter-sterilized IBA for 10 to 14 days. Rooted shoots were transferred to rock wool blocks in a glasshouse (18-20^{o}C) and allowed to acclimatize for two weeks. Plants were further grown for four to five weeks before inoculation with *A. solani* and received standard fertilization. Both 'Solentos' and LA2157, an EB resistant (HRC86.329) and a susceptible (HRC90.145) genotype (Poysa and Tu 1996), which served as controls in resistance tests, were also clonally propagated *in vitro*. One set of F2 clones was allowed to self-pollinate to produce F3 seeds for use in a field test.

Early blight resistance evaluation

1. F2 glasshouse test

The complete F2 evaluation consisted of two series of four tests; each test was considered a block in the statistical analyses. In each test, one plant of 44 F2 clones, and two plants of each parent and control genotype were tested. Leaflets were inoculated with *A. solani* isolate 60, which was cultured and applied using the droplet test method (Chapter 2). Abaxial surfaces of 12 terminal leaflets of four leaves were inoculated with droplets. Two droplets of 10 µl of 4 x 10^3 conidia ml^{-1} agar 0.1% were applied on each leaflet, making up a total of 24 inoculation sites on each plant. EB lesion size (length × width) was measured with a ruler at 4, 7, 10, and 14 days post inoculation (DPI).

The area under the lesion expansion curve (AULEC) was calculated using the following formula:

$$\text{AULEC} = \sum_{i=1}^{n-1} \{([R_{i+1} + R_i]/2) \times (t_{i+1} - t_i)\}$$

where R_i is the lesion size at the *i*th observation, t_i is the time (days after planting) at the *i*th observation, and *n* is the total number of observations. The AULEC values were then converted to the relative AULEC (RAULEC) by dividing each value by the period from the date of the first appearance of appreciable EB lesions, which was 2 DPI to the date of disease evaluation, and by the maximum lesion size recorded up to the final evaluation date. The theoretical maximum RAULEC value therefore is 100%.

Lesions that did not grow beyond 1 mm^2 were counted at 7 DPI. The percentage of these small lesions (PSmL) was strongly correlated with lesion size (LS; $r^2 = 0.82$).

2. F3 field test

Seeds were obtained from 156 F2 plants. Eight-week-old seedlings were transplanted in a field in Wanayasa (600 m altitude), West Java, Indonesia at a within-row distance of 35 cm and a between-row distance of 90 cm on raised beds (30 cm high, 30 cm wide). The field test consisted of two blocks. Each block contained an 8-plant plot of each F3 family and the P1 (‘Solentos’), and six 8-plant plots of each P2 (LA2157), HRC 90.145 and HRC 86.329. The field was bordered with cv. Ratna (East-West Seed Indonesia), a susceptible *S. lycopersicum* cultivar. Beds were covered with black polyethylene mulch to prevent the growth of weeds and watered with sub-surface irrigation. Standard recommendations of fertilizer and growth regulator were applied. Insecticidal spray was done as necessary and a fungicide was applied once to prevent damping-off disease (*Pythium* spp.).

Each plant was artificially inoculated six times on 13 and 20 December 2004, 3, 17, and 24 January, and 7 February 2005. Inocula were obtained from infected leaves which were fragmented in a blender, sieved through cheesecloth and diluted 10 times. At each inoculation about 30 to 60 litres of inoculum was sprayed to the field.

Plants were individually rated for EB severity seven times at weekly interval from December 30, 2004 until February 9, 2005, on a scale between 0 and 7, where 0 = no symptoms, 1 = trace to 1%, 2 = 2 to 5%, 3 = 6 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 = 76 to 100% of total foliage on middle third of canopy infected (Christ 1992). Stem infection was rated once on 7 February using a scale between 0 and 4, where 0 = no infection, 1 = minute (up to 1 mm in diameter) and few lesions, 2 = minute, scattered lesions, 3 = slightly larger (>1 to 3 mm in diameter) and scattered lesions, and 4 = many sunken, well-developed lesions, covering >50% stem surface (modified from Barksdale 1971). Percentage of EB index (PEBI) and percentage of stem lesion index (PStLI) for each plot were calculated using the following formula:

$$\text{Percentage of disease index} = \frac{\text{sum of all ratings}}{\text{number of plants} \times \text{maximum rating grade}} \times 100$$

The percentage of EB indices were used to calculate the area under the disease progress curve (AUDPC) analogous to the AULEC calculation and converted to the relative AUDPC (RAUDPC) using a similar method as for AULEC.

DNA isolation and marker analysis

For the SSR and SNP analysis, DNA was isolated from freeze-dried leaves using cell lysis and protein/polysaccharide precipitation methods according to Fulton *et al*. (1995) followed by DNA binding and elution using the DNAeasy® Plant Mini Kit column (Qiagen, Venlo, The Netherlands). DNA for AFLP analysis was prepared by Keygene N.V. from fresh, young leaves (Vos et al, 1995).

Thirty six SSR markers obtained from Sol Genomics Network (http://www.sgn.cornell.edu), Smulders *et al*. (1997), Areshchenkova and Ganal (1999; 2002), Bredemeijer et al. (2002) and P. Arens (unpubl. results) were used in this population. PCR were done in 20-µl volumes containing 10 ng of genomic DNA, 0.2 µM each of forward and reverse primers, 2 µl of 10X Goldstar reaction buffer, 2.5 mM $MgCl_2$, 0.1 mM each of dNTP, and 0.4 units of GoldstarTaq DNA polymerase (Eurogentec, Maastricht, the Netherlands). DNA amplification was performed in a PTC-100 or PTC-200 thermocycler (MJ Research Inc., Waltham, Mass.) using a profile of 3-

min pre-denaturation at 94°C followed by 35 to 40 cycles of 30 s at 94°C, 30 s at 50 or 55°C, 45 s at 72°C, and finalized by a 10-min extension at 72°C. The PCR products were separated on 2.5 to 3.5% agarose gel (w/v) and visualized by ethidium bromide staining or separated on a 6% polyacrylamide gel and stained as described in the Promega Silver Staining Kit (Promega).

Twenty-six SNP markers, developed from tomato RFLP probes or gene sequences present in the public nucleotide databases, were available at Plant Research International from previous research (C. G. van der Linden and B. Vosman, unpubl. results). SNP polymorphisms were detected using SNaPshot following the protocol of ABI Prism SNaPshot Multiplex Kit Protocol (Applied Biosystems). PCR was performed in a 25-µl volume consisting of 10 ng DNA, 0.4 µM each of forward and reverse primer, 2.5 µl of 10X PCR buffer, 0.2 mM each of dNTP, and 0.3 units of HotStarTaq DNA polymerase (Qiagen). Amplification was carried out in a PTC-100 or PTC-200 thermal cycler, programmed for 15 min at 96°C for initial denaturation and 40 cycles consisting of 30 s at 96°C, 45 s at 50°C, and 90 s at 72°C, followed by a final 10-min extension at 72°C. After amplification, PCR products were purified with shrimp alkaline phosphatase (SAP) and *Exo*I for removal of dNTPs and primers. Up to 10 different PCR products were pooled and single base-extended with SNaPshot primers and with fluorescent-labeled ddNTPs on a thermal cycler. Prior to analysis on an ABI 3700 sequencer (Applied Biosystems), samples were purified with SAP and *Exo*I to remove unincorporated ddNTPs. Data were analysed using Genotyper 3.6 (PE Biosystems).

AFLP analysis was performed by Keygene B.V. as previously described in Vos *et al*. (1995). The primer combinations used were P11M48, P11M50, P11M51, P11M60, P11M62, P13M47, P13M49, P13M61, P14M50, P14M51, P14M60, and P15M62. AFLP markers were scored codominantly.

Linkage analysis

The genetic map was constructed using JoinMap® 3.0 (van Ooijen and Voorrips 2001). Grouping of the markers was initially done with a minimum LOD-score of 3.0. The recombination threshold was set at 0.49 and the Kosambi mapping function was used to convert recombination frequencies into map distances.

QTL mapping

The MapQTL® 4.0 software package (van Ooijen et al. 2002) was used to identify QTL for all traits. First the interval mapping procedure was performed to identify the major QTL. For each trait a 1,000x permutation test was performed to identify the LOD threshold corresponding to a genome-wide false discovery rate of 5% (P<0.05). Markers

with LOD scores exceeding the threshold were used as cofactors in multiple-QTL-model (MQM) mapping procedures. If new QTLs were identified, the linked markers were added to the cofactor list and the analysis was repeated. If the LOD value of a marker dropped below the threshold in the new model, it was removed from the cofactor list and the MQM was rerun. This procedure was repeated until the cofactor list became stable. The final LOD scores and 2-LOD support intervals were determined using Restricted MQM.

Statistical analysis

All data were analysed using GenStat® 6.0 (Payne et al. 2002). The phenotypic data were transformed if necessary to achieve a normal error distribution. Unbalanced treatment structure of ANOVA and general ANOVA was used to analyze the F2 and F3 phenotypic data, respectively.

Main effects and epistatic interactions between all pairs of markers that were used as cofactors in QTL mapping were analyzed using general linear regression. Regression was performed by first fitting the main additive effect of each locus in the model. Loci with small and non-significant effects were dropped from the model and regressions were repeated, leaving only loci with significant effects at P=0.05. Next, dominance effects were fitted and new regressions were performed by dropping non-significant loci. Interactions between loci, starting from the lower to the higher order of interactions, were examined in a similar manner.

Results

Linkage map

For the construction of a genetic linkage map 176 F2 plants were genotyped with SSR and AFLP markers, whereas up to 171 plants were genotyped with SNP markers. Out of 393 polymorphic markers, 370 (33 SSR, 21 SNP and 316 AFLP) could be mapped on the 12 tomato chromosomes, resulting in a linkage map spanning 1179 cM (average density 1 marker per 3 cM), which is similar to the *S. lycopersicum* x *S. pennellii* (syn. *L. pennellii*) high density map (1276 cM; Tanksley *et al.* 1992). Twenty-one markers which showed linkage to chromosome 1, 3, 6 and 7 could not be placed in best positions with a “jump threshold” of 5. Two markers were completely unlinked to all others. The number of markers mapped per chromosome ranged from 17 (chromosome 5) to 53 (chromosome 1). Linkage group length ranged from 70 cM (chromosome 9) to 143 cM (chromosome 1). A high marker density was observed in regions where centromeres have been mapped (Tanksley et al. 1992). The maps of chromosomes 5, 6, 10, 11 and 12 contained gaps longer than 20 cM. The order and placement of SSR and SNP markers were generally in a

good agreement with the *S. lycopersicum* x *S. pennellii* reference map (Tanksley *et al.* 1992, Sol Genomics Network http://www.sgn.cornell.edu). The exceptions are CT259, ID285-3, SSR86, and ASR1, which according to the tomato reference map are on chromosomes 4, 3, 4, and 1, respectively, but were mapped on chromosome 1, 2, 3, 4 in our population. The orientation of linkage group 4 is unknown, since two SSR reference markers (TMS22 and EST259379) were originally co-mapped on *S. lycopersicum* x *S. pennellii* map (Areshchenkova et al. 2002). These two markers were separated at 6.6 cM distance in our population. The complete map can be obtained from the corresponding author.

Distorted segregation

A high proportion of the mapped markers (51%) deviated significantly from the expected 1:2:1 segregation ratio for F2 generation at $P<0.05$. Distorted segregation was observed on all chromosomes. On chromosomes 1, 2, 4, 7, 8, and 9 more than 45% of the markers were skewed; this usually occurred only in part of the chromosome. The distortion on chromosome 1 was caused by a surplus of heterozygotes and *S. arcanum* homozygotes on the short arm of the chromosome. Markers on chromosomes 9 displayed a higher frequency of heterozygotes, while distortions on chromosome 2, 4, 7 and 8 were caused by an excess of *S. arcanum* homozygotes.

Phenotypic evaluation

In order to achieve approximately normal error distributions of the traits scored in the F2 glasshouse tests, a log transformation was required for LS and RAULEC, whereas an arcsine transformation was applied to the PSmL data. The ANOVA analyses revealed significant block effects. For the F3 field data, EB assessment at 48 days after transplanting (DAT) for PEBI and at 75 DAT for RAUDPC were used, since the parents and control genotypes were most clearly distinguished at these dates. No transformation was required for PEBI and RAUDPC data, whereas an arcsine transformation was applied to the PStLI data.

