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Full Length Research Paper

Genetic analysis of resistance to early blight disease in tomato

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Early blight is a fungal pathogen that causes destructive necrotic and chlorotic symptoms on leaves, stems and fruits of tomato plants. Host resistance remains the most desirable control strategy against the early blight disease. In this study, the responses of 6 cultivated and 3 wild tomato accessions were tested with 3 isolates of the fungal pathogen. To investigate inheritance of the resistance, resistant plants of NCEBR2 and NCEBR4 genotypes were crossed with susceptible NC84173 tomato line, and their F_1, F_2 and BC_1 populations were established. In the established populations, resistance differences were significant (P>0.05) for NCEBR2 × NC8413 and NC84173 × NCEBR4 parents, F_1 and BC_1. However, no significance (P>0.05) was obtained in their F_2 population to early blight pathogen. The data from these populations revealed that early blight resistance in NCEBR2 and NCEBR4 was quantitatively controlled by more than one gene or quantitative trait locus under controlled glasshouse environment.

Key words: Tomato, early blight disease, genetic analysis.

INTRODUCTION

Foliage pathogens cause economic crop reductions in tomatoes (*Solanum lycopersicum*). One of the most destructive common fungal diseases caused by *A. solani* affects primarily the leaves, stems, flowers and fruits of tomatoes. The leaf spots are generally from dark brown to black, often numerous and enlarging with concentric rings. Lower leaves are attacked first, and then disease progresses upward and affected leaves turn yellow and dry up. Stems lesions can develop on seedling, and may form canker and kill the plant. The *A. solani* can attack fruits when they approach maturity at the stem end where the symptoms may be small and sunken or may enlarge to cover most of the fruit (Agrios, 1997; Chaerani and Voorrips, 2007; Rotem, 1994).

The fungal pathogen has dark brown mycelium with simple erect conidiophores bearing chains of conidia. Large conidia are dark pear shaped and multicellular, and are detached easily by air currents. The *A. solani* Sorauer can survive on tomato debris or in seeds during winter period (Agrios, 1997; Rotem, 1994). The early blight pathogen produces several toxins to infect tomato plants. Among these toxins alternatic acid and solanapyrone induce necrotic symptoms with encircled chlorosis and these toxins enhance the pathogen infection and the development of necrotic symptoms of *A. solani* (Langsdorf et al., 1990).

Control of early blight is difficult when high temperature and humidity conditions are prevalent: 3 to 5 year crop rotation, routine fungicide applications, and use of disease-free seedlings are able to control the fungal pathogen. Fungicide treatments are generally the most effective control measures; however, they are not only costly but also create problems on environment, human health in all areas of the world (Herriot et al., 1986). Resistant cultivars are the most convenient way to control early blight disease. However, there are limited resistance sources available to produce strong resistant plants to the fungal pathogen because of quantitative expression and polygenic inheritance of the resistance (Thirthamallappa and Lohitaswa, 2000; Chaerani et al., 2007). Additionally, the early blight pathogen has complex physiological, morphological and ecological characters, allowing genetic variation in *A. solani* during infection process (Chaerani and Voorrips, 2007).

Extensive genetic studies on the inheritance of early blight resistance revealed different resistance sources

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from wild tomato accessions such as *Solanum habrochaites* and *S. pimpinellifolium* resulted in the same conclusion that the resistance is a quantitative trait with recessive and partially dominant polygenes, conferring resistance with complicated epistatic effects (Thirthamallappa and Lohitaswa, 2000; Maiero et al., 1990). All the earlier studies have been conducted in open fields with uncontrollable conditions and presence of other pathogens. Identification of resistance sources, genetic inheritance of tomato plants and spray inoculated pathogenicity tests must be combined with optimum environmental conditions to study resistance to the fungal pathogen in greenhouses. The present study aimed to identify resistant tomatoes, to dissect inheritance of resistance and to map resistance loci in tomatoes against the fungal early blight disease under controllable green-house conditions.

