Characterization of novel sources of resistance to *Phytophthora infestans* (late blight) in organic fresh market tomato

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List of abbreviations

ANOVA	Analysis of variance
AUDPC	Area under the disease progress curve
AVRDC/WVC	The Asian Vegetable Research and Development Center/World Vegetable Center
Ch.	Chromosome
CI	Confidence interval
CIM	Composite Interval Mapping
cM	Centimorgan
Cv.	Cultivar
Df.	Degree of freedom
DNA	deoxyribonucleic acid
FAO	Food and Agriculture Organization
LB	late blight
LOD	Logarithm of the odds
LSD	Least Significant Difference
Mean Sq.	Mean square
OOTP	Organic Outdoor Tomato Project
Pi	*Phytophthora infestans*
QTL	Quantitative Trait Locus
SNP	single-nucleotide polymorphism
Sum Sq.	Sum of squares
TGRC, UC	Tomato Genetics Resource Center, University of California
Var.	Variety

1. Introduction

1.1 Tomato

Tomato (*Lycopersicon esculentum*) is the most widely grown vegetable food crop in the world. The total production of tomatoes exceeded 177 million tonnes in 2016 (FAOSTAT 2016) from 4.78 million ha. It is considered as one of the most valuable vegetables in the world due to its flavour and diverse use as a fresh vegetable in cooking and value-added processed foods (Preedy & Watson 2008).

Originated and first domesticated in South West America and Mexico (Bai & Lindhout 2007), Tomato gained its worldwide importance later when it was introduced to Europe in the early 16th century. Nowadays, tomato is the most consumed vegetable worldwide (Foolad *et al.* 2008) and it's been cultivated around the globe regardless the climatic differences.

China leads the world tomato production with 56 million tonnes in 2016 (FAOSTAT 2016), followed by India and USA with 18 and 14 million tonnes respectively. 60% of the total tomato production is coming from Asia followed by Americas and Europe with 14.7% and 13.7 % (Fig. 1.1) while the Arab and Mediterranean countries leads in terms of per capita tomato consumption with 40 to 100 Kg per year (Bergougnoux 2014).

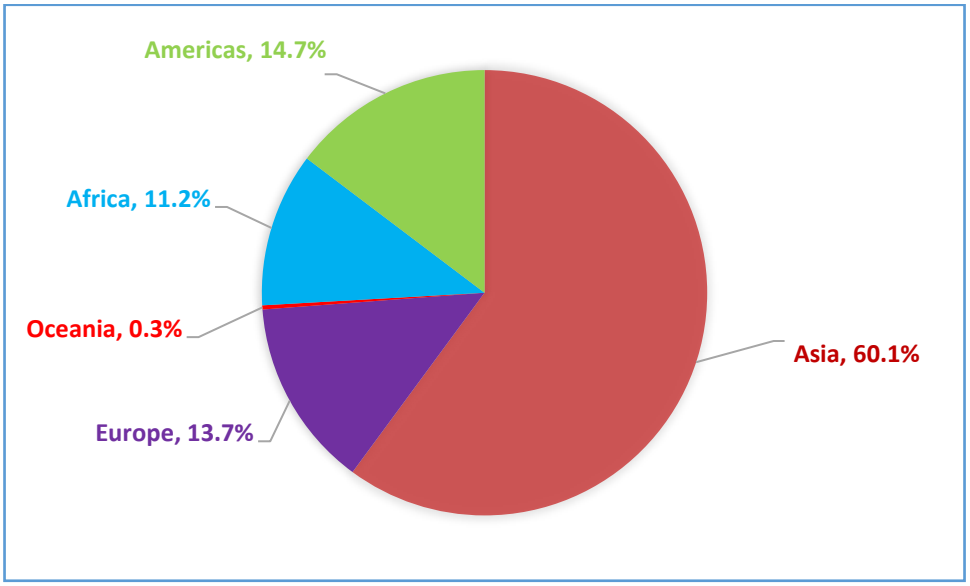


Fig. 1.1 World tomato productions by regions (FAO Stat 2016)

Botanically tomato is a fruit berry belonging to the family Solanaceae, originated in the tropical and subtropical mountainous regions of the Americas, part of present-day Peru, Bolivia, Chile and Ecuador. Tomatoes grow best in high altitudes with low humidity and high luminosity (Preedy & Watson 2008). The optimum tomato growing temperature ranges between 18-28^{o}C (Preedy & Watson 2008). The optimum pollination temperature requirement is also of same scale and minimum temperature for fruit set is 15^{o}C (George 2009).

1.2 Late blight and *Phytophthora infestans*

Late blight (LB) caused by *Phytophthora infestans* (Mont.) de Bary is a major cause of crop loss in tomato around the globe (Foolad *et al.* 2008 & Nowicki *et al.* 2013). It spreads quickly and cause complete yield loss under favourable conditions (Foolad *et al.* 2008).

P. infestans life cycle

P. infestans (Mont.) de Bary is an oomycete with sexual and asexual reproduction as part of the life cycle. For sexual reproduction, the mycelia of A1 and A2 mating types should come in contact. Sexual reproduction results in the production of oospores which have the capability to survive unfavourable conditions outside host tissue and germinate when the environmental conditions become favourable (Foolad *et al.* 2008).