All the resistance traits measured showed a continuous distribution with the population mean skewed towards resistance (Figure 1). The phenotypic distributions of LS, RAULEC, PSmL and PStLI showed a bimodal frequency distribution. In the F2 data transgressive segregation occurred in both directions, whereas in the F3 data transgression was observed towards resistance only.

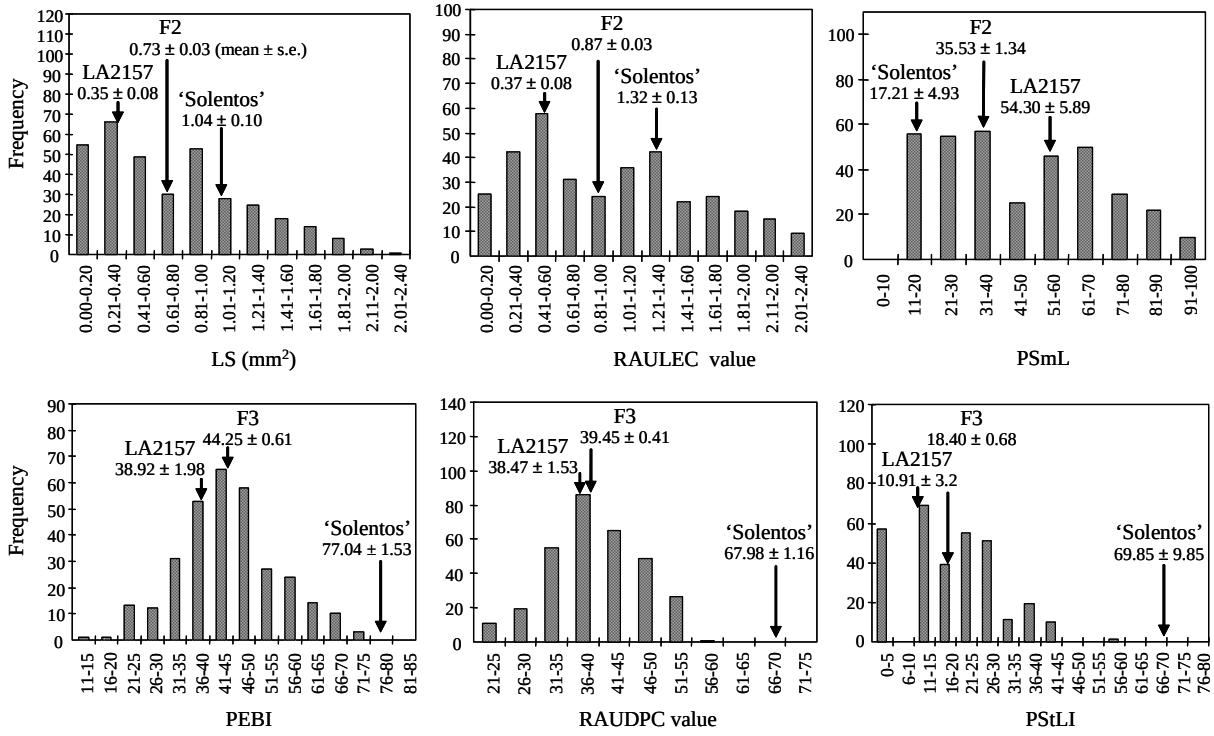

Figure 1 Frequency distribution for lesion size (LS), percentage of small lesions (PSmL), and relative area under the lesion expansion curve (RAULEC) in an F2 population; percentage of early blight index (PEBI), relative area under the disease progress curve (RAUDPC), and percentage of stem lesion index (PstLI) in a population of F3 lines. The F2 population was tested in a glasshouse in the Netherlands with a single *A. solani* isolate; the F3 population in a field in Indonesia with mixed field isolates.

QTL analysis

1. F2 glasshouse test

Four QTLs were identified from the glasshouse data on chromosomes 2, 5, 7, and 9 (Table 1, Figure 2). The QTLs for the three traits overlapped in all cases except that no significant QTL was found for PSmL on chromosome 5. This co-location is consistent with a higher correlation coefficient between LS and RAULEC (r^2 = 0.95) than between PSmL and LS (r^2 = 0.82) or between PSmL and RAULEC (r^2 = 0.86).

For LS, the four QTLs explained in total 39% of the phenotypic variance and individual QTLs accounted for 7.7 to 13.2% of the phenotypic variance. For RAULEC each QTL explained 8.1 to 15.8% of the phenotypic variance, whereas each QTL for PSmL accounted for 7.1 to 16.4% of the phenotypic variance. The QTL on chromosome 7 was the most important in explaining the phenotypic variation, regardless the type of traits measured. Beneficial alleles were contributed by the susceptible parent at the QTLs on chromosomes 2 and 7.

All QTLs exhibited significant additive gene action ($P<0.05$ or $P<0.001$), but dominant effects were also displayed by the QTL on chromosome 2 for PSmL and PStLI ($P<0.05$), the QTL on chromosome 7 for LS ($P<0.001$) and PSmL ($P<0.05$) and the QTL on chromosome 9 for LS and RAULEC ($P<0.001$). No between-locus interactions were found for the QTLs detected in the glasshouse.

Table 1 QTLs for early blight and stem lesion resistance identified by multiple-QTL-models mapping (MQM) method

Chr	Trait	Test	Cofactor	Position (cM)	Coverage (cM)[a]	LOD score[b]	%expl	Add	Dom
1	RAUDPC	F3, field	P14M60-276P	137.7	31	4.13	7.0	2.28***	-0.59
2	LS	F2, glasshouse	P11M48-082E	37.0	59	5.55	9.4	-0.19***	0.10
2	RAULEC	F2, glasshouse	P15M62-073P	45.1	59	5.03	8.3	-0.21***	0.08
2	PSmL	F2, glasshouse	P13M49-435E	38.8	59	5.82	11.1	9.97***	-7.81*
2	PEBI	F3, field	P11M60-276E	89.0	25	3.35	7.6	-3.63***	-0.68
2	RAUDPC	F3, field	P14M51-146E	81.0	25	8.54	15.5	-4.15***	0.99
2	PStLI	F3, field	P13M49-352P	64.1	39	4.94	8.0	-5.57**	2.91*
5	LS	F2, glasshouse	P14M51-055P	57.4	38	4.53	7.7	0.15***	-0.02
5	RAULEC	F2, glasshouse	P14M51-055P	57.4	41	4.75	8.1	0.18***	-0.03
5	RAUDPC	F3, field	P14M51-055P	57.4	44	5.68	9.8	2.85***	0.17
5	PStLI	F3, field	P14M50-537P	59.1	64	4.84	7.8	4.24***	-0.03
6	PEBI	F3, field	P13M49-231E	51.2	30	3.61	8.10	3.72**	-2.00
6	RAUDPC	F3, field	P11M48-266E	31.2	35	6.12	10.8	3.40***	0.12
7	LS	F2, glasshouse	P15M62-349P	36.1	19	7.51	13.2	-0.22***	0.08***
7	RAULEC	F2, glasshouse	P15M62-349P	36.1	19	8.92	15.8	-0.29***	0.11
7	PSmL	F2, glasshouse	P15M62-349P	36.1	22	8.23	16.4	11.40***	-5.51*
9	LS	F2, glasshouse	P14M50-081E	17.4	25	5.25	8.9	0.17***	-0.14***
9	RAULEC	F2, glasshouse	P14M50-081E	17.4	23	5.41	9.1	0.21***	-0.15***
9	PSmL	F2, glasshouse	P11M48-065E	29.0	31	3.86	7.1	-7.81***	1.84
9	PEBI	F3, field	P11M60-109P	44.9	23	6.52	15.3	5.31***	-0.41
9	RAUDPC	F3, field	P11M60-109P	44.9	23	8.76	15.8	3.55***	1.35
9	PStLI	F3, field	P14M50-072P	37.1	10	16.39	31.1	8.24***	-4.37**

Abbreviation: Chr = chromosome number; % expl. = explained part of the phenotypic variance; Add = additive effect; Dom = dominance effect; LS = lesion size (mm^2), PSmL = percentage of small lesions; PEBI = percentage of early blight index; RAUDPC = relative area under the disease progress curve; RAULEC = relative area under the lesion expansion curve; PstLI = percentage of stem lesion index.

[a]based on 2-LOD support interval obtained from restricted MQM mapping.

[b]LOD thresholds obtained from 1000x permutation tests for a genome wide significance ($P<0.05$) were 3.90, 3.60, 3.80, 3.70, 3.80, and 3.90 for LS, PSmL, RAULEC, PEBI, RAUDPC, and PStLI, respectively.

*significant at $P<0.05$, **at $P<0.01$, and ***at $P<0.001$, according to t-test.

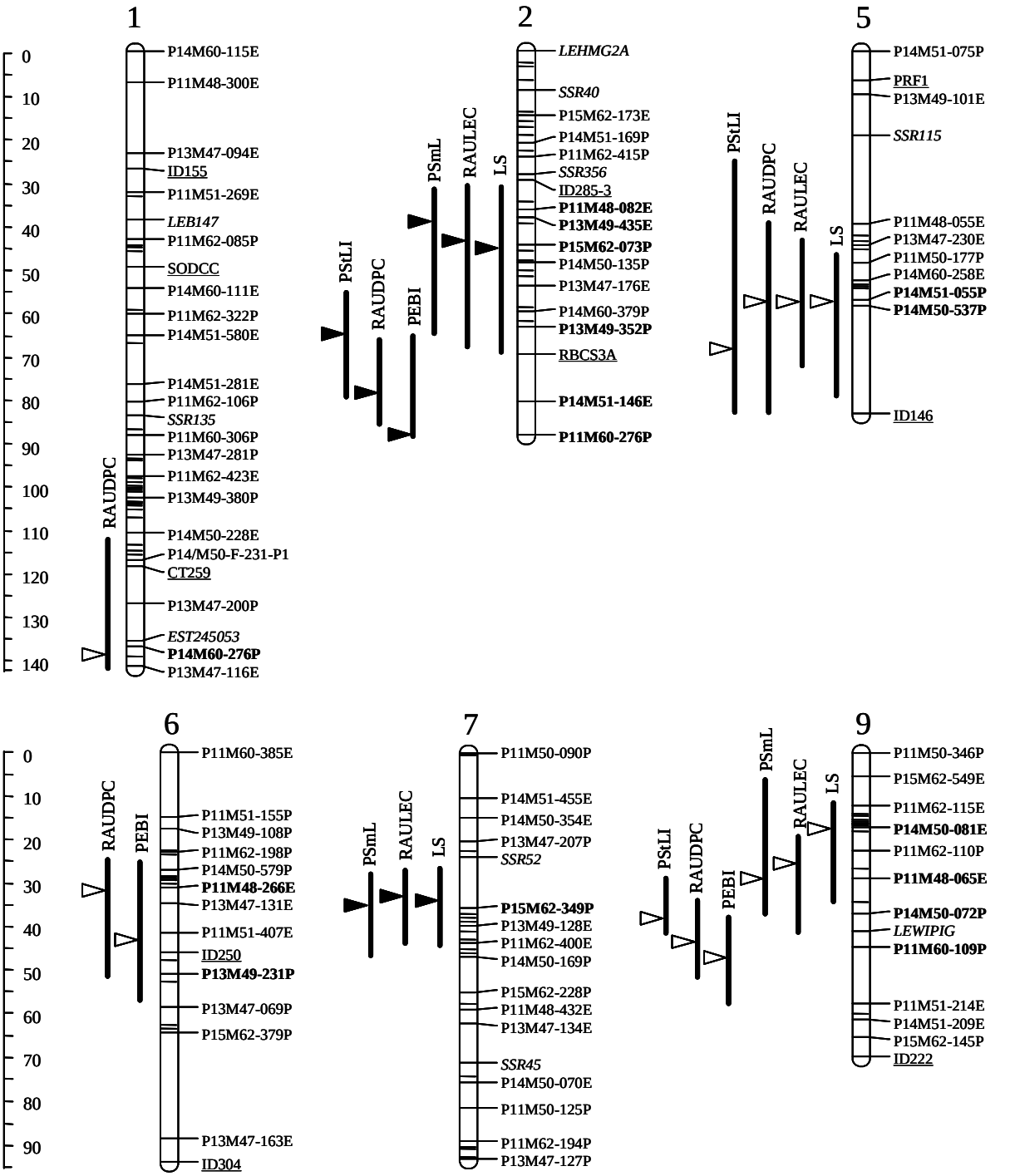

Figure 2 Map positions of QTLs for resistance to leaf and stem lesion of EB disease depicted on a skeletal map based on an F2 population of the cross *S. lycopersicum* cv. 'Solentos' × *S. arcanum* LA2157. QTLs are represented by bars covering 2-LOD support intervals obtained by restricted multiple-QTL-method (RMQM) mapping. Triangles indicate the position of the LOD peaks; solid and open triangles indicate that the resistance alleles were contributed by the susceptible and resistant parent, respectively. Markers in bold face were used as cofactors in mapping. SSR markers are italicized and SNP markers are underlined. Lesion size (LS), the relative area under the lesion expansion curve (RAULEC), and percentage of small lesions (PSmL) were assessed in the F2 population inoculated with a single *A. solani* isolate in a glasshouse in the Netherlands; percentage of EB index (PEBI), the relative area under the disease progress curve (RAUDPC) and percentage of stem lesion index (PstLI) were assessed in the F3 population inoculated with mixed field isolates in a field in Indonesia.