MATERIAL AND METHODS

Alternaria solani isolates

The fungal pathogen isolates were collected from tomato growing areas of the plants showing typical early blight symptoms on leaves. Three (2, 5, and 6) isolates were chosen among 25 and then these 3 fungal isolates were further characterized with using Koch postulates on tomato leaves. The early blight pathogen cultures were maintained every 20 days with periodic transfers on tomato juice agar plates and incubated in darkness at 28°C. These fungal isolates produce many spores and have the highest aggressiveness on all tested wild materials and cultivars of tomatoes.

Plant material

In the pathogenicity tests, 7 tomato lines and 4 wild tomato accessions were used to assess their phenotypic reactions. Tomato seeds of all tomato lines (from EBR1 to EBR6), except NC84173 were kindly provided by Prof. Dr. Randolph G. (Horticultural Science, North Carolina State University, Raleigh, USA). The wild tomato accessions (LA1392, LA1404, LA1406) and NC84173 tomato line seeds were obtained from Tomato Genetics Resource Center (Department of Plant Sciences, University of California at Davis, USA). The tomato seeds were sown in 6 × 30 × 50 cm plastic seed trays containing turf (Potground, Klasmann, Germany). The seed trays were placed in a glasshouse at 24 ± 5°C temperature with 50% relative humidity, 16/8 h day and night conditions, respectively. Germinated seedlings were placed on turf containing pots at 4 to 5 real leaf stages of tomato plants.

The tomato cultivars and wild accessions were selected on the basis of their resistance to tested EB pathogens and their pedigree or their geographic origin were clearly described as mention earlier (Gardner and Shoemaker, 1999; Gardner, 2000).

Inoculum's preparation and inoculation

Fungal isolates were grown on tomato juice agar containing 90 ml commercial tomato juice (Dimes Co., Tokat-Turkey), 810 ml tap water. The solution's pH was adjusted to 5.5 to 6.0 with diluted HCl acid. Then 15 g agar (Lab M, United Kingdom) was added and mixed continuously, the solution filled up to 1000 ml with adding tap water. The prepared tomato juice agar (TJA) was autoclaved for 20 min at 121°C.

The *A. solani* isolates were grown on TJA at 28°C for 3 days, and 5 mm diameter mycelial plugs were taken from the edge of the fungal colonies which were used for subcultivation. Spores of fresh grown early blight pathogen were harvested by flooding the plates with distiled sterile H_2O and the spore concentrations were counted using a heamocytometer. Final fungal spore concentration was adjusted to 2×10^6 spore ml^{-1} and Tween 20 (Merck, Germany) was added to properly cover 4 leaf-stage of tomato seelings. The fungal suspension was applied on to plant leaf surfaces with three replications at the same day, and the relative humidity was increased from 75 to 80% with additional sterile distilled water pulverizations to ensure succesful inoculum load for the infection. Disease progress was monitored 5 weeks after post inoculation.

Disease assessments

Leaves of the plants sprayed and inoculated with spore suspension were evaluated for early blight (EB) symptoms at 7 day intervals. At evaluation, each plant was rated for EB symptoms using modified Horsfall-Barrat rating scheme (Foolad and Lin, 2001; Bock et al., 2010). In this system, 11 step rating scales (1 = 0 to 3%, 2 = 3 to 6%, 3 = 6 to 12%, 4 = 12 to 25%, 5 = 25 to 50%, 6 = 50 to 75%, 7 = 75 to 87%, 8 = 87 to 94%, 9 = 94 to 97%, 10 = 97 to 100%, 11 = 100%) were used for evaluation, where 0 indicating no visible symptom of EB infection and 100 indicating complete defoliation. For each tomato plant *a*rea *u*nder the *d*isease *p*rogress *c*urve (AUDPC) was calculated as:

$$AUDPC = \sum_{i=1}^{n} \left[\left(R_{i+1} + R_i \right) / 2 \right] . \left[t_{i+1} - t_i \right]$$

Where, R is the rating estimated proportion of defoliated tissue at the ith observation; t_i is the time (days) since previous rating at the ith observation and n is the total number of observation (Tooley and Grau, 1984). A single rating was assigned to each plant at each evaluation. The AUDPC values and the final percentage defoliation for all plants and constructed populations were used to measure resistance levels and estimated heritability for EB resistance. A linear relationship existed between estimated disease and actual disease.