Asexual reproduction starts when sporangia, spore producing structure, come in contact with host tissue. The germination of sporangia is favoured by cool and humid conditions and germination occurs when plant tissue is covered with film of water at low temperature (Fry 2008b). Zoospores released from the sporangia move freely using flagella, causing rapid infection (Walker and van West 2007). Zoospore release occurs at temperatures around 10-15^{o}C (Melhus 1915) [cited in Fry (2008b)]. Zoospores penetrate the plant tissue producing germ tubes when plant tissue is covered with a film of water. A single lesion (in potato) can produce up to 300,000 sporangia per day, which leads to a rapid infection spread (Govers 2005).

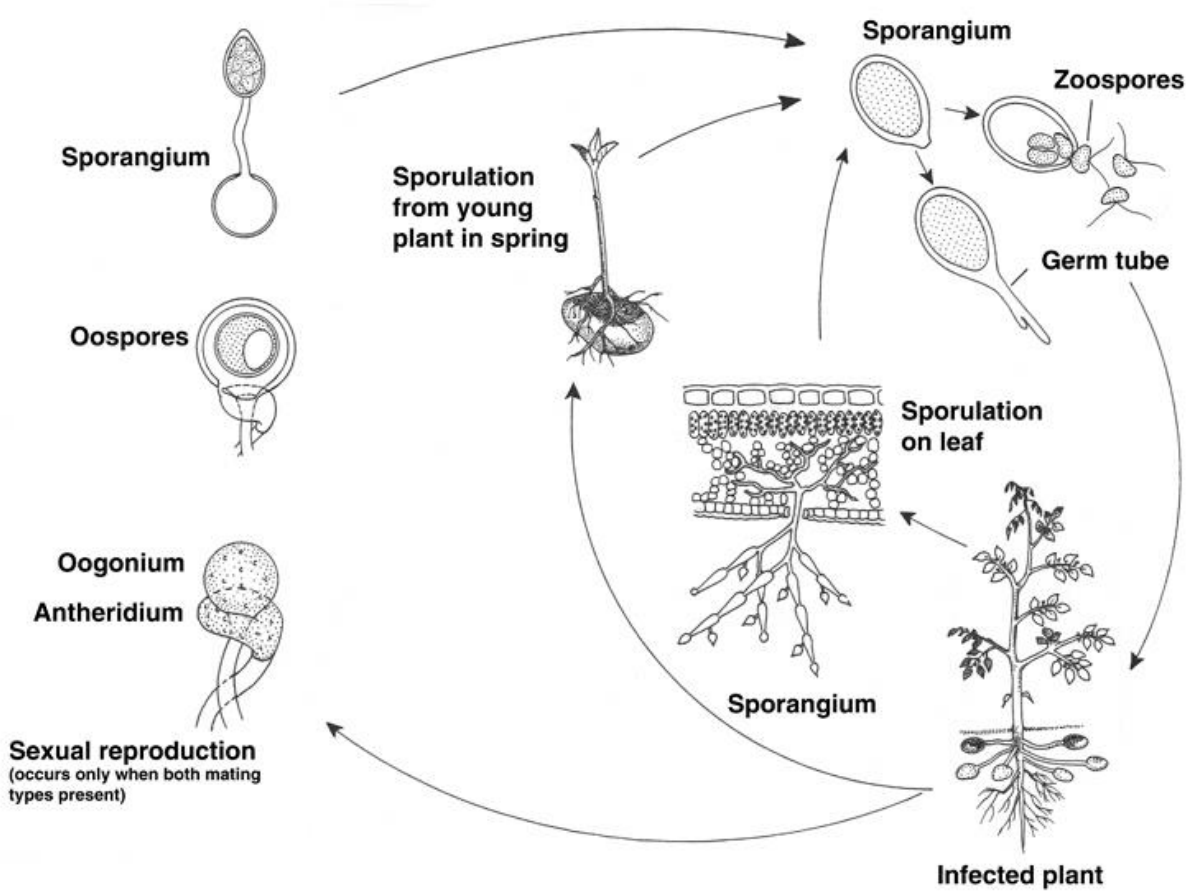


Fig. 1.2 Life cycle of *P. infestans*

(Drawing by Vickie Brewster)

https://www.apsnet.org/edcenter/intropp/HungryPlanet/Chapter1/Pages/ImageGallery.aspx

1.3 History and present status of late blight resistance breeding in tomato

Late blight resistance breeding has been a hot subject among tomato breeders due to its rapid spreading and evolving capacity (Drenth *et al.* 1994). Three major resistance genes, viz., Ph-1, Ph-2 and Ph-3, has been identified in wild tomato species *S. pimpinellifolium* and successfully introgressed into commercial cultivars (Foolad *et al.* 2008). Apart from these, a few more proposed genes are discussed below.

1.3.1 Ph-1

Ph-1 is the first identified late blight resistance gene in tomato in early 1950s. It was first identified in *S. pimpinellifolium* accessions West Virginia 19 and West Virginia 731 (Bonde and Murphy 1952; Gallegly and Marvel 1955). Ph-1 is a dominant gene located at the distal end of Chromosome 7 (Peirce 1971). Later Ph-1 was successfully introduced into commercial cultivars such as New Yorker, Rockingham, Nova. Ph-1 was completely resistant over the dominant *P. infestans* race T-0. But the resistance provided by Ph-1 was soon overcome by the evolution of new virulent *P. infestans* strains (Walter and Conover 1952). Today, Ph-1 is no more considered resistant and used in breeding programmes (Mutschler *et al.* 2006; Foolad *et al.* 2014).