2. F3 field test

One main QTL on chromosome 9 was identified for PEBI (Table 1, Figure 2). Two QTLs with smaller effects on chromosomes 2 and 6, of which LOD values were below the threshold value (3.80), were included in the analyses since these QTLs also associated with RAUDPC (see below). The markers at these QTLs when used as cofactors increased the LOD value of the main QTL from 4.91 to 6.52 and the explained phenotypic variation from 13.5 to 15.3%. They also increased each other's LOD value although not significantly, from 3.00 to 3.35 (chromosome 2) and from 3.06 to 3.61 (chromosome 6).

Five QTLs for RAUDPC were identified on chromosomes 1, 2, 5, 6 and 9. Collectively these QTLs explained 59% of the phenotypic variance and they all showed additive gene action ($P<0.001$). A proportion of more than 10% of the phenotypic variances was explained by the QTLs on chromosome 2, 6, and 9. Except for the QTL on chromosome 2, all QTLs inherited resistance alleles from the resistant parent. The QTLs on chromosomes 2, 6, and 9 were also associated with PEBI. This is in agreement with a high correlation between the phenotypic values of the two disease traits ($r^2 = 0.80$).

Resistance to stem lesions was associated with three QTLs on chromosomes 2, 5, and 9. The QTL on chromosome 9 was the most important for resistance to stem lesions since by itself it explained over 30% of the phenotypic variance. The 2-LOD support intervals of the stem lesion QTLs partly or completely overlapped those of three QTLs for RAUDPC or PEBI.

Irrespective of the type of disease syndrome and the trait measured, the QTL on chromosome 9 was the most important in the field. For each trait measured, it explained the largest proportion of the phenotypic variance.

Additive genetic effects were prevalent for the QTLs detected in the field, while the QTL on chromosome 9 also displayed a dominant genetic effect ($P<0.001$) on stem lesion resistance. Digenic epistatic interactions of the type additive × additive ($P<0.05$) were found for RAUDPC between the QTLs on chromosomes 2 and 9.

Discussion

Linkage analysis

Deviation from the expected segregation ratio is a common feature of tomato interspecific crosses, often with the extent of skewness being higher on wider crossess. A skewness rate of 50% was reported in a *S. lycopersicum* × S. *cheesmaniae* (syn. *L. cheesmanii*) F2 population (Patterson et al. 1991), and up to 80% in a *S. lycopersicum* × *S. pennellii* F2 population (de Vicente and Tanksley 1993). Less skewed segregation (8 to 10%) was exhibited in crosses with *S. pimpinellifolium* (syn. *L. pimpinellifolium*), a species closely related with the cultivated tomato (Chen et al. 1999; Grandillo et al. 1996). A distortion

rate (55%) similar to our result was previously reported by van Heusden et al. (1999) using a different subset of F2 progeny from the same cross with *S. arcanum* LA2157. The aberrant segregation on chromosomes 2, 4, 7, and 8 towards *S. arcanum* alleles was also previously reported by van Heusden et al. (1999). Additionally, an excess in heterozygotes was observed on chromosome 9, as was also observed by Fulton et al. (1997) in a cross with *S. arcanum* LA1708 (syn. *L. peruvianum* LA1708). In our population, QTLs for EB resistance were observed both in regions with skewed segregations (chromosomes 2, 6, 7, and 9) and in regions without skewed segregation (chromosomes 1 and 5).

QTL analysis

We assessed EB resistance at the single plant level in the F2 population in glasshouse tests using inoculation with a single isolate and compared these data to the F3 data from a field test under artificial inoculations with mixed field isolates. Six QTLs were detected, two of which (the QTLs on chromosomes 2 and 7) inherited the resistant allele from the susceptible parent. This is not uncommon and has been reported in many plant species (e.g. Young et al. 1993; Lefebvre and Palloix 1996; Pilet et al. 1998). For EB resistance in tomato, Zhang et al. (2003) also detected a QTL on chromosome 3 for which the resistance allele was inherited from the susceptible parent. The presence of QTLs with opposite effects to those predicted by the parents may be responsible for the occurrence of individuals with transgressive phenotypes (de Vicente and Tanksley 1993; Dirlewanger et al. 1994).

Notwithstanding the differences in experimental techniques (pathogen isolates, inoculation method and resistance assessment criteria) and environmental conditions between the disease tests, we detected three EB QTLs in the glasshouse (chromosomes 2, 5, and 9) which coincided with QTLs for resistance traits in the field. Two QTLs were detected with a significant effect only on the field-test trait RAUDPC on chromosomes 1 and 6, with the second also having an elevated but non-significant LOD score for PEBI. One QTL on chromosome 7 was the major QTL affecting all glasshouse test traits, while it showed no effect on the field test traits. Especially the QTL on chromosome 9 is interesting: it is the major QTL detected for all traits in the F3 field test, and it is also an important QTL in the F2 glasshouse tests.

Plant conditions, including developmental stage and physiological state may affect the expression of QTL for resistance. Young et al. (1993) observed two QTLs for resistance to powdery mildew in mungbean which were significant at 65 days after planting and detected a different QTL at 85 days after planting. In our study, the QTL on chromosome 7 showed an effect in glasshouse tests with young plants (56 to 63 days after planting), whereas the QTLs on chromosomes 1 and 6 were only effective in the field at

later plant stage (90 to 110 days after sowing). The QTL on chromosome 7, which inherited the favorable alleles from the susceptible parent, might not have a true effect on EB resistance. As the susceptible parent is a cultivated, semi-determinate *S. lycopersicum* variety and much better adapted to the glasshouse test environment than the resistant, indeterminate *S. arcanum* parent, this suggests that the QTL on chromosome 7 may affect the condition of the plants in the glasshouse rather than the resistance itself. Thus, plants carrying the *S. lycopersicum* allele would in general be more vigorous and therefore better able to withstand infection, which overshadows the effect of their genotype at the "true" resistance QTLs. The fact that well-fertilized plants are more resistant than plants starved for nutrients and that young plant generally show more apparent resistance to EB than older plant (Rotem 1994) support the notion that plant condition can affect EB resistance. Whether this speculation is true or not, the QTL on chromosome 7 is not an interesting target for breeders, as it doesn't show an effect on EB severity in the field.

The detection of common QTLs at different experimental locations may be hampered by genotype × environment or genotype × isolate interactions as was observed in some studies, e.g. by Lübberstedt et al. (1999). We do not preclude the presence of such interactions in EB resistance that might further explain the discrepancy between the F2 glasshouse and F3 field tests; however, such interactions could not be determined in this study. In the two environments different isolates were used, so that the effects of the isolates and experimental conditions were confounded.

Comparison with classical genetic and molecular mapping studies of EB resistance

The current research is the first genetic study of EB resistance using *S. arcanum* as a donor parent. Our results concur with previous classical genetic and molecular mapping studies using *S. habrochaites* (syn. *L. hirsutum*) or derived materials and *S. pimpinellifolium*, which indicate that EB resistance is under polygenic control. Additive genetic effects were predominant (Nash and Gardner 1988a; Maiero et al. 1990a; Foolad et al. 2002b; Thirthamalappa and Lohithaswa 2000; Zhang et al. 2003); in some cases also dominant effects (Nash and Gardner 1988a; Thirthamalappa and Lohithaswa 2000) as well as epistatic interactions (Maiero et al. 1990a; Nash and Gardner 1988a; Thirthamalappa and Lohithaswa 2000) were observed.

Although we used a different resistance source, the 2-LOD support intervals of five of our QTLs overlapped with the QTL regions detected by Foolad et al. (2002b) and Zhang et al. (2003). The QTL on chromosome 7, which we detected only in the glasshouse test using a single isolate, was not detected in the field studies by Foolad et al. (2002b) and Zhang et al. (2003) using a mixture of two isolates from Pennsylvannia, U.S.. The smaller number of QTLs detected in our study may be due to a higher LOD threshold

employed (3.6 to 3.9 depending on the trait) compared to the previous mapping study using an *S. habrochaites* source which used a LOD threshold of 2.4 (Foolad et al. 2002b). Both studies revealed no major QTLs for EB resistance, but rather showed that resistance is controlled by several QTLs with small effects: 7 to 16% explained variance in our study, and 4 to 22% in Foolad et al. (2002b). The number of QTLs (7) detected by Zhang et al. (2003) using selective genotyping on a backcross population with *S. habrochaites* as donor was similar to the number of QTLs (6) we identified. A larger mapping population and more replications could possibly uncover more QTLs for EB resistance, but probably no major QTLs will be found.

Previous studies showed that stem lesion resistance was found in the same sources as EB resistance but the genetic relationship was not investigated (Barksdale and Stoner 1973, 1977; Stancheva et al. 1991a, b). In the present study three EB resistance QTLs coincided with stem lesion resistance QTLs; one QTL on chromosome 9 even had a major effect on the stem lesion resistance (31%).

Breeding implications

For breeding purposes QTLs with large additive effects, which are stable across environments and which do not depend on epistatic interactions, are most desirable. QTLs which meet these criteria perfectly were not found in the current study. Nevertheless, it would be useful for breeders to make use of the QTLs on chromosomes 2 and 9 as they are effective in both environments and are the most important according to the field test results. Genotypes homozygous for the 'Solentos' allele at the QTL on chromosome 2 or for the LA2157 allele at the QTL on chromosome 9 showed enhanced resistance as measured by different parameters (Table 2). A further increase in resistance was generally observed in the double homozygotes. It is possible that the favorable QTL allele on chromosome 2 is already present in most tomato material; in that case only the QTL on chromosome 9 would have to be introgressed. For introgression purposes a more precise determination of the QTL positions will be needed. This could be achieved through the development of a population of plants or lines, each containing parts of the *S. arcanum* QTL regions in a cultivated tomato background.

Table 2 Mean values for resistance parameters of F2 plants and the derived F3 lines based on the QTL genotypes on chromosome 2 and 9. The QTL on chromosome 2 inherited the resistant alleles from the susceptible parent 'Solentos'.

		QTL on chromosome 9											
		LS (mm2)			Mean	RAULEC			Mean	PSmL			Mean
		aa[a]	ab	bb		aa	ab	bb		aa	ab	bb	
QTL	aa	0.96[b]	0.43	0.45	0.44	0.99	0.65	0.75	0.72	41.20	48.10	48.84	47.91
on chr		(1)[c]	(11)	(5)	(23)	(2)	(13)	(10)	(26)	(1)	(11)	(5)	(17)
2	ab	1.05	0.63	0.70	0.71	1.32	0.90	1.01	0.99	24.97	38.07	40.39	35.59
		(11)	(43)	(21)	(89)	(12)	(45)	(17)	(76)	(14)	(38)	(21)	(80)
	bb	1.10	0.81	0.71	0.85	1.25	1.10	0.97	1.09	29.47	30.70	38.05	31.91
		(12)	(32)	(10)	(62)	(4)	(31)	(12)	(48)	(10)	(37)	(14)	(63)
Mean		1.04	0.68	0.66		1.35	0.95	0.90		27.65	35.82	41.50	
		(26)	(99)	(44)		(26)	(99)	(44)		(26)	(98)	(45)	

		QTL on chromosome 9											
		PEBI			Mean	RAUDPC			Mean	PStLI			Mean
		aa	ab	bb		aa	ab	bb		aa	ab	bb	
QTL	aa	45.94	40.60	37.75	40.48	39.34	35.11	32.71	35.27	19.32	7.68	7.57	9.76
on chr		(5)	(24)	(11)	(40)	(2)	(12)	(8)	(25)	(2)	(7)	(5)	(15)
2	ab	51.64	44.49	38.52	44.76	45.04	39.70	36.43	39.98	32.16	17.43	11.25	18.26
		(18)	(39)	(17)	(76)	(19)	(46)	(18)	(89)	(15)	(49)	(22)	(89)
	bb	51.98	48.61	44.81	47.60	41.69	42.30	39.00	41.38	33.23	18.71	15.53	21.01
		(4)	(13)	(11)	(28)	(5)	(19)	(11)	(38)	(7)	(11)	(11)	(29)
Mean		50.01	44.17	40.08		43.31	39.63	36.11		30.38	17.05	12.35	
		(30)	(81)	(40)		(30)	(81)	(40)		(30)	(76)	(44)	

Abbreviation: chr = chromosome

[a]aa = homozygous 'Solentos'; ab = heterozygous 'Solentos'/LA2157; bb = homozygous LA2157.