Establishment of backcross populations

For each plant crossed, single F_1 hybrid plant was used as the emasculated parent, receiving pollens from the donor parent NC84173, the hybridized plants produced F_1, F_2, and backcross (BC) populations. At least 2 backcross populations were developed to locate the genomic regions considering response to *A. solani*. A single F_1 hybrid plant was used as the pollen receiver parent to hybridize plants of 'NC84173' and produced BC_1 seeds. The BC_1 populations were sprayed-inoculated with spore suspensions of 2 and 6 isolates of *A. solani*. Starting from inoculation day, at 7 day intervals thereafter, the inoculated tomato plants were scored for EB symptoms using the mentioned Horsfall-Baratt rating scheme for six times. The final evolution rating was considered as the final disease severity.

DNA isolation

DNA was isolated using the protocol of Doyle and Doyle (1990) that was determined to be suitable for DNA isolation of all tomato cultivars including F_1, F_2 and BC_1 populations. To extract DNA, a middle size (3 by 5 cm) leaf was sampled from each plant and

Table 1. Phenotypic reactions of 7 tomato cultivars to 3 isolates of *A. solani* pathogen.

Tomato cultivars	Isolate 2		Isolate 5		Isolate 6	
	Intermediate		Intermediate		Intermediate	
	Susceptibility	Resistance	Susceptibility	Resistance	Susceptibility	Resistance
NCEBR1		X	X			X
NCEBR2		X		X		X
NCEBR3		X	X			X
NCEBR4		X		X		X
NCEBR5	X			X	X	
NCEBR6		X	X		X	
NC84173	X		X		X	
LA1392		X	X		X	
LA1404		X	X			X
LA1406		X	X		X	

placed in an Eppendorf tube with adding extraction buffer. The DNA was recovered by eluting with 100 µl of sterile distilled water.

Polymerase chain reaction (PCR) amplifications

PCR was conducted in 20 µl volumes containing 50 ng of template DNA, 5 × PCR buffer (Promega Corp.), 4 mM $MgCl_2$, 100 µM each of dATP, dCTP, GTP and dTTP, 20 ng of primer, and 1.20 units of Taq DNA polymerase. Thermocycling conditions consisted of a single cycle of 5 min at 94°C, 40 cycles of 1 min at 94°C and 1 min at 72°C, and annealing temperatures varied from 35 to 60°C with 1 or 2 min. Amplified product from all PCR assays were separated on 1.5% agarose gels including ethidium bromide (0.5 µg ml^{-1}) for 2 h at 6 V cm^{-1} constant voltage in TBE buffer according to Sambrook et al., (1989).

Statistical analysis

Using the SPSS statistical package (SPSS for Windows 17.0), an analysis of variance (ANOVA) was performed on the AUDPC values to estimate genetic resistance, and the Tukey multiple range test was used to compare the differences between means.
Chi-square tests were used to determine the goodness of fit of observed numbers of plants in each population to expected segregation ratios for the phenotypic AUDPC values.

RESULTS

No monogenic resistance in tested tomato cultivars

The pathogenicity test results with 7 tomato lines against 3 isolates of *A. solani* showed intermediate resistance and susceptibility (Table 1). The NCEBR2 and NCEBR4 tomato lines were intermediate resistant and NC84173 tomato cultivar was intermediate susceptible to all 3 isolates of the early blight pathogen respectively (Table 1). None of the tomato cultivars was fully resistant to early blight isolates because all the tested tomato lines exhibited variable level of susceptibility from low to high incidence of necrotic spots on inoculated leaves. The isolates 5 and 6 of EB were most virulent and less virulent isolate 2 caused disease on 4, 3 and 2 lines among tested 7 tomato lines respectively (Table 1).