1.3.2 Ph-2

Ph-2 is the second late blight resistant gene reported in tomato. It was discovered in *S. pimpinellifolium* accession West Virginia 700 (Gallegly 1960). It showed a monogenic and dominant nature in inheritance studies done by Turkensteen (1973). Later, studies done by Moreau *et al.* (1998) unveiled that Ph-2 is a partially dominant gene, the F_1 progenies in the study showed an intermediate resistance between resistant and susceptible parents. The gene was subsequently mapped between CAPS markers dTG422 and dTG63, at the bottom of Chromosome 10 (Panthee & Foolad 2012). Interestingly, the resistance conferred by Ph-2 was found to be more effective in the early stages of plant development rather than cropping period (Kole 2007). Ph-2 had performed better against all the isolates, under experimental conditions, where Ph-1 was completely susceptible and Ph-3 was broken by many isolates (Brusca 2003). Ph-2 in combination with Ph-3 gives a stronger resistance (Wagner 2012; Nowicki *et al.* 2013). Numerous cultivars carrying Ph-2 have been developed, including West-Virginia 63 (Gallegly 1964), Caline (Goodwin *et al.* 1995), Legend, Centennial, Macline, Pieraline, Herline, Fline, Flora Dade, Heinz 1706, Campbell 28, Europeel (Foolad *et al.* 2014).

1.3.3 Ph-3

Ph-3 was the third late blight resistant gene in tomato. Ph-3 was derived from *S. pimpinellifolium* accession L3708, which was observed to exhibit strong resistance to a number of *P. infestans* strains (Black *et al.* 1996a; Black *et al.* 1996b; AVRDC 2005). Studies indicated that the resistance was conferred by a single gene (Black *et al.* 1996b). It has been identified as a partially dominant major gene on chromosome 9, between CAPS markers TG328 and TG591 (Robbins *et al.* 2010; Chen *et al.* 2014). Ph-3 is widely used in late blight resistance programmes due to its high level of resistance (Mutschler *et al.* 2006). The zygosity also plays a role in the level of resistance provided by Ph-3 (Kim and Mutschler 2006), for e.g. heterozygous lines exhibiting complete resistance, as the homozygous, to US-11, but almost complete susceptibility to US-7. This was also reported by Wagner (2012) and Chen *et al.* (2014). Ph-3 has been mapped to the long arm of chromosome 9 near RFLP marker TG591a (Chunwongse *et al.* 2002) and later fine-mapped in the 0.5 cM genomic region, between Indel_3 and P55 molecular markers (Zhang *et al.* 2013).

1.3.4 Ph-4

Ph-4 gene was identified in *S. habrochaites* accession LA1033 (AVRDC 1998). Further investigations revealed that the resistance conferred in LA1033 was by multiple quantitative trait loci (QTLs) (Lough 2003; Kim and Mutschler 2000). This obstructed the further characterization of Ph-4 (Zhang *et al.* 2014).

1.3.5 Ph-5-1 and Ph-5-2

Two genomic regions identified on chromosome 1 and chromosome 10 were tentatively named as Ph-5-1 and Ph-5-2 respectively (Merk *et al.* 2012; Merk and Foolad 2012; Nowicki *et al.* 2012). The resistance provided by these regions are as strong as Ph-2 and Ph-3 combined (Merk *et al.* 2012). The gene on Chromosome 1 was mapped between markers SSRW11 and cTOE7J7 and the gene on Chromosome 10 between TMA0040 and SSR223 (Merk *et al.* 2012). Further fine mapping of Ph-5-2 is needed to confirm that it is a distinct gene, since it has been identified in the same region as Ph-2 (Merk *et al.* 2012). Cultivars having Ph-5 genes

in combination with Ph-2 and Ph-3 are under investigation (Foolad *et al.* 2008; Nowicki *et al.* 2012).

1.3.6 Late blight resistant quantitative traits in tomato

Apart from the aforementioned R genes, a number of late blight resistant QTL have been identified in tomato and its wild relatives. Majority of these QTLs were identified in *S. pimpinellifolium* (Bonde and Murphy 1952; Peirce 1971; Foolad *et al.* 2014) and *S. habrochaites* accessions (Lobo and Navarro 1986; Brouwer and Clair 2004; Johnson *et al.* (2012). Aside, QTLs were found in *S. lycopersicum* (Johnson *et al.* 2014) and *S. pennellii* (Smart *et al.* 2007) accessions also. A list of the QTLs and details are explained in Stroud (2015). Panthee *et al.* (2017) has been reported detection of additional QTLs on Chromosome 6, 8 & 12. Incorporation of QTLs from *S. habrochaites* and *S. pennellii* into commercial cultivars hampered since these QTLs were frequently associated with undesirable horticultural characteristics (Brouwer and Clair 2004; Nowakowska *et al.* 2014).

1.4 Late blight (*P. infestans*) control measures

Late blight is conventionally controlled by cultural practices and protective measures.