[b]Values are log (x+1) transformation for LS (lesion size) and RAULEC (relative area under the lesion expansion curve) and arcsine √(x/100) transformation for PSmL (percentage of small lesions) and PStLI (percentage of stem lesion index).

[c]Figures in parentheses are the number of F2 plants or F3 lines.

Acknowledgements

We thank Gerda Uenk, Hanneke van der Schoot, and Wendy van 't Westende for help and advice in the marker analyses; Dirk Budding and Remmelt Groenwold for assistance with *in vitro* plant culture; Daan Jaspers and Geurt Versteeg for plant care in the glasshouse. Nurul Hidayati, Vita Anggraini and Sularno of East-West Seed Indonesia are gratefully acknowledged for conducting the field trial. Financial support was provided by the Royal Netherlands Academy of Arts and Sciences in the framework of the Scientific Programme Indonesia–Netherlands.

Chapter 4

General discussion

Tomato early blight

Early blight (EB) is widely distributed in the world and can cause substantial yield loss of tomato in endemic areas (Chapter 1). The disease appears first on the lower leaves and intensifies as the plant matures. The frequent application of fungicides needed to control the disease might be reduced if cultivars with a sufficient level of resistance and satisfactory horticultural characteristics become available.

Many studies on the biology, epidemiology, and genetic variation of the fungal pathogen, *Alternaria solani,* have been published. Nevertheless, the important question concerning the existence of physiological races remains unanswered. Conversely, also no evidence is available of race- or isolate-specific resistance in the host. The study of physiological races is complicated by the multinucleate nature of the pathogen, which prevents the establishment of genetically uniform isolates. However, even if race-specific interactions do occur, these are unlikely be mediated by R-genes with major effects, as so far no evidence for major resistance genes have been found.

Various screening test methods have been developed, which differ with respect to test environment (field, glasshouse, or laboratory), biological materials (detached leaflets, intact young or old plants) and inoculum (conidia, mycelium or toxin). In few cases different methods have been compared. Reasonable correlations have been found between various types of glasshouse and field tests. In Chapter 3 a genetic study is described which uncovered partly the same and partly different QTLs using a glasshouse and a field test.

Several wild related species of tomato harbor resistance to EB but only *S. habrochaites* has been utilized for developing EB resistant cultivars through classical breeding. The major constraints in EB resistance breeding are the quantitative nature and polygenic inheritance of the resistance, which also causes serious linkage drag if no closely linked selection markers are available.

The complex genetic control of EB resistance in several sources of resistance has been studied using quantitative genetic methods. The loci underlying the resistance have been further dissected using a QTL mapping approach in *S. habrochaites* (Foolad et al. 2002; Zhang et al. 2003) and more recently in *S. arcanum* (described in Chapter 3).

Resistance test methods and screening of tomato accessions

The most widely used method of screening involves spraying plants with suspensions of conidia and visually estimating disease severity based on the amount of damaged leaf area. This type of disease assessment is subjective and not easily transferable across experiments. Locke (1948) was the first to devise a droplet inoculation method, which allows precise measurements of lesion growth. However, he used detached leaflets in his experiments, which resulted in poor correlations with field test results. Consequently this method was hardly used. We adopted this inoculation method, but used young intact plants in a glasshouse environment rather than detached leaflets following the protocol by Nash and Gardner (1988) with some improvements. We used this method to screen collections of tomato accessions including related species and a segregating population. This test method has several advantages: 1) it yields objective results, independent of the observer; 2) the scores represent a concrete quantity (lesion area) rather than an ill-specified disease index; 3) it allows to recover even susceptible plants for further use, as the disease remains confined only to the inoculated leaves during the test period; 4) it allows a detailed assessment of other epidemiological parameters such as lesion growth rate and the percentage inoculations that result only in very small spots, which were used to map resistance genes in Chapter 3**;** and 5) it can be extended to inoculations on petioles (resulting in defoliation by petiole lesions), stems and fruits so resistance in all these organs can be assessed separately.

From a series of experiments three conclusions emerged:

- The first two true leaves above the cotyledons are not suitable for inoculation due to early senescence, especially in non-glasshouse-adapted wild species;
- always a wide range of EB lesion sizes was found irrespective of resistance level, from minute flecks ($\leq$ 1 mm^2), to almost complete leaf blight; the lesion size followed an exponential-like distribution. The mean of this distribution was an indicator for the resistance level. Minute lesions occurred at a lower rate on susceptible than on resistant genotypes; this offers a quicker way of assessing resistance by calculating the percentage of small lesions, rather than measuring all lesions. In Chapter 3 the percentage of small lesions was used to map QTLs for resistance, with essentially the same results as mapping lesion size.
- Lesion size of droplet-inoculated plants did not correlate well with disease index of spray-inoculated plants. This was to a large extent caused by significant defoliation of spray-inoculated plants due to accidental lesions developing on petioles.

The droplet inoculation method can be applied to study epidemiological parameters for EB resistance, for which very limited information is available in tomato (e.g. O'Leary and Shoemaker 1983), and to characterize differential aggressiveness among *A. solani* isolates. The method also has potential for physiological studies. For

example, it could be applied to study the hypothesis that young leaves show a transient resistance to EB.

Several tomato lines previously reported as resistant (Poysa and Tu 1996) turned out to be susceptible to an Indonesian *A. solani* isolate used in our study. This warrants the verification of resistance to local isolates before introducing breeding lines or cultivars developed elsewhere into local breeding programs.

Mapping QTLs for EB resistance

The QTL mapping study described in Chapter 3 is the first such study involving a resistance source from *S. arcanum*. QTL mapping was performed in an F2 population phenotyped in a glasshouse test and in the derived population of F3 lines phenotyped in the field; in the two test environments different EB resistance parameters were scored. Some QTLs identified in the two environments overlapped while others were specific for either environment. While two QTLs specific for the F3 field test had relatively low LOD scores and might be spurious, one QTL specific for the F2 glasshouse test was highly significant. Although the causes of the discrepancies remain unknown, both plant age related expression of resistance or differential adaptation to the glasshouse environment might be involved.

At two QTLs, including the glasshouse-specific QTL mentioned above, the susceptible parent contributed the alleles for resistance; this is not uncommon and the same phenomenon was also observed in another EB resistance mapping study where *S. habrochaites* PI 126445 was used as sources of resistance (Zhang et al. 2003). One QTL perfectly overlapped in both F2 and F3, whereas two others partially overlapped. Among these three QTLs, the QTLs on chromosomes 2 and 9 are considered good targets for resistance breeding since they showed large effects in the glasshouse and in the field for EB resistance and also for stem lesion resistance. Introgression into a tomato background would be needed only for the QTL on chromosome 9 since the QTL on chromosome 2, which inherited the resistant alleles from the susceptible parent, might already be present in tomato.

Perspective for early blight resistance breeding in Indonesia

Given the low number of QTLs detected in *S. arcanum* LA2157 it should be feasible to introgress these into elite tomato breeding lines. The QTL mapping in transient F2 and F3 populations serves as an initial analysis of the effects of QTLs at particular positions. To show that a QTL will be effective in an adapted Indonesian tomato background, permanent near-isogenic lines (NILs) should be developed. The detailed procedure is described in the following and the working plan is depicted in Figure 1. F3 lines

containing the target QTL alleles have been selected for this purpose. Next, each plant must be genotyped using the AFLP markers flanking the target QTLs. For an easier monitoring of introgression these flanking markers can be converted into simple PCR markers. Individuals retaining the target chromosomal segments but carrying a minimal number of non-target segments are selected and backcrossed to a recurrent parent, preferably a tomato line adapted to Indonesian conditions. The backcross procedure is repeated with marker-assisted selection (MAS) in every cycle. When the desired recombinants are obtained, e.g. in the BC3, one round of selfing follows to produce BC3S1 progenies in which segregants homozygous for the donor allele of the QTL are selected. Each line is genotyped with more markers to estimate size of the introgression segment. The use of additional published tomato PCR-based markers can aid in saturating the interval of interest to precisely delineate the introgressed segment. This procedure will yield a series of NILs consisting of plants each with a different single homozygous introgression containing one target QTL. The NILs are tested to confirm the putative QTL NIL effect. NILs can further be used to study genotype × environment and genotype × isolate interactions and components and mechanism of resistance.

Fine mapping is needed to reduce the linkage drag associated with introgressing the QTL. It may also reveal whether the QTL effects are caused by closely linked loci or by a single locus. This can be done by crossing NIL(s) with an elite tomato and selfing the resulting F1(s) or backcrossing to obtain F2 or BC1 progenies. Marker assays are performed to identify plants containing recombinants in the QTL region. Recombinants are phenotyped to check if the remaining part of the QTL regions still has an effect on resistance.

The MAS approach has not yet been incorporated in public tomato breeding programs in Indonesia, even though the technology has been acquired for rice breeding since the last decade. The major obstacle, a classical problem for many developing countries, is the higher cost which would not readily attract governmental funding. However, since the phenotypic expression of EB resistance is highly influenced by environmental factors and resistance is conferred by several genes each with relatively small effects the application of MAS is justified, efficient and cost effective. The application of MAS in each cycle of introgression facilitates the precise selection of the desired QTL and therefore enhances the development of resistant cultivars. The QTL mapping study described in this thesis and the produced population of F3 lines are proposed as a starting point for an EB resistance marker-assisted breeding program in Indonesia.