The results of the pathogenicity tests demonstrated that LA1392, LA1404 and LA1406 wild tomato accessions were susceptible to the EB pathogen isolate 5. However, all the tested wild tomato accessions were resistant to the isolate 2 (Table 1). The LA1404 was resistant to the isolate 6, meanwhile LA1392 and LA1406 wild tomato accessions were susceptible to the same isolate 6 (Table 1).

The final values of area under the disease progress curve (AUDPC) for the parental lines and established two F_1, F_2 and BC_1 progenies are presented in Table 2. The moderately resistant lines NCEBR2, NCEBR4 and intermediate susceptible NC84173 parents did not exhibit extreme responses to EB 2 and 5 isolates (Table 2). The mean AUDPC value for the susceptible parent (NC84173) was only 1.5 times higher than that for the resistant parents (NCEBR2 and NCEBR4). Plants of F_1 generation were similar to the resistant parents. There was less variation within for AUDPC range in the F_1 generation of NCEBR2 × NC84173 than AUDPC range in the F_1 generation of NC84173 × NCEBR4 (Table 2).

There were statistically significant differences among the BC_1 plants in EB disease resistance, with the AUDPC values ranging from 2.1 to 27.6 and 7 to 56 for established populations respectively (Table 2). The averages of AUDPC values were very similar in both resistant parents, NCEBR2, NCEBR4 and in their established BC_1 populations (Table 2). It was important that F_1 AUDPC average value was much lower than the AUDPC of NCEBR2. However, the F_1 AUDPC average value was slightly higher than the AUDPC of NCEBR4

Table 2. Two isolates of early blight (EB) caused disease severity and area under disease progress curve (AUDPC) for 7 parents and two established F_1, F_2 and BC_1 populations from NCEBR2 (P1) and NCEBR4 (P2) hybridized to intermediate susceptible parent NC83174 (P3), respectively.

Generation	Total number of plant	EB isolate 2		EB isolate 5	
		AUDPC		AUDPC	
		Average	Range	Average	Range
NCEBR1	50	22.4 ± 1.3	3.5 - 38.5	25.0 ± 0.8	16 - 38.5
NCEBR3	50	22.4 ± 1.3	3.5 - 38.5	34.4 ± 1.2	20 - 49.0
NCEBR5	50	28.7 ± 1.5	7 - 45.5	38.4 ± 1.5	12 - 52.5
NCEBR6	50	21.0 ± 1.4	3.5 - 38.5	41.3 ± 1.2	20 - 49
NC84173 (P2)[1]	50	25.9 ± 1.7	14 - 45.5	41.7 ± 1.6	16 - 56
NCEBR2 (P1)[2]	50	16.8 ± 1.0	3.5 - 28	14.7 ± 0.8	4 - 24.5
F_1 (P1 × P2)	60	9.2 ± 0.8	1.5 - 21.5	ND[4]	ND
F_2	120	8.9 ± 0.7	1.5 - 21.3	ND	ND
BC_1	293	13.8 ± 1.0	2.1 - 27.6	ND	ND
NCEBR4 (P3)[3]	50	16.8 ± 1.0	3.5 - 28	14.9 ± 0.8	3.3 - 26.9
F_1 (P2 × P3)	20	18.7 ± 2.3	10.5 - 52,5	ND	ND
F_2	20	5.09 ± 1.0	10.5 - 17.5	ND	ND
BC_1	150	13.24 ± 0.9	7 - 56	ND	ND

P2: Pollen donor and receiving parent, P1: Pollen-receiving parent, P2: Pollen donor parent, ND: Not determined

resistant parent (Table 2), demonstrating that different quantitative trait controls resistance.

Phenotypic differences

Statistical analyses revealed significant differences ($P > 0.05$) between susceptible and resistant genotypes for resistance using calculated AUDPC values following pathogenicity tests with two isolates of EB pathogen (Table 3). The resistance differences were significant ($P > 0.05$) for parents (NCEBR2 × NC84173 and NC84173 × NCEBR4), F_1 and BC_1 populations (Table 3).