1.4.1 Protective cultivation

Spores are the main sources of disease spread in case of late blight. Protective cultivation take measures to keep the plants away from possible contact with spores as well as pathogen favourable conditions like rain and dew. This can effectively achieved by growing tomatoes in glass houses or poly tunnels (Collins 2013; Nelson 2008).

1.4.2 Application of fungicides

Fungicides are one of the effective controlling measures and widely used. Fungicides are basically copper based or synthetic in nature. Both are hazardous to environment and beneficial soil microorganisms. Prolonged use of copper based fungicides results in copper accumulation in soil (Wightwick *et al.* 2008; Komarek *et al.* 2010) which can even lead to

modest human toxicity (Fishel 2005). Metalaxyl, Mancozeb, Fluazinam and Carbamates are the most commonly used synthetic fungicides. Extensive use of fungicides leads to evolution of resistant *P. infestans* isolates. Evidence of Metalaxyl resistant races is an example. (Day *et al.* 2004; Matson *et al.* 2015).

1.4.3 Resistance by genes

Genetic resistance qualitative as well as qualitative loci are the ideal manoeuvre to resist late blight infection. This is the most economical way of disease control for farmers. There is no need of investment in the form of greenhouses or fungicides, as well as environment friendly.

1.5 Limitations of control measures under organic cultivation

Unlike conventional farming, organic cultivation requires a lot of special needs. Late blight (LB) control by cultural practices alone is insufficient (Chen *et al.* 2008). Protective cultivation is very common among organic growers. But due to high capital investment, it is not always affordable, especially for small scale farmers. Usages of fungicides are restricted under organic conditions. In addition to that, fungicide measures cannot be used when tomatoes are harvested for the fresh market leading to fungicide residues (Horneburg and Myers, 2012). Under the special circumstances of organic agricultural practices, it is most befitting to use resistant cultivars.

1.6 Need of search for new sources of resistances

P. infestans has been described as a pathogen with "high evolutionary potential" (Raffaele *et al.* 2010). Sexual reproduction, mating between A1 and A2, results in oospore (Judelson, 1997) and leads to the emergence of new races which could overcome host resistance (Drenth *et al.* 1994). Ph-3 is the most effective, commercially available, resistant gene against *P. infestans*. The resistance conferred by Ph-3 has been reported broken (Chunwongse *et al.* 2002; Miranda *et al.* 2010). Metalaxyl resistant races are also reported in

the recent past (Day *et al.* 2004; Matson *et al.* 2015). Considering the evolutionary potential of the pathogen, it is necessary to search for new sources of resistances.

1.7 Role of gene pyramiding in increased resistance

Combination of multiple resistant genes is always been a good disease control measure. There have been reports that Ph-2 in combination with Ph-3 gives a stronger resistance (Wagner 2012; Nowicki *et al.* 2013). Pyramiding several genes could provide a more durable resistance than deploying just a single one (Foolad *et al.* 2008). 'Mountain Magic', 'Mountain Merit' and 'Defiant PhR' are some of the commercial cultivars with Ph-2 and Ph-3 genes. They are found to be most effective cultivars against late blight infection (Gardner & Panthee 2012; Hansen *et al.* 2014).

2. Objectives of the study

Objectives

1. Identification of sources of resistances involved and pyramiding.
2. Identification of QTL for late blight field resistance.
3. Screening for potential new sources of resistance to late blight.

3. Evaluation of late blight field resistance in tomato F_2 populations from diverse sources of resistance

3.1 Introduction

Tomato late blight (LB) caused by *Phytophthora infestans* (Mont.) de Bary is a major cause of crop as well as economic loss around the globe in temperate and humid environments (Foolad *et al*., 2008; Nowicki *et al*., 2013). The asexual and sexual life cycles of *P. infestans* and its capacity to rapidly overcome plant resistance genes makes it a pathogen difficult to control (Foolad *et al*., 2008; Nowicki *et al*., 2012). The later factor has led researchers to describe *P. infestans* as a pathogen with a "high evolutionary potential" (Raffaele *et al*., 2010).

Late blight control by cultural practices alone is difficult and *P. infestans* could be more problematic where tomatoes are grown continuously on the same field, like the highland tropics of Africa, South America, Asia (Chen *et al*., 2008) and Europe (Andrivon *et al*., 2011; Brurberg *et al*., 2011). Chemical control is an effective method (Fry, 2008) but may also lead to the development of resistant isolates of the pathogen (Gisi and Cohen, 1996; Gisi *et al*., 2011), in addition chemical measures cannot be used when tomatoes are harvested in short intervals for the fresh market leading to fungicide residues (Horneburg and Myers, 2012). It is also found ineffective when the environmental conditions are favourable for disease incidence and spread (Gisi *et al*., 2011; Zwankhuizen *et al*., 2000). Development and cultivation of resistant cultivars may be an efficient way to control the pathogen.

3.2 Materials and Methods

3.2.1 Plant materials

Available sources of resistance against late blight are used as parents. Seven genotypes that had shown some level of resistance to late blight in field trials in the Organic Outdoor Tomato Project during the preceding years were chosen from three distinct groups based on their origin. 1. The Organic Outdoor Tomato Project (OOTP), 2. commercial cultivars and 3. exotic genotypes (Tabe 3.1).