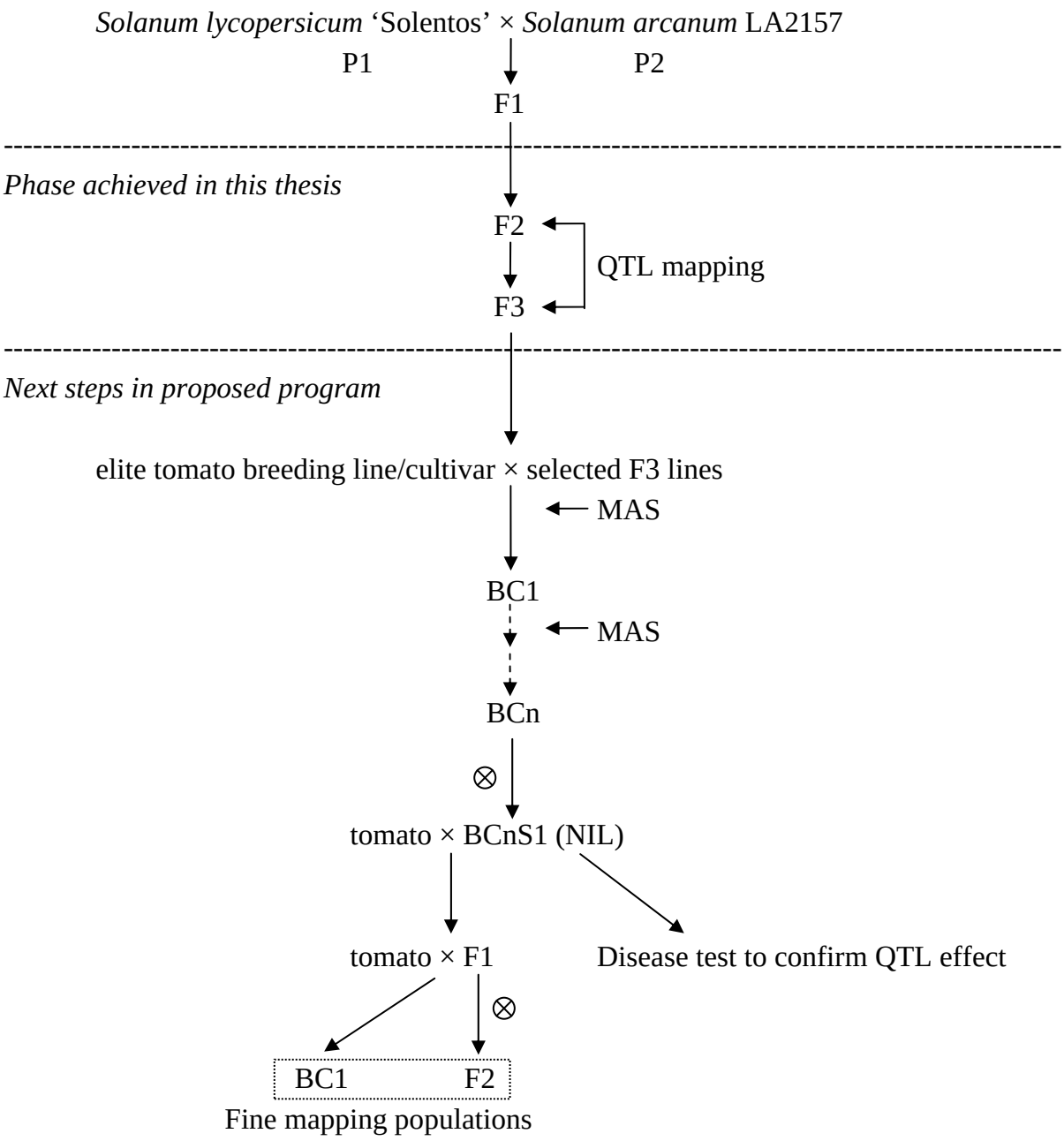

Figure 1 Schematic diagram for generation of NILs and fine-mapping of early blight resistance QTLs.

References

Agrios GN (2005) Plant Pathol, 5th edition. Elsevier Academic Press, London, pp 454-455

Apandi ES (1979) The effects of interval of fungicides against *Alternaria solani* on *Solanum lycopersicum* (tomato). Laporan Hasil Penelitian Kegiatan Hama dan Penyakit 3: 10-14

Areshchenkova T, Ganal MW (1999) Long tomato microsatellites are predominantly associated with centromeric regions. Genome 42: 536-544

Areshchenkova T, Ganal MW (2002) Comparative analysis of polymorphisms and chromosomal location of tomato microsatellite markers isolated from different sources. Theor Appl Genet 104: 229-235

Asandhi AA, Sastrosiswojo S (1988) Research on vegetables in Indonesia. In: MacLean BT (ed) Vegetable reseach in Southeast Asia: AVDRC-ADB workshop on collaborative vegetable research in Southeast Asia, AVDRC 1-6 August. Asian Vegetable Research and Development Center (AVRDC), Taipei, pp 95-99

Aveling TAS, Snyman HG, Rijkenberg FHJ (1993) Morphology of infection of onion leaves by *Alternaria porri*. Can. J. Bot. 72: 1164-1170

Banerjee MK, Chhabra ML, Saini PS (1998) Responses of tomato cultivars to *Alternaria* blight. Tests of Agrochemicals and Cultivars 19: 50-51

Barksdale TH (1969) Resistance of tomato seedlings to early blight. Phytopathology 59: 443-446

Barksdale TH (1971) Field evaluation for tomato early blight resistance. Plant Dis Rep 55: 807-809

Barksdale TH, Stoner AK (1973) Segregation for horizontal resistance to tomato early blight. Plant Dis Rep 57: 964-965

Barksdale TH, Stoner AK (1977) A study of the inheritance of tomato early blight resistance. Plant Dis Rep 61: 63-65

Barrat RW, Richards MC (1944) Physiological maturity in relation to Alternaria blight in tomato. Phytopathology 34: 997 (Abstr.)

Basu PK (1974a) Existence of chlamydospores of *Alternaria porri* f. sp. *solani* as overwintering propagules in soil. Phytopathology 61: 1347-1350

Basu PK (1974b) Measuring early blight, its progress and influence on fruit losses in nine tomato cultivars. Can Plant Dis Surv 54: 45-51

Bhatia IS, Uppal DS, Bajaj KL (1972) Study of phenolic contents of resistant and susceptible varieties of tomato (*Lycopersicum esculentum*) in relation to early blight disease. Indian Phytopath 25: 231-235

Bonde R (1929) Physiological strains of *Alternaria solani*. Phytopathology 19: 533-548

Bos G, Kartapradja R (1977) Tomato variety trials on Java with emphasis on yield potential, adaptability to environment and tolerance to pests and diseases. Bulletin Penelitian Hortikultura 5: 93-113

Bredemeijer GMM, Cooke RJ, Ganal MW, Peeters R, Isaac P, Noordijk Y, Rendell S, Jackson J, Röder MS, Wendehake K, Dijks M, Amelaine M, Wickaert V, Bertrand L, Vosman B (2002) Construction and testing of a microsatellite database containing more than 500 tomato varieties. Theor Appl Genet 105: 1019-1026

Brian PW (1952) The phtytotoxic properties of alternaric acid in relation to the etiology of plant disease caused by *Alternaric acid* (Ell. & Mart.) Jones & Grout. Ann Appl Biol 39: 308-321

Brüggemann W, Linger P, Wenner A, Koornneef M (1996) Improvement of post-chilling photosynthesis in tomato by sexual hybridization with a *Lycopersicon peruvianum* line from elevated altitude. Adv Hort Sci 10: 215-218

Bussey MJ, Stevenson WR (1991) A leaf disk assay for detecting resistance to early blight caused by *Alternaria solani* in juvenile potato plants. Plant Dis 75: 385-390

Castro MEA, Zambolim L, Chavez GM, Cruz CD, Matsuoka K (2000) Pathogenic variability of *Alternaria solani*, the causal agent of tomato early blight. Summa Phytopathologica 26: 24-28

Charlton KM (1953) The sporulation of *Alternaria solani* in culture. Trans. Br. Mycol. Soc. 36: 349-355

Chen FQ, Foolad MR (1999) A molecular linkage map of tomato based on a cross between *Lycopersicon esculentum* and *L. pimpinellifolium* and its comparison with other molecular maps of tomato. Genome 42: 94-103

Christ BJ (1991) Effect of disease assessment method on ranking potato cultivars for resistance to early blight. Plant Dis 75: 353-356

Coquoz Jl, Buchala AJ, Meuwly Ph, Métraux JP (1995) Arachidonic acid induces local but not systemic synthesis of salicylic acid and confers systemic resistance in potato plants to *Phytophthora infestans* and *Alternaria solani*. Phytopathology 85: 1219-1224

Darakov OB (1995) Gametophytic selection of tomatoes for resistance to early blight disease. Sex Plant Reprod 8: 95-98

Datar VV, Lonkar SG (1985) Inheritance of resistance in tomato early blight. J Mah Agric Univ 10: 357-358

Datar VV, Mayee CD (1981) Assessment of losses in tomato yield due to early blight. Indian Phytopath 34: 191-195

De Vicente MC, Tanksley SD (1993) QTL analysis of transgressive segregation in an interspecific tomato cross. Genetics 134: 585-596

Dirlewanger E, Isaac PG, Ranades S, Belajouza M, Cousin R, de Vienne D (1994) Restriction fragment length polymorphism analysis of loci associated with disease resistance genes and developmental traits in *Pisum sativum* L. Theor Appl Genet 88: 17-27

Douglas DR, Pavek JJ (1971) An efficient method of inducing sporulation of *Alternaria solani* in pure culture. Phytopathology 61: 239

Douglas DR, Pavek JJ (1972) Screening potatos for field resistance to early blight. Am Potato J 49, 1-7

Ellis MB, Gibson IAS (1975) *Alternaria solani* no 45 set 48. Commonwealth Mycological Institute, Kew, Surrey, England

Esquivel EA (1984) *Pleospora solani* sp. nov, teleomorphosis of *Alternaria solani* (Ell. & Mart.) Jones & Grout. Phytopathology 74: 1014 (Abstr.)

Faris JD, Li WL, Liu DJ, Chen PD, Gill, BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. Theor Appl Genet 98: 219-225

Fernandez A, Solorzano E, Peteira B, Fernandez E (1996) Peroxidase induction in tomato leaves with different degrees of susceptibility to *Alternaria solani*. Revista de Proteccion Vegetal 11: 79-83

Foolad MR, Lin GY (2001) Heritability of early blight resistance in a *Lycopersicon esculentum* × *Lycopersicon hirsutum* cross estimated by correlation between parent and progeny. Plant Breed 120: 173-177

Foolad MR, Ntahimpera N, Christ BJ, Lin GY (2000) Comparison of field, greenhouse, and detached-leaflet evaluations of tomato germ plasm for early blight resistance. Plant Dis 84: 967-972

Foolad MR, Subbiah P, Ghangas GS (2002a) Parent-offspring correlation estimate of heritability for early blight resistance in tomato, *Lycopersion esculentum* Mill. Euphytica 126: 291-297

Foolad MR, Zhang LP, Khan AA, Niño-Liu D, Lin GY (2002b) Identification of QTLs for early blight (*Alternaria solani*) resistance in tomato using backcross populations of a *Lycopersicon esculentum* × *Lycopersion hirsutum* cross. Theor Appl Genet 104: 945-958

Fulton TM, Chunwongse J, Tanksley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13: 207-209

Fulton TM, Nelson JC, Tanksley SD (1997) Introgression and DNA marker analysis of *Lycopersicon peruvianum*, a wild relative of the cultivated tomato, into *Lycopersicon esculentum*, followed through three successive backcross generations. Theor Appl Genet 95: 895-902

Gardner RG (1988) NC EBR-1 and NC EBR-2 early blight resistant tomato breeding lines. HortScience 23: 779-781

Gardner RG (1990) Greenhouse disease screen facilitates breeding resistance to tomato early blight. HortScience 25: 222-223

Gardner RG (2000) 'Plum Dandy', a hybrid tomato, and its parents, NC EBR-5 and NC EBR-6. HortScience 35: 962-963

Gardner RG, Shoemaker PB (1999) 'Mountain Supreme' early blight-resistant hybrid tomato and its parents, NC EBR-3 and NC EBR-4. HorstScience 34: 745-746

Gilchrist DG, Grogan RG (1975) Production and nature of a host-specific toxin from *Alternaria alternata* f.sp. *lycopersici*. Phytopathology 66: 165-171

Grandillo S, Tanksley SD (1996) Genetic analysis of RFLPs, GATA microsatellites and RAPDs in a cross between *L. esculentum* and *L. pimpinellifolium*. Theor Appl Genet 92: 957-965

Gwary DM, Nahunnaro H (1998) Epiphytotics of early blight of tomatoes in Northeastern Nigeria. Crop Prot 17: 619-624

Hammond-Kosack KE, Jones JDG (1996) Resistance gene-dependent plant defense responses. Plant Cell 8: 1773-1791

Henning RG, Alexander LJ (1959) Evidence of existence of physiologic races of *Alternaria solani*. Plant Dis Rep 43: 298-308

Herriot AB, Haynes Jr FL, Shoemaker PB (1986) The heritability of resistance to early blight in diploid potatoes (*Solanum tuberosum* subsp. *phureja* and *stenotonum*). Am Potato J 63, 229-232

Holley JD, Hall R, Hofstra G (1983) Identification of rate-reducing resistance to early blight in potato. Can J Plant Pathol 5: 111-114

Horsfall JG, Barrat RW (1945) An improved grading system for measuring plant diseases. Phytopathology 35:655 (Abstr.)