The mean AUDPC value was 26.95 for NCEBR2, 12.74 for NCEBR4 resistant parents and 41.53 for NC84173 susceptible parent at 35 days post inoculation, respectively (Table 3). Their crosses to resistant parents significantly decreased the AUDPC in the F_1 populations (Table 3). On the other hand, there were no significant differences between resistant parents and their F_2 plants (Table 3), indicating presence of resistance genes.

Frequency distribution of the AUDPC values of disease reactions to established populations were summarised in Figure 1. The calculated AUDPC values ranged from 22 to 28 and from 7 to 21 for NCEBR2 and NCEBR4 resistant parents, respectively. However, the AUDPC values ranged between 29 and 56 for NC84173 susceptible parents (Figure 1).

Genetic characterization of EB resistance

Tomato cultivars NC84173, NCEBR2 and NCEBR4 showed significant differences in the responses to EB pathogen (Table 2): NC84173 had high susceptibility while NCEBR2 and NCEBR4 lines had high levels of resistance ($P < 0.01$). The results demonstrated that EB resistance in NCEBR2 and NCEBR4 were quantitatively controlled by more than one gene or QTL (Figure 1).

QTL mapping

To map resistance loci in BC_1 population of NCEBR2 × NC84173, 70 simple sequence repeat (SSR), 23 cleaved amplified polymorphic sequence (CAPS), 1 insertion-deletion polymorphism (INDEL), and 5 resistant gene anologs (RGA) marker were tested to find polymorphism between the parents. Among tested 99 markers, only 11 markers (11%) were identified as polymorphic between the parents (Data not shown). Hence, the established map consisted of 11 markers covering entire haploid tomato genome (1409 centi Morgan: cM) with 128 cM intervals.

The SSR polymophic markers between the parents were located on the following chromosomes: the TOM196 was on chromosomme 1, the SSR22 was on chromosomme 2, the SSR63 was on chromosomme 8, SSR383 was on chromosomme 9, SSR318 and SSR248 were on chromosome 10 and the TOM144 was on chromosome 11, respectively. The CAPS markers located as: LEOH18 was on chromosome 4 and RX3-L1 was on chromosome 5. However, CAPS marker LEOH57 and INDEL marker CT107371 found in multiple gene families could not be mapped to a specific chromosomme.

Table 3. Two early blight pathogens inoculated parents and their F_1, F_2 and BC_1 populations' AUDPC values.

Lines	Statistical value	Area under disease progress curve (Day post inoculation: DPI)					
		0	7	14	21	28	35
NC84173 (♀)	Mean	0.00	5.37	12.90	22.10	29.97	41.53
	Std. dev.	0.00	3.39	4.96	7.46	9.07	6.98
	N	30	30	30	30	30	30
EBR4 (♂)	Mean	0.00	1.20	2.66	5.18	8.54	12.74
	Std. dev.	0.00	1.50	2.09	2.69	2.68	3.33
	N	25	25	25	25	25	25
F_1	Mean	0.00	2.10	6.30	15.75	29.40	40.25
	Std. dev.	0.00	1.41	4.48	7.22	10.24	10.56
	N	20	20	20	20	20	20
F_2	Mean	0.00	0.60	0.70	3.50	8.05	12.60
	Std. dev.	0.00	1.26	1.47	0.00	1.69	3.38
	N	20	20	20	20	20	20
BC_1	Mean	0.00	1.18	4.13	10.99	20.21	29.70
	Std. dev.	0.00	1.47	3.46	6.09	9.83	11.27
	N	150	150	150	150	150	150
NCEBR2 (♀)	Mean	0.00	3.35	12.75	18.45	23.80	26.95
	Std. dev.	0.00	0.47	3.62	2.28	4.61	1.69
	N	30	30	30	30	30	30
F_1	Mean	0.00	1.50	4.08	6.42	12.83	21.58
	Std. dev.	0.00	1.64	2.63	4.09	4.24	5.61
	N	36	36	36	36	36	36
F_2	Mean	0.00	1.50	4.08	6.65	10.97	21.35
	Std. dev.	0.00	1.53	3.20	3.48	5.87	9.25
	N	60	60	60	60	60	60
BC_1	Mean	0.00	2.14	7.12	12.48	20.00	27.58
	Std. dev.	0.00	1.36	3.68	6.083	8.07	9.96
	N	293	293	293	293	293	293

ANOVA tests applied to the calculated values and Tukey multiple range test was used to compare the differences between means.