Table 3.1 Specifications of parent genotypes; breeder, fruit weight & colour and origin

Parent cultivars	***Breeder***	***Fruit weight (g)****	***Fruit colour***	***Background***
Golden Current	Unknown	5.4	Yellow	Donated by Dreschflegel to the OOTP
Resi	OOTP	17.3	Red	Developed from the accession called Resi Gold which was donated to the OOTP in 2003 by Samenarchiv Gerhard Bohl
Rote Murmel	Unknown	5.7	Red	Donated by Dreschflegel to the OOTP
Phantasia F_1	De Ruiter	122.7	Red	De Ruiter
Philovita F_1	De Ruiter	19.4	Red	De Ruiter
LBR 11	World Vegetable Center	135.4	Red	Donated by a private seed saver. Original accession from World Vegetable Center (Taiwan) was not available because of technical reasons.
NC 37	Unknown	122.2	Red	Supplied by Yigal Cohen, Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel

* Data from field season 2016, Reinshof

Thirteen F2 populations resistant x resistant, to explore the genetic base of resistance and to pyramid the resistances, were produced (Table 3.2) in the organic greenhouse, University of Göttingen.

Table 3.2 F_2 populations, indicating parent families, year of trials, mapping population and number of seed lots

X: Populations evaluated only in 2016, XX: Populations evaluated in 2016 & 2017, $: Mapping population, #: number of seed lots.

F_2 Populations		***Commercial cultivars***		***Exotic***	
		Philovita F_1	*Phantasia F_1*	*NC-47*	*LBR 11*
Organic Outdoor Tomato Project	*Rote Murmel*		XX #3	X #2	XX #3
	Golden Currant			X #4	XX #5
	Resi	XX #3	XX $ #5	X #3	XX #4
Commercial cultivars	*Philovita F_1*			X #3	XX #3
	Phantasia F_1			X #3	X #3

3.2.2 Experimental setup

Field trials were conducted at two locations in Central Germany. Reinshof (51.503985, 9.923220), experimental farm of Georg-August-University Göttingen and land of the private organic seed producer Culinaris at Ballenhausen (51.4555728, 9.966503). In 2016, all populations were grown at Reinshof, except Resi x Phantasia F_1 which was grown at Ballenhausen. In 2017, six populations were grown at Reinshof. The populations resembled an

increased resistance were selected to distinguish between the most resistant F_2 individuals and the better parent.

In 2016, sowing for Reinshof and Ballenhausen took place on May 10th and June 8th, respectively in multi-pot trays QP 96 (Hermann Meyer KG, Germany). Trays were evenly filled with Bio Kräutererde (HAWITA GRUPPE GmbH, Germany) substrate. Every pot was seeded with 2-3 seeds and kept in the greenhouse (Day Night, 16:8 h and 22°C (day) & 18°C (night)). A week after germination, all but one seedling per pot was removed. The seedlings were potted on June 22nd and 23rd in plastic pots of 500 ml volume using the potting mixture Bio-Topferde (HAWITA GRUPPE GmbH, Germany). The potted plants were moved to a polyhouse. Planting took place on June 29th at Reinshof and on July 15th at Ballenhausen.

In 2017, sowing took place on May 8th. Plants were potted on May 31st in Bio Kräutersubstrat (Klasmann-Deilmann GmbH, Germany). Replication 1 was planted on June 26th and replication 2 the next day. Prior to planting, plants were kept in plastic boxes filled with water for 5 minutes. No irrigation was done afterwards.

3.2.3 Experimental design

At Reinshof the experiment was planned as a randomized complete block design. Each population was divided into two replications with an equal number of individuals. Each block represents a row of 26 F_2 individuals and 3 plants per parent. Each replication was supplied with an almost equal number of plants from each seed lot. The plants were planted at a distance of 1.5 m within the row and 2.5 m between rows.

The experiment design at Ballenhausen for the population Resi x Phantasia F_1 was slightly different. A total of 42 F_2 individuals were used in the trial and was distributed over 6 rows. Each row was planted with 7 F_2 individuals and two of each parent. Every third plant in every row was one of the parents. 5 out of the 6 rows belonged to each seed lot and the 6th row was a mixture of plants from all the 5 seed lots. The trial dimension was adjusted to 1.5 m within and between rows. Plants were grown without any pruning. Eventually, the plants grew as small bushes of about 1.5 m diameter.

3.2.4 Trial maintenance

In 2016, weeds between the rows were controlled by tractor with rotavator a week after planting. It was followed by weeding with front hoe two weeks later. Weeding within the row was done on August 3rd and 4th by hand hoe. In Ballenhausen, the weed density was not severe. Weeds around the plants were removed using a hand hoe in the first week of September. In 2017, first weeding at Reinshof took place on July 24th using a tractor rotavator. It was followed by hand hoeing on August 3rd around the plants and hand rotavator was used to remove weeds between the plants on the next day. Last weeding took place on September 8th by hand.