Johanson A, Thurston HD (1990) The effect of cultivar maturity on the resistance of potato to early blight caused by *Alternaria solani*. Am J Pot 67: 615-623

Johnson KB, Teng PS (1990) Coupling a disease progress model for early blight to a model of potato growth. Phytopathology 80: 416-425

Kalloo G, Banerjee MK (1993) Early blight resistance in *Lycopersicum esculentum* Mill. transferred from *L. pimpinellifolium* (L.) Mill. and *L. hirsutum* f. *glabratum* Mull. Gartenbauwissenschaft 58:238-239

Keinath A, DuBose VB, Rathwell PJ (1996) Efficacy and economics of three fungicide application schedules for early blight control and yield of fresh-market tomato. Plant Dis 80: 1277-1282

Langsdorf G, Furuichi N, Doke N, Nishimura S (1990) Investigations of *Alternaria solani* infections: detection of alternaric acid and a susceptibility-inducing factor in the spore-germination fluid of *A. solani*. J Phytopathol 128: 271-282

Langsdorf G, Park P, Nishimura S (1991) Investigations on *Alternaria solani* infections: effect of alternaric acid on the ultrastructure of tomato cells. Ann. Phytopath. Soc. Japan 57: 32-40

Lawrence CB, Joosten MHAJ, Tuzun S (1996) Differential induction of pathogenesis related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. Physiol Mol Plant Pathol 43: 361-377

Lawrence CB, Singh NP, Qiu J, Gardner RG, Tuzun S (2000) Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solani* and may function as an elicitor release mechanism. Physiol Mol Plant Pathol 57: 211-220

Lefebvre V, Palloix A (1996) Both epistatic and additive effects of QTL are involved in polygenic induced resistance to disease: a case study, the interaction pepper-*Phytophthora capsici* Leonian. Theor and Appl Genet 93: 503-511

Locke SB (1948) A method for measuring resistance to defoliation diseases in tomato and other *Lycopersicon* species. Phytopathology 38: 937-942

Locke SB (1949) Resistance to early blight and Septoria leaf spot in the genus Lycopersicon. Phytopathology 39, 829-836

Lodha PC (1977) Reaction of some tomato cultivars to culture filtrate of *Alternaria solani* (Ell. et Mart.) Jones et Grout. Phytopathologia Mediterranea 16: 36-37

Lübberstedt T, Xia XC, Tan G, Liu X, Melchinger AE (1999) QTL mapping of resistance to *Sporisorium reiliana* in maize. Theor Appl Genet 99: 593-598

Lukens RJ (1960) Conidial production from filter paper cultures of *Helminthosporium vagans* and *Alternaria solani*. Phytopathology 50: 867-868

Lynch DR, Wastie RL, Stewart HE, MacKay GR, Lyon GD, Nachmias A (1991) Screening for resistance to early blight (*Alternaria solani*) in potato (*Solanum tuberosum* L.) using toxic metabolites produced by the fungus. Potato Res 34: 297-304

Madden L, Pennypacker SP, MacNab (1978) FAST, a forecast system for *Alternaria solani* on tomato. Phytopathology 68, 1354-1358.

Maiero M, Bean GA, Ng TJ (1991) Toxin production by *Alternaria solani* and its related phytotoxicity to tomato breeding lines. Phytopathology 81: 1030-1033

Maiero M, Ng TJ, Barksdale TH (1989) Combining ability estimates for early blight resistance in tomato. J Am Soc Hort Sci 114: 118-121

Maiero M, Ng TJ, Barksdale TH (1990a) Genetic resistance to early blight in tomato breeding lines. HortScience 25: 344-346

Maiero M, Ng TJ, Barksdale TH (1990b) Inheritance of collar rot resistance in the tomato breeding lines C1943 and NC EBR-2. Phytopathology 80: 1365-1368

Manohara D (1971) Penyakit-penyakit pada tanaman famili Solanaceae di Lembang dan Pacet. Fakultas Pertanian Institut Pertanian Bogor, Bogor, pp 33

Marcinkowska J (1982) Fungi of the genus Alternaria occurring on tomato. Acta Agrobotanica 34: 261-276.

Martin FW, Hepperly P (1987) Sources of resistance to early blight, *Alternaria solani*, and transfer to tomato, *Lycopersicon esculentum*. J Agric Univ Puerto Rico 71: 85-95

Martinez SP, Snowdon R, Pons-Kuhnemann (2004) Variability of Cuban and international populations of *Alternaria solani* from different hosts and localities: AFLP genetic analysis. Eur J Plant Pathol 110: 399-409

Mauch-Mani B, Slusarenko AJ (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonium lyase enzymes in the resistance of Arabidopsis to *Peronospora parasitica*. Plant Cell 8: 203-212

Mehrotra RS, Areja KR (1990) An introduction to mycology. New Age Publishers, pp 648

Mehta P, Vyas KM, Saksena SB (1975) Pathological studies on fruit rot of tomato caused by *Alternaria solani* and *A. tenuis*. Indian Phytopath 28: 247-252

Montemurro N, Visconti A (1992) *Alternaria* metabolites – chemical and biological data. In: Chelkowski J, Visconti A (eds) *Alternaria* biology, plant disease and metabolites. Elsevier, Amsterdam, pp 449-558

Moore WD (1942) Some factors affecting the infection of tomato seedlings by *Alternaria solani*. Phtyopathology 32: 399-403

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497

Nash AF, Gardner RG (1988a) Heritability of tomato early blight resistance derived from Lycopersicon hirsutum PI 126445. J Am Soc Hort Sci 113: 264-268

Nash AF, Gardner RG (1988b) Tomato early blight resistance in a breeding line derived from *Lycopersicon hirsutum* PI 126445. Plant Dis 72: 206-209

Neergaard P (1945) Danish species of *Alternaria* and *Stemphylium*: taxonomy, parasitism, economic significance. Oxford University Press, London, pp 260-287

Nicholson RL, Hammerschmidt R (1992) Phenolic compound and their role in disease resistance. Annu Rev Phytopathol 30: 369-389

O'Leary DJ, Shoemaker PB (1983) Components of resistance for tomato early blight. Phytopathology 73: 803 (Abstr.)

Opeña RT, van der Vossen HAM (1993) *Lycopersicon esculentum* Miller. In: Siemonsma JS, Piluek K (eds) Plant resources of South-East Asia No 8 vegetables. Pudoc Scientific Publishers, Wageningen, pp 199-205

Padhi NN, Rath GC (1973) Sporulation of *Alternaria solani* in pure culture. Indian Phytopath 26: 495-502

Pandey KK, Pandey PK, Kallo G, Banerjee MK (2003) Resistance to early blight of tomato with respect to various parameters of disease epidemics. J Gen Plant Pathol 69: 364-371

Paravicini E (1923) Kartoffelkrankkheiten in Niederlandisch-Ost-Indien. Centralb Bakt Abt Bot 24: 61-79

Pasche JS, Wharam CM, Gudmestad NC (2004) Shift in sensitivity of Alternaria solani to Q_oI fungicides. Plant Dis 88: 181-187

Paterson AH, Damon S, Hewitt JD, Zamir D, Rabinowitch HD, Lincoln SE, Tanksley SD (1991) Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments. Genetics: 127: 181-197

Patterson CL (1991) Importance of chlamydospores as primary inoculum of *Alternaria solani*, incitant of collar rot and early blight on tomato. Plant Dis 75: 274-278

Payne RW, Harding SA, Murray DA, Soutar DM, Baird DB, Welham SJ, Kane AF, Gilmour AR, Thompson R, Webster R, Wilson GT (2002) GenStat® for Windows™ 6th Edition. VSN International, Oxford

Pelletier JR, Fry WE (1989) Characterization of resistance to early blight in three potato cultivars: incubation period, lesion expansion rate, and spore production. Phytopathology 79: 511-517

Peralta IE, Knapp S, Spooner DM (2005) New species of wild tomatoes (*Solanum* section *Lycopersicon*: Solanaceae) from Northern Peru. Sys Bot 30: 424-434

Peréz S, Martinéz (1999) Infection of tomato cultivars by *Alternaria solani* (E & M) J & G. Revista de Proteccion Vegetal 14: 1-5

Petrunak DM, Christ BJ (1992) Isozyme variability in *Alternaria solani* and *A. alternata*. Phytopathology 82, 1343-1347

Pierre RE, Millar RL (1965) Histology of pathogen – suscept relationship of *Stemphylium botryosum* and alfalfa. Phytopathology 55: 909-914

Pilet ML, Delourme R, Foisset N, Renard M (1998) Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. Et de Not., in winter rapeseed (*Brassica napus* L). Theor Appl Genet 96: 23-30

Pound GS, Stahmann MA (1951) The production of a toxic material by *Alternaria solani* and its relation to the early blight disease of tomato. Phytopathology 41: 1104-1114

Poysa V, Tu JC (1996) Response of cultivars and breeding lines of *Lycopersicon* spp. to *Alternaria solani*. Can Plant Dis Surv 76: 5-8

Rant AD (1915) De droge vlekkenziekte bij de aardappel op Java. Teysmannia 26: 285

Reynard GB, Andrus CF (1945) Inheritance of resistance to the collar-rot phase of *Alternaria solani* on tomato. Phytopathology 35: 25-36

Rotem J (1966) Variability in *Alternaria porri* f. sp. *solani*. Israel J Bot 15: 48-57

Rotem J (1994) The genus *Alternaria* biology, epidemiology, and pathogenicity, 1st ed. The American Phtyopathological Society, St. Paul, Minnesota, pp 48, 203

Rotem J, Reichert I (1964) Dew – a principal moisture factor enabling early blight epidemics in a semiarid region of Israel. Plant Dis Rep 48: 211-215

Sandbrink JM, van Ooijen JW, Purimahua C, Vrielink R, Verkerk M, Zabel P, Lindhout P (1995) Localization of genes for bacterial canker resistance in *Lycopersicon esculentum* using RFLPs. Theor Appl Genet 90: 444-450

Sands DC, Lukens RJ (1974) Effect of glucose and adenosine phosphates on production of extracellular carbohydrases of *Alternaria solani*. Plant Physiol 54: 666-669

Schlagnhaufer CD, Arteca RN, Pell EJ (1997) Sequential expression of two 1-aminocyclopropane-1-carboxylate synthase genes in response to biotic and abiotic stresses in potato (*Solanum tuberosum* L.) leaves. Plant Mol Biol 35: 683-688

Schlegel RH (2003) Encyclopedia dictionary of plant breeding and related subjects 1st ed. Food Product Press, New York, pp 320

Semangun H (1989) Penyakit-penyakit tanaman hortikultura di Indonesia, 1st ed. Gadjah Mada University Press, Yogyakarta, pp 120-123

Shahin EA, Shepard JF. 1979. An efficient technique for inducing profuse sporulation of *Alternaria* species. Phytopathology 69 (6): 618-620.

Sherf AF, MacNab AA (1986) Vegetable diseases and their control. John Wiley and Sons, New York, pp 634-640

Shtienberg D, Blachinsky D, Kremer Y, Ben-Hador G, Dinoor A (1995) Integration of genotype and age-related resistance to reduce fungicide use in management of *Alternaria* diseases of cotton and potato. Phytopathology 85, 995-1002

Sinden SL, Goth RW, O'Brien MJ (1972) Effect of potato alkaloids on the growth of *Alternaria solani* and their possible role as resistance factors in potatoes. Phytopathology 63, 303-307

Smulders MJM, Bredemeijer G, Rus-Kortekaas W, Arens P, Vosman B (1997) Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. Theor Appl Genet 97: 264-272

Solorzano E, Fernandez A, Peteira B, Fernandez E (1996) Polyphenol oxidases and phenylalanine ammonium lyases induction in tomato leaves infected with *Alternaria solani*. Revista de Proteccion Vegetal 11: 153-157

Spletzer ME, Enyedi AJ (1999) Salicylic acid induces resistance to *Alternaria solani* in hydrophonically grown tomato. Phytopathology 89: 722-727

Stall RE (1958) An investigation of nuclear number in *Alternaria solani*. Am J Bot 45: 657-659

Stall RE, Alexander LJ (1957) Heterocaryotic variation in *Alternaria solani*. Phytopathology 47: 34 (Abstr.)