DISCUSSION

We used pathogenicity tests for evaluation of early blight resistance in 7 tomato lines and 3 wild tomato accessions. Prepared spore concentrations were applied to the plants and disease progresses were rated according to Horsfall-Baratt rating scheme (Foolad and Lin, 2001; Bock et al., 2010). This method was reliable to assess disease progress on the inoculated plants. The Horsfall-Baratt scale divided the percent scale into 11 logarithmic based severity intervals between 0 and 100%. Recently, it has been shown that the interpretation of the scale describing an apparent logarithmic relationship between estimated and actual disease severity has not properly explained in the case of controversy (Bock et al., 2010). However, a linear relationship existed between estimated disease and actual disease, while the distribution of diseased leaves and the number of classes in the disease scale affected the accuracy of the estimated AUDPC values in tested plants. The assumption that the parents and their established populations were skewed populations rated with few grades could result in inaccurate AUDPC values for disease severity.

General classical studies on the inheritance of EB

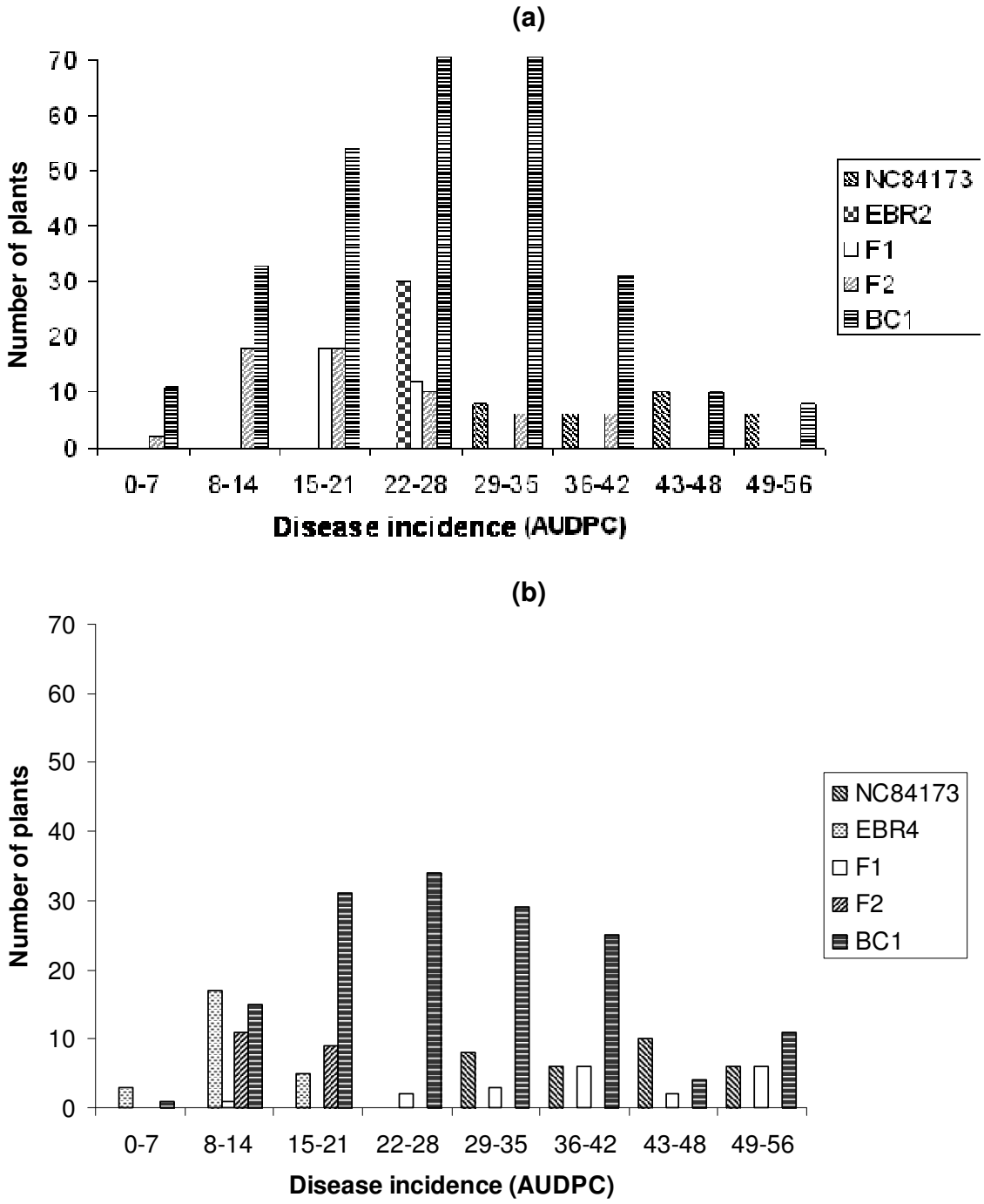

Figure 1. Frequency distributions of AUDPC values for established NCEBR2 × NC84173 (a) and NC84173 × NCEBR4 populations (b).

resistance reached the same conclusion that the resistance is a quantitative trait that is controlled polygenically (Chaerani and Voorrips, 2007). Our results from two individually established F_1, F_2 and BC_1 populations have confirmed the above conclusion. The homozygous tomato NCEBR2, NCEBR4 lines have shown quantitative resistance mediated by multiple genes or QTLs with each providing a partial increase in resistance (Tables 2 and 3). The results verified previous studies (Nash and Gardner, 1988; Maiero et al., 1990) that additive genetic components demonstrated from small to moderate role in the quantitative expression of resistance. The obtained intermediate resistance in NCEBR2 and NCEBR4 lines crossed to susceptible parent NC84173 allowed us to map resistance using BC_1 generation. The used parents were genetically closed: 99