3.2.5 Phenotyping

The field phenotyping started with scoring of first mature fruit. The scoring was done according the same scale for LB as described below. First scoring of LB started by at least half of the trial plants showed symptoms of infection. Scorings were done at intervals of one to two weeks depending on the infection progress. The disease severity was scored on a scale of 1-9, 1 equals no infection and 9 means dead plant. Details of scoring scale is described in table 3.3. The scorings were used to calculate Area Under Disease Progress Curve (AUDPC) using the following equation (Kranz, 1996).

$$\text{AUDPC} = \sum_{i=0}^{n-1} \left(\frac{x_{i+1} + x_i}{2}\right)(t_{i+1} - t_i)$$

Where, x_i is the score at time i, t_i is the day of the ith observation, and n is the number of scores.

3.2.6 Statistical analysis

Data adjustment

The AUDPC values of each individual were adjusted per population. The mean of each population (two blocks) and of respective individual blocks were calculated. The difference in

mean values between individual blocks and whole population were also calculated. The AUDPC values of each individuals of respective blocks were adjusted by adding or subtracting the mean difference.

The Pearson correlation coefficients between leaf and fruit infection and analysis of variance were done using 'R' (R 3.3.2 for Macbook).

Table 3.3 Key for the assessment of damages by late blight (*P. infestans*) on leaves and fruits of tomatoes in field experiments (adapted from Horneburg & Becker 2011).

	Leaf infections
1	No infections
2	First symptoms as grey-green to brown leaf spots
3	Symptoms obvious. Yellowing or browning of some leaves or small leaf spots up to 50% of plant mass
4	Increased yellowing or browning, or small leaf spots to 75% of plant mass
5	Plant severely affected
6	Yellowing or browning to 50% of plant mass
7	Yellowing or browning to 75% of plant mass
8	Entire plant yellow to brown, all leaves infected
9	All leaves dead
	Fruit infections (*including small, immature fruit)s*
1	No infections
2	Up to 12.5% of the fruits with grey-green to brown spots
3	Up to 25% of the fruits with typical dark spots
4	Up to 37.5% of the fruits with typical dark spots
5	Up to 50% of the fruits with typical dark spots
6	Up to 62.5% of the fruits with typical dark spots
7	Up to 75% of the fruits with typical dark spots
8	More than 75% of the fruits with typical dark spots
9	All fruits infected

Confidence interval (CI) and Least Significant Difference (LSD)

A 95% confidence interval of both parents, from the mean value of each parent has been shown as error bars on the scatter plot figures. CI was calculated as per the following equation. Where X is the mean, Z is the value from the standard normal distribution table for the selected confidence level and SE is the standard error.

$$CI = \quad \bar{X} \pm (Z * SE)$$

The LSD was calculated using the following formula.

$$LSD = t\sqrt{2s^2/n}$$

Where t is the t value for respective degree of freedom, S^2 is the variance and n equals the number of individuals.

LSD of each parent was calculated. The mean of these LSDs was shown as the LSD_{05} of each F_2 individuals in the graph.

3.3 Results

3.3.1 Performance of the parents

Parent genotypes belong to the group OOTP, viz. Golden Current, Resi and Rote Murmel, showed different levels of resistance. Golden Current was found to be more susceptible over the period of infection. Resi and Rote Murmel showed a medium level of resistance, showed a better performance at the initial days and the infections increased periodically. The genotypes of the categories Commercial and Exotic are very similar in field resistance. All the parent genotypes exhibited the same trend in both the years (Fig. 3.1).