Stancheva I (1988) Evaluating the susceptibility of tomato to *Alternaria solani* by using the cultural filtrate of the pathogen. Rasteniev'dni-Nauki 25:71-76

Stancheva I (1990) Intraspecies differentiation of *Alternaria solani* according to aggressiveness degree. Genetika-i-Selektsiya 27:60-64

Stancheva I (1991) Inheritance of the resistance to injuries on the growth mass caused by *Alternaria solani* in the tomato. Genetika-i-Selektsiya 24:232-236

Stancheva I, Lozanov I, Achkova Z (1991a) Sources of resistance to *Alternaria solani* in the tomato in wild growing species of the genus *Lycopersicon*. Genetika-i-Selektsiya 24: 126-130

Stancheva I, Lozanov I, Stamova L (1991b) Correlations between the resistance to different injuries from *Alternaria solani* in the tomatoes. Genetika-i-Selektsiya 24: 51-55

Strandberg JO (1992) *Alternaria* species that attack vegetable crops: biology and options for disease management: In: Chelkowski J, Visconti A (eds) *Alternaria* biology, plant disease and metabolites. Elsevier, Amsterdam, pp 175-208

Tanksley SD, Ganal MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messegeur R, Miller JC, Miller L, Patterson AH, Pineda O, Roder MS, Wing RA, Wu W, Young ND (1992) High density molecular linkage maps of the tomato and potato genome. Genetics 132: 1141-1160

Thipyapong P, Steffens JC (1997) Tomato polyphenol oxidase: differential response of the polyphenol oxidase F promoter to injuries and wound signals. Plant Physiol 115: 409-418

Thirthamalappa, Lohithaswa HC (2000) Genetics of resistance to early blight (*Alternaria solani* Sorauer) in tomato (*Lycopersicum esculentum* L.) Euphytica 113: 187-193

Vakalounakis DJ (1983) Evaluation of tomato cultivars for resistance to *Alternaria* blight. Ann Appl Biol 102: 138-139

Van der Goot P (1924) Overzicht der voornamste ziekten van het aardappelgewas op Java. Inst voor Plantenziekten 18: 42

Van der Waals JE, Korsten L and Aveling TAS (2001) A review of early blight of potato. African Plant Prot 7: 1-12

Van der Waals JE, Korsten L, Slippers B (2004) Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa. Plant Dis 88: 959-964

Van Hall CJJ (1925) Ziekten en plagen der cultuurgewassen in Nederlandsch-Indie in 1924. Meded Inst voor Plantenziekten 67:53

Van Heusden AW, Koornneef M, Voorrips RE, Bruggemann W, Pet G, Vrielink-van Ginkel R, Chen X, Lindhout P (1999) Three QTLs from *Lycopersicon peruvianum* confer a high level of resistance to *Clavibacter michiganensis* ssp. *michiganensis*. Theor Appl Genet 99: 1068-1074

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

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

Veremis JC, van Heusden AW, Roberts PA (1999) Mapping a novel heat-stable resistance to *Meloidogyne* in *Lycopersicon peruvianum*. Theor Appl Genet 98: 274-280

Villareal RL (1980) Tomato in the tropics. Westview Press, Boulder, Colorado, pp 3-10

Visker MHPW, Keizer LCP, van Eck HJ, Jacobsen E, Colon LT, Struik PC (2003) Can the QTL for late blight resistance on potato chromosome 5 be attributed to foliage maturity type? Theor Appl Genet 106: 317-325

Vloutoglou I (1999) Evaluation of tomato cultivars and hybrids for resistance to *Alternaria solani* infection. Tests of Agrochemicals and Cultivars 20: 48-49

Vloutoglou I, Kalogerakis SN (2000) Effects of inoculum concentrations, wetness duration and plant age on development of early blight (*Alternaria solani*) and on shedding of leaves in tomato plants. Plant Pathol 49: 339-345

Vos P, Hogers R, Bleeker M, Rijans M, van der Lee T, Hornes M, Frijters A, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acid Res 23: 4407-4414

Walker JC (1952) Diseases of vegetable crops, 1st ed. MacGraw-Hill Book Company, Inc. New York, pp 471-474

Weir TL, Huff DR (1998) RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato. Mycologia 90: 813-821

Wellman FL (1943) A technique to compare virulence of isolates of *Alernaria solani* on tomato leaflets. Phytopahtology 41: 698-706

Young ND, Danesh D, Menancio-Hautea, Kumar L (1993) Mapping oligogenic resistance to powdery mildew in mungbean with RFLPs. Theor Appl Genet 87: 243-249

Zhang LP, Lin GY, Niño-Liu, Foolad MR (2003) Mapping QTLs conferring early blight (*Alernaria solani*) resistance in a *Lycopersicon esculentum* × *L. hirsutum* cross by selective genotyping. Mol Breed 12: 3-19

Zhang R (2004) Genetic characterization and mapping of partial resistance to early blight in diploid potato. Ph.D. Thesis, The Pennsylvania State University (Abstr.)

Summary

Tomato early blight (EB) caused by the fungus *Alternaria solani* is a field disease with a worldwide distribution, including Indonesia. The disease is currently controlled using frequent applications of fungicides. The use of resistant cultivar would be an attractive way to reduce fungicide application. The aims of the research are to support breeding of EB resistance cultivars for the Indonesian market, by identifying EB resistance genes effective in Indonesia and developing markers for MAS.

In Chapter 1 the literature on *A. solani*, EB and resistance is reviewed. On the fungal side pathogenicity and genetic variation have been widely investigated. However, the existence of different physiological races has not been convincingly demonstrated or disproved. Reliable methods of screening are available for use in resistance breeding. Intensive screening of tomato accessions worldwide has shown that strong resistance is not available in the cultivated tomato but only in wild species. Resistance to *A. solani* is expressed quantitatively and is polygenically inherited. For that reason classical breeding has not been able to achieve high levels of resistance, and undesirable traits from the donors have been introgressed as well. Recently QTLs have been mapped in a few resistance sources, which could facilitate transferring such quantitative resistance genes and circumvent the problem of unfavourable linkage drag once markers tightly linked to the QTLs have been obtained.

In Chapter 2 a resistance test method involving droplet inoculation is described, which is an adaptation of an existing but hardly-used technique. This method has advantages in comparison with the more widely used spray inoculation method, including a clear distinction between lesions caused by *A. solani* and necroses of leaf loss due to other causes, and an objective measurement of damage in contrast to subjective scoring. In the droplet inoculation method leaflets of intact plants are inoculated with droplets of an *A. solani* conidial suspension in water or an agar solution. Lesions are assessed quantitatively by measuring the length and the perpendicular width. In this way objective and accurate assessments, which are prerequisites for QTL analysis, can be achieved. This inoculation method was used to screen a collection of 54 tomato accessions for resistance to an Indonesian *A. solani* isolate.

S. arcanum LA2157 is highly resistant to *A. solani* in the glasshouse screening tests. The genetics of the resistance in this wild relative of tomato was further studied using a QTL mapping approach (Chapter 3). The mapping population consisted of 176 F2 plants. A linkage map consisting of 12 linkage groups covering 1179 cM was based on 379 markers (33 SSR, 21 SNP and 316 AFLP markers). All linkage groups could be assigned to the 12 tomato chromosomes. About half of the markers showed deviation from the expected 1:2:1 segregation ratio. The F2 population was phenotyped in a

glasshouse in Netherlands with an Indonesian isolate of *A. solani*. EB resistance was evaluated with respect to lesion size (LS) and related parameters (relative area under the lesion expansion curve [RAULEC] and percentage of small lesions [PSmL]). The derived F3 lines (156) were tested in a field in Indonesia. Percentage of EB index (PEBI) was assessed at six times and relative area under the disease progress curve (RAUDPC) was calculated. A total of six QTLs with a range of LOD scores 3.6 to 16.4 were mapped on chromosomes 1, 2, 5, 6, 7 and 9. Three of the QTLs showed effects in both tests despite differences in experimental method and observed traits. For the first time, three QTLs for resistance affecting the development of stem lesions, another disease symptom caused by the fungus on the main and secondary stems of the plant, were identified, which completely overlapped with QTL regions for early blight resistance. Two QTLs on chromosomes 2 and 9, which explained 7 to 16% of the phenotypic variation for EB resistance and 31% for stem lesion resistance, are recommended to be used in tomato breeding programs for resistance to *A. solani*.

Samenvatting

De ziekte Early Blight (EB) in tomaat wordt veroorzaakt door de schimmel *Alternaria solani*. De ziekte komt wereldwijd voor, ook in Indonesië. Voor de bestrijding worden frequent fungiciden toegepast. Het gebruik van resistente rassen zou een aantrekkelijke manier zijn om het fungicidengebruik te reduceren. Het doel van dit onderzoek is om de veredeling van EB-resistente rassen voor de Indonesische markt te ondersteunen, door genen voor resistentie tegen EB te identificeren die effectief zijn in Indonesië en door selectiemerkers voor MAS te ontwikkelen.

In hoofdstuk 1 wordt een overzicht van de literatuur over *A. solani*¸ EB en resistentie gegeven. De pathogeniteit en genetische variatie van de schimmel zijn uitgebreid onderzocht. Er zijn echter geen overtuigende bewijzen beschreven voor het al of niet bestaan van verschillende fysio's. Er zijn betrouwbare methoden beschikbaar voor het toetsen van resistentie. Uitgebreide toetsen van tomaten-accessies uit de hele wereld hebben aangetoond dat er geen sterke resistentie voorkomt binnen de cultuurtomaat, maar wel in wilde verwanten. Resistentie tegen *A. solani* komt kwantitatief tot expressie en berust op meerdere genen. Daardoor is het via klassieke veredeling nog niet gelukt om een hoog niveau van resistentie te realiseren, en zijn er naast resistentie ook ongewenste eigenschappen uit de donors ingekruist. Recent zijn er QTLs uit enkele resistentiebronnen in kaart gebracht. Wanneer er nauw gekoppelde selectiemerkers verkregen worden zal dit het inkruisen van dergelijke kwantitatieve resistentiegenen, zonder gekoppelde ongewenste eigenschappen, vergemakkelijken.

In hoofdstuk 2 wordt een toetsmethode beschreven die gebaseerd is op druppel-inoculatie, een aanpassing van een bestaande, maar tot nu toe nauwelijks toegepaste methode. Deze methode heeft voordelen in vergelijking met de veelgebruikte spray inoculatie, waaronder een duidelijk onderscheid tussen lesies veroorzaakt door *A. solani* en necroses of bladverlies ten gevolge van andere oorzaken, en een objectieve meting van de aantasting in plaats van subjectieve schatting. Bij de druppel-inoculatiemethode worden blaadjes van intacte planten geïnoculeerd met een suspensie van *A. solani* conidia in water of in een agar-oplossing. De lengte en breedte van de lesies wordt gemeten, zodat nauwkeurige en objectieve gegevens verkregen worden, wat een voorwaarde is voor een QTL analyse. Deze inoculatiemethode is gebruikt voor het toetsen van een collectie van 54 tomaten-accessies op resistentie tegen een Indonesisch isolaat van *A. solani*.

S. arcanum LA2157 is sterk resistent tegen *A. solani* in kastoetsen. De genetica van de resistentie in deze wilde verwant van de cultuurtomaat is verder onderzocht door QTLs te karteren (hoofdstuk 3). De karteringspopulatie bestond uit 176 F2 planten. De moleculaire-merker kaart omvatte 12 koppelingsgroepen met een totale lengte van 1179 cM en was gebaseerd op 379 merkers (33 SSR, 21 SNP en 316 AFLP merkers). Alle

koppelingsgroepen konden worden toegewezen aan chromosomen van tomaat. Ongeveer de helft van de merkers vertoonde een significante afwijking van de verwachte 1:2:1 splitsingsverhouding. De F2 populatie werd gefenotypeerd in een kas in Nederland met een Indonesisch isolaat van *A. solani*. EB resistentie werd bepaald aan de hand van lesiegrootte (LS) en daaraan gerelateerde parameters (relatief oppervlakte onder de lesiegroeicurve [RAULEC] en percentage kleine lesies [PSmL]). De uit de F2 planten verkregen F3 lijnen (156) werden in een Indonesisch proefveld getoetst. Hier werd op zes momenten de percentuele EB index (PEBI) bepaald, en het relatief oppervlak onder de ziekte-ontwikkelingscurve (RAUDPC) werd berekend. In totaal werden zes QTLs met LOD-scores van 3.6 tot 16.4 gevonden op chromosomen 1, 2, 5, 6, 7 en 9. Drie van de QTLs werden in beide toetsen gevonden, ondanks de verschillen in experimentele methoden en waargenomen kenmerken. Voor de eerste keer werden ook QTLs gevonden voor een ander symptoom van de schimmel: de ontwikkeling van stengellesies op hoofd- en zijstengels. Deze QTLs overlapten geheel met de QTL-gebieden voor EB resistentie. Twee QTLs op chromosomen 2 en 9, die 7 tot 16% van de fenotypische variatie voor EB resistentie en 31% van de variatie voor stengellesies verklaarden, worden aanbevolen voor gebruik in veredelingsprogramma's gericht op de ontwikkeling van rassen met resistentie tegen *A. solani*.