tested markers gave 11% polymorphism between resistant and susceptible parents; the identified 11 polymorphic markers can be located at 128 cM intervals on the haploid genome of resistant plant. Considering primers used to detect polymorphism between resistant NCEBR2 and susceptible NC84173 parent, the resistant tomato genome could not be included in this study because of its lack of polymorphic markers in some regions. Therefore, it is possible that resistance loci could be found in these parts of the genome and could not be detected in this study.

For early blight resistant tomatoes, wild tomato germplasms should be used as genetic resources for classical or marker assisted breedings. In this study, 3 wild tomato accessions were tested to idenfy resistance reactions to early blight pathogens (Table 1). The wild tomato accession LA1392 (*Solanum habrochaites*, LA 1404 and LA1406) which are *Solanum cheesmaniae* gave polygenic resistance to tested EB isolates (Table 1).

Our results show that none of the cultivated and wild tomatoes was fully resistant to all tested EB pathogens at controlled greenhouse environment. The absence of durable resistance is consistent with previous findings (Foolad et al., 2002; Chaerani and Voorrips, 2007). Each cultivated and wild tomato accession displayed a specific pattern of interaction with early blight isolates (Table 1), and this strongly suggested that the mechanisms for resistance to early blight pathogens differ between accessions. Incomplete and quantitative resistance was found in tested tomatoes against three isolates of early blight pathogen (Table 1). It seems that resistance to *A. solani* isolates is not race specific and not mediated by genes with major effect. The 3 fungal *A. solani* isolates could have high genetic variation based on their phenotypes in which the most avirulent isolate, 2, produced great amount of spores, however, the virulent isolate 5 and isolate 6 produced significantly less spores. The driven force of genetic variability could be due to heterokaryosis, variation in morphology and toxin productions (Agrios, 1997; Langsdorf et al., 1990). This genetic variability could allow the pathogen to react to changing environments spontaneously.

A considerable amount of information is available between tomato and *A. solani* interactions. Further genetic studies should be established for revealing host complete resistance and existence of several pathogen races.

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