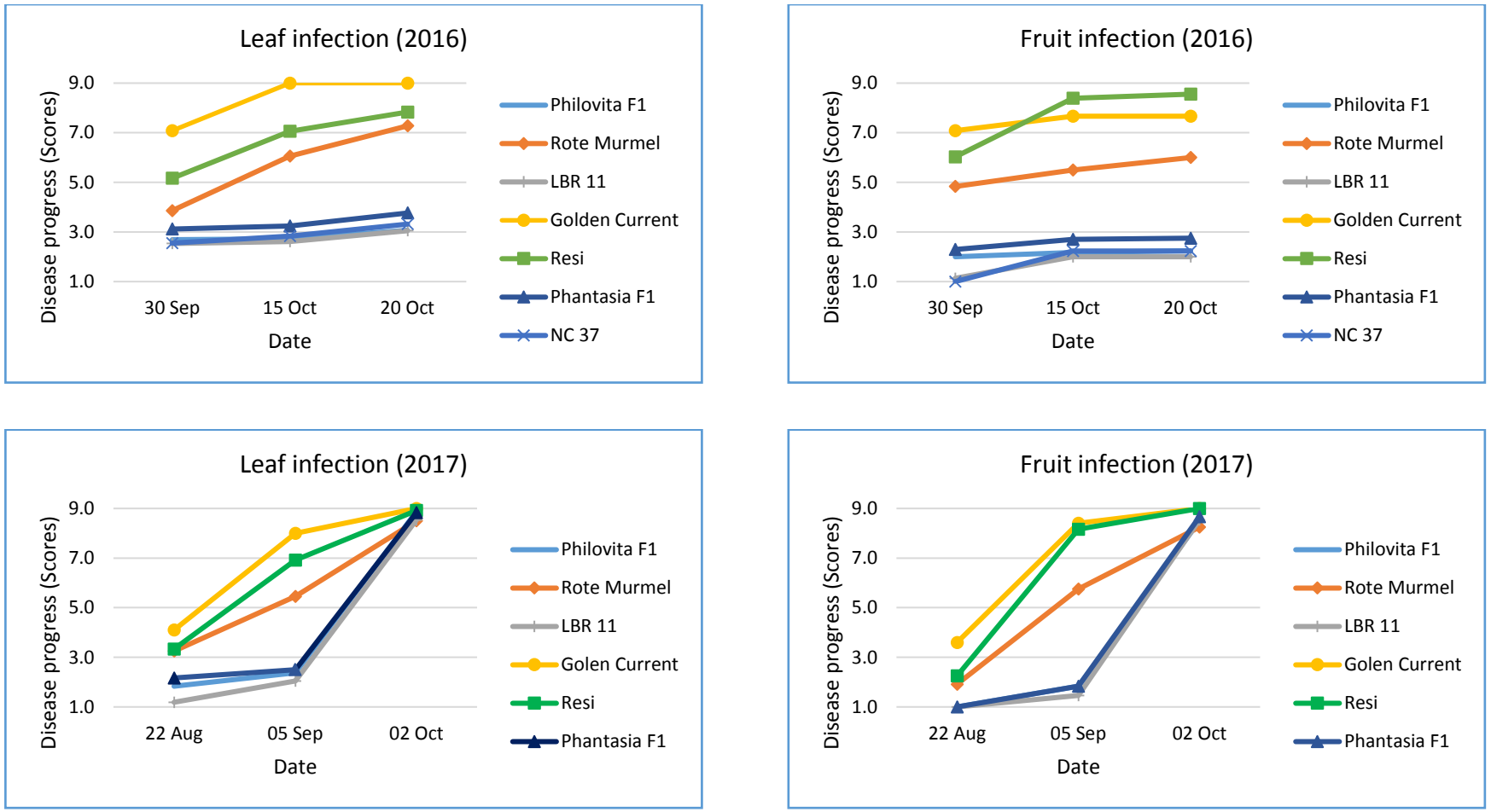


Fig 3.1 progress in leaf & fruit infection (individual disease scores) over the seasons (parents) 2016 & 2017 Reinshof

Table 3.4 ANOVA of 7 parent genotypes for the years 2016 & 2017 (n=12)

Sources of variations	***Degree of freedom***	***Sum of Squares***	***Mean Square***	***F value***	***Pr(>F)***	***Least significant difference (5%)***
Genotype (Leaf)	6	117243	19540	369.736	< 2e-16***	34.45
Year	1	155312	155312	2938.744	< 2e-16***	
Genotype x Year	5	2053	411	7.769	0.000139***	
Residuals	26	1374	53			
Genotype (Fruit)	6	176721	29454	86.218	6.62E-16***	37.88
Year	1	160508	160508	469.851	< 2e-16***	
Genotype x Year	5	3591	718	2.102	0.0972.	
Residuals	26	8882	342			

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

3.3.2 Performance of F_2 populations

OOTP x Commercial

The group comprises three crosses viz. Resi x Philovita F_1, Rote Murmel x Phantasia F_1 and Resi x Phantasia F_1 (Fig. 3.2.11, 3.2.12 and 3.2.13). As described above, the parents used in these crosses from OOTP are of medium late blight resistant and on the other hand Philovita F_1 and Phantasia F_1 exhibit the strongest resistance in the parents' group. All the crosses in this group showed a clear segregation into two groups. The F_2 individuals formed one group with the most resistant parent and the other near to the less resistant parent.

The year 2017 was ideal for late blight screening at Reinshof. Increased rainfall and optimum temperature favoured late blight infection. F_2 individuals more resistant than the better resistant parent was distinguishable under the field conditions (Fig. 3.3.4, 3.3.5 and 3.3.6).

The comparison with in the year is not possible since the cross Resi X Phantasia F_1 was grown at a different location in 2016. Instead, the comparison between the years is possible. The F_2 population resulted from the above mentioned cross forms two different groups, one is around the most resistant parents, Phantasia F_1, and the second one is a susceptible group. In the other crosses also, a same kind of pattern was seen in both the years. But in the crosses with Rote Murmel, some of the F_2s were seen in the near vicinity of Rote Murmel. This kind of segregation was seen also in other crosses with Rote Murmel.

OOTP x Exotic

This group represents the majority of crosses evaluated in the year 2016. When the parents belong to the OOTP group exhibit a medium resistance, both the exotic parents were on highly resistant side. The performance of the F_2s was different among the populations. Both the crosses with Rote Murmel showed the same kind of pattern (Fig. 3.2.1 and 3.2.4). In both cases, mean values for leaf infection was close to the exotic parent, though it was a slight difference in case of the cross with LBR 11. While the fruit infection values were nearest to Rote Murmel in both the crosses. In the crosses with Golden Current, mean values of both the traits were closer to respective values of NC 37 and LBR 11 (Fig. 3.2.2 and 3.2.6). The crosses with Resi showed an opposite scenario in each cross, the mean value of both the traits were close to NC 37 in one cross while it was close to Resi in the cross with LBR 11 (Fig. 3.2.3 and 3.2.5).
In 2017, all the three crosses with LBR 11 were repeated. In all the three populations, the mean values for both the traits were close to the LBR 11. This pattern was seen only in the population resulted from cross with Rote Murmel in 2016 (Fig. 3.3.1, 3.3.2 and 3.3.3).