Ringkasan

Becak kering (BK) disebabkan oleh jamur *Alternaria solani* adalah penyakit pada tomat di lapangan yang sebarannya luas di dunia, termasuk Indonesia. Saat ini BK dikendalikan dengan aplikasi fungisida berfrekuensi sering. Penggunaan kultivar tahan merupakan cara yang ampuh untuk penjarangan aplikasi fungisida. Tujuan dari percobaan ini adalah untuk mendukung pemuliaan kultivar tahan BK untuk pasar Indonesia, dengan mengidentifikasi gen-gen ketahanan BK yang efektif di Indonesia dan mengembangkan penanda untuk program MAS.

Pada Bab 1 kepustakaan tentang *A. solani*, BK dan ketahanan diulas secara menyeluruh. Patogenisitas dan keragaman genetika jamur telah diteliti secara luas. Akan tetapi, keberadaan ras-ras fisiologik yang berbeda belum secara meyakinkan ditunjukkan ataupun dibantah. Metode terpercaya untuk penyaringan ketahanan telah tersedia untuk pemuliaan. Penyaringan asesi tomat secara intensif di seluruh dunia telah memperlihatkan bahwa ketahanan yang tinggi tidak ditemukan pada tomat budidaya tetapi hanya ada pada spesies liar. Ketahanan terhadap *A. solani* diekspresikan secara kuantitatif dan diwariskan secara poligenik. Karena itu kultivar dengan level ketahanan yang tinggi belum diperoleh melalui pemuliaan secara klasik, disamping juga sifat-sifat yang tidak diinginkan dari donor turut terbawa. Baru-baru ini QTL telah terpetakan pada beberapa sumber ketahanan, yang bisa membantu pemindahan gen-gen ketahanan kuantitatif itu dan mengatasi masalah “linkage drag” yang tidak diinginkan jika penanda yang terpaut erat dengan QTLs telah diperoleh.

Pada Bab 2 sebuah metode uji ketahanan berupa inokulasi droplet diuraikan, yang diadaptasi dari sebuah metode yang telah ada tetapi hampir tidak pernah digunakan. Metode ini mempunyai beberapa keuntungan dibandingkan dengan metode inokulasi semprot yang lebih sering dipakai, antara lain mampu membedakan dengan jelas antara becak yang disebabkan oleh *A. solani* dan nekrosis pada daun gugur yang diakibatkan oleh penyebab lain, dan mampu mengukur kerusakan dengan lebih jelas dibandingkan dengan cara skoring yang subyektif. Pada metode inokulasi droplet ini anak-anak daun pada tanaman diinokulasi dengan suspensi konidia *A. solani* dalam air atau larutan agar. Becak dinilai secara kuantitatif dengan mengukur panjang dan lebarnya. Dengan cara ini penilaian yang obyektif dan teliti, yang merupakan persyaratan untuk analisis QTL, bisa diperoleh. Metode inokulasi ini telah digunakan untuk menyaring ketahanan 54 asesi tomat terhadap sebuah isolat *A. solani* dari Indonesia.

Solanum arcanum LA2157 sangat tahan terhadap *A. solani* di rumah kaca. Genetika ketahanan pada kerabat liar tomat ini dipelajari lebih jauh menggunakan pendekatan pemetaan QTL (Bab 3). Populasi pemetaan terdiri dari 176 tanaman F2. Sebuah peta keterpautan terdiri dari 12 kelompok pautan yang mencakup 1179 cM telah

dibuat berdasarkan pada 379 penanda molekuler (33 SSR, 21 SNP dan 316 AFLP). Seluruh kelompok pautan itu dapat ditentukan nomor kromosomnya ke dalam 12 kromosom tomat. Setengah dari penanda-penanda itu menyimpang dari perbandingan segregasi 1:2:1. Populasi F2 diuji ketahanannya terhadap sebuah isolat tunggal *A. solani* di sebuah rumah kaca di Belanda. Ketahanan terhadap BK dievaluasi berdasarkan ukuran becak dan parameter yang berkaitan dengannya (daerah relatif dibawah kurva perluasan becak [RAULEC] dan persentase becak berukuran kecil [PSmL]). Galur-galur F3 yang diperoleh (156) diuji di sebuah lapangan percobaan di Indonesia. Persentase indeks BK dinilai pada enam titik waktu yang selanjutnya digunakan untuk menghitung daerah relatif dibawah kurva perkembangan penyakit [RAUDPC]). Sebanyak enam QTL dengan kisaran nilai LOD dari 3.6 hingga 16.4 terpetakan pada kromosom 1, 2, 5, 6, 7 dan 9. Tiga dari QTL tersebut memperlihatkan pengaruh pada kedua lingkungan uji walaupun ada perbedaan metode percobaan dan parameter penyakit yang diamati. Untuk pertama kalinya tiga QTL untuk ketahanan yang mempengaruhi perkembangan becak batang, sebuah gejala penyakit disebabkan jamur tersebut pada batang utama dan sekunder tanaman, telah diidentifikasi, yang daerah QTL-nya tumpang tindih secara menyeluruh dengan daerah QTL untuk ketahanan terhadap BK. Dua QTL pada kromosom 2 dan 9, yang menerangkan 7 sampai 16% dari keragaman fenotipik untuk ketahanan BK dan 31% untuk ketahanan becak batang, disarankan untuk digunakan dalam program pemuliaan tomat untuk ketahanan terhadap *A. solani.*

Acknowledgements

It was far beyond my imagination that someday I could do Ph.D. studies in Holland. I am therefore very grateful to people who have given this opportunity: Dr. Gerard Grubben, the consultant for the Biotechnology Research Indonesia-Netherlands (BIORIN) program, Dr. Djoko Damardjati and Dr. Sugiono Moeljopawiro, the former directors of my institute back home: Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD).

The completion of this study would have been impossible without the support from many people. First, I am deeply indebted to my daily supervisor Roeland who prepared the framework of this Ph.D. project. His excellent guidance and patience in explaining the correct usage of scientific terms and teaching some fundamental knowledge have helped my learning process and increased my understanding in plant disease resistance, genetics and breeding as well as statistical analysis. Many times I wanted to give up with the experimental results but he encouraged me to put more thoughts on it. His thorough corrections on my confusing 'Inglish' (Indonesian-English) writing style had greatly improved the contents and readability of this thesis. I am sorry for the frequent changes I made after the corrections; as well as the research itself the manuscripts also evolved in such a way. I am also thankful for his effort to arrange a quick MVV procedure and the extension of my allowance in the past few months.

Equal great appreciation goes to my promotor Piet for his invaluable comments on the chapters and suggestions for data analyses. He was always interested in my monthly research progress although several times I came to his office with barely any new results in my hands. Towards the end of my contract he put immense efforts to smooth the finalization of this thesis.

I should like to express my sincere appreciation to PRI staff who directly contributed to my Ph.D. research. It was a pleasure to work with Remmelt, who was always very enthusiastic to show me the correct way to work aseptically and to show me around the glasshouse complex. I thank René and his former IGD staff members (Hanneke, Gerda U, Wendy, Danny and Paul) for their technical guidance and assistance during my work in the molecular labs. In almost every occasion René was always interested to see my silver-stained gels and SNP analysis and gave practical solutions whenever I was stuck. Dirk's help in the tissue culture lab and glasshouse was a big relieve; his jokes have made things easy to execute. I am grateful to Greetje and Paul Dijkhuis for their advice in *in vitro* culture; and also to the glasshouse staff (Dick, Daan and Geert) for plant maintenance. Teun and Herti lent their hands in transporting *in vitro* plant materials. To Sjaak many thanks for your suggestions in marker system and offering help whenever you caught me stuck in front of my screen.

I am grateful to Pim Lindhout for reading and offering useful criticisms on Chapter 1 and also advices on research plans during the Resistance Group meeting. I owe thanks to Vagner, Susanna, Paolo, Ludmila and my father for their help in making available the English translations of some foreign research articles.

I wish to express my gratitude to several people in East-West Seed Company Indonesia (Nurul Hidayati, Arnaud Aardse and Rien Rodenburg) and ENZA who have participated in the field experiments and molecular marker analysis and to Dr. Eri Sofiari (Indonesian Vegetables Research Institute, IVEGRI) and Dr. Suhardi (Indonesian Ornamental Plants Research Institute, IOPRI) for their contributions in the early stage of this project.

To my BIORIN Ph.D. student fellows: Pak Ence, Vivi and Bu Tetty many thanks for your help with social matters; our journey exploring Holland, dinner and our bicycle activities were indeed unforgettable; Rudi and Sigit thanks for your willingness to share your scientific knowledge. May we extend our friendship to a fruitful scientific collaboration in our country as was challenged to us by Evert Jacobsen. Our BIORIN projects would not run well without the excellent coordination by Dr. Huub Löffler.

Oene, Leontine, and Zifu thanks for being wonderful office mates and your kind offer to get me a cup of coffee during the breaks. I thank Jan Knuiman for his kind help with computer software and Kees for help with literature search. Yvonne, your ‘kue lapis legit’ has inspired one of my propositions; thanks a lot.

Surrounded by Indonesian atmosphere during the regular social gatherings and other activities with the large Indonesian Student Association in Wageningen (PPI-W) made me as if being at home. Therefore I thank Pak Ruandha, Pak Muli, Bu Oke, Bu Retno, Hady, Bu Titiek, Adhian, mbak Ina, mbak Endah, Irin, Nono, Dwi, Amy, Pak Agus and also the other prominent members.

I extend my gratitude to the head and staff members of the Consular and Education sections of the Indonesian Embassy for their help in extending the validity of my service passport; to Dr. Sutrisno (the current ICABIOGRAD director), and work colleagues there, especially Dr. Budihardjo Sugiharto, for arranging documents to extend my study abroad.

Finally, I’d like to take this opportunity to thank Dr. M. Kosim Kardin, who has been very influential during my career as a young researcher.

December 2005

Curriculum vitae

The author was born in Malang, Indonesia, on 6 April 1967. She obtained a B.Sc. degree in Plant Pest and Disease from Bogor Agricultural University (IPB) in 1991. In the same year she got a temporary job at the Phytopathology Department of Bogor Research Institute for Food Crops (BORIF) of the Ministry of Agriculture of Indonesia. Two years later she became a full employee at the same institute, which in 1995 was changed into Research Institute for Food Crops Biotechnology (RIFCB) and in 2004 has been renamed as Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD). Her main responsibility in the former institute was to run the nematology section. During 1993 to 1995 she participated in joint research with Japan International Research Center for Agricultural Sciences (JIRCAS) on investigation of plant parasitic nematodes threatening upland crops and with St. Patrick College, Ireland, on the use of entomopathogenic nematodes for biological control of rice stem borers. Two international publications have been earned from these fruitful co-operations. From 1995 to 1997 she participated in biological control studies of fungal diseases led by work colleagues. In 1997 she got a British Chevening Award scholarship to study MSc course in Molecular Plant Pathology for one year at the University of East Anglia Norwich, UK. After returning from UK she continued working on nematological research projects. In May 2001 she started Ph.D. studies at Plant Research International.

PE&RC Ph.D. education statement

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

Review of Literature (4 credits)

- Tomato early blight (2001-2005)

Post-Graduate Courses (2.6 credits)

- Autumn School “Disease resistance in plants” (2002)
- Basic and Advanced Statistics (2004/2005)

Deficiency, Refresh, Brush-up and General Courses (4.8 credits)

- Selection methods (2001)
- Breeding for resistance against diseases and pests (2001)
- EndNote (2001)
- Scientific Writing (2003)

Ph.D. Discussion Groups (7.3 credits)

- Resistance Group Laboratory of Plant Breeding (2001-2005)
- Cluster Meeting Plant Research International (2001-2005)
- Businesss Unit Meeting Plant Research International (2001-2005)
- Genetic resources and diversity in production ecology group (2002)
- Semi annual and annual meeting BIORIN project Wageningen (the Netherlands), Bogor (Indonesia) (2001-2004)

PE&RC Annual Meetings, Seminars and Introduction Days (1 credit)

- PE&RC annual meeting “Food Insecurity” (2001)

International Symposia, Workshops and Conferences (2 credits)

- Plant Genomics European Meeting 4, CBSG (2005)

Laboratory Training and Working Visits (3 credits)

- East-West Seed Company Indonesia, Field Work (2002)
- East-West Seed Company Indonesia, Field Work (2005)

Notes:

Notes:

Cover illustration: a duotone-image of a tomato leaf showing target board-like early blight lesions generated by droplet inoculation method (original photograph by Roeland Voorrips)