It was very clear that each OOTP genotypes having a same pattern of segregation with both the exotic genotypes. Both the crosses with Rote Murmel, the F_2 population is mainly grouped into two clusters, one with the most resistant parent (exotic) and another group which is more susceptible than both the parents. While we can see a few individuals in the range of Rote Murmel. In the crosses with Golden Currents, both the populations form two very clear clusters, one with the exotic parents and the other with Golden Current. The same segregation

pattern can be seen in case of the crosses with Resi also, but much spread. All the three crosses were repeated in 2017, under a very high disease pressure and the same segregation patterns were well visible.

Commercial x Exotic

This group attributes four crosses viz. Philovita F_1 x NC 37, Phantasia F_1 x NC 47, Phantasia F_1 x LBR 11 and Philovita F_1 x LBR 11 (Fig. 3.2.7, 3.2.8, 3.2.9, and 3.2.10). Both commercial and exotic genotypes were the most resistant parent's groups in the field. The mean values of both the traits were close to the commercial parents. The same phenomenon was repeated in the year 2017 with the repeated population of Philovita F_1 x LBR 11.

All the four parents fell on the most resistant side of the plot and formed a single group together. The F_2s were segregated into two different groups, a.k.a. resistant and susceptible. The only repeated cross in this group for the year 2017 was Philovita F_1 x LBR 11 and which followed the same pattern (3.3.7).

3.3.3 Correlations between leaf and fruit infection

All the crosses showed a positive correlation between leaf and fruit infection. In the year 2016, the correlation coefficient ranged from 0.53 to 0.85 while in the year 2017 (7 populations) it ranged from 0.70 to 0.94 (Table 3.3). Parents are not included in the following table.

Table 3.5 Pearson correlation coefficient between leaf and fruit infection in 2016 & 2017 (52 F2 plants / population)

F_2 Population	**Correlation coefficient (2016)** *(P< 0.05)*	**Correlation coefficient (2017)** *(P< 0.05)*
Rote Murmel x Phantasia F_1	0.80	0.84
Resi x Phantasia F_1	0.85*	0.85
Resi x Philovita F_1	0.82	0.94
Golden Current x NC37	0.64	-
Golden Current x LBR11	0.67	0.87
Rote Murmel x NC37	0.82	-
Rote Murmel x LBR11	0.82	0.76
Resi x NC37	0.59	-
Resi x LBR11	0.83	0.78
Philovita F_1 x NC37	0.53	-
Philovita F_1 x LBR11	0.69	-
Phantasia F_1 x NC37	0.56	-
Phantasia F_1 x LBR11	0.76	0.70

* 42 F_2 plants / population

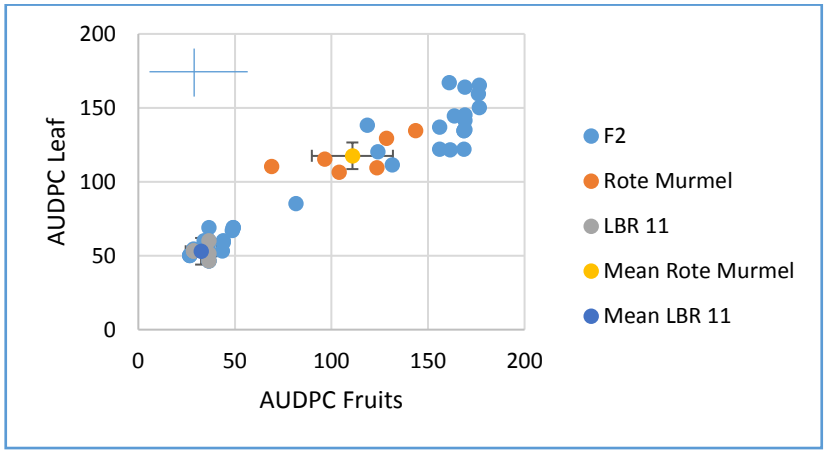


Fig. 3.2.1

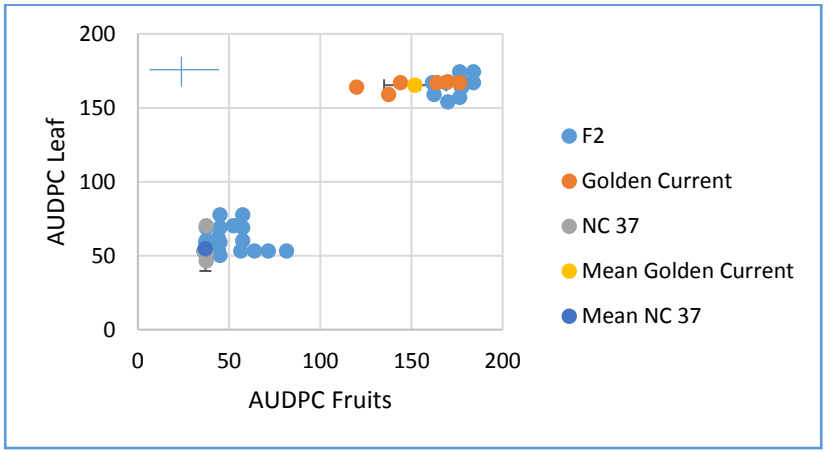


Fig. 3.2.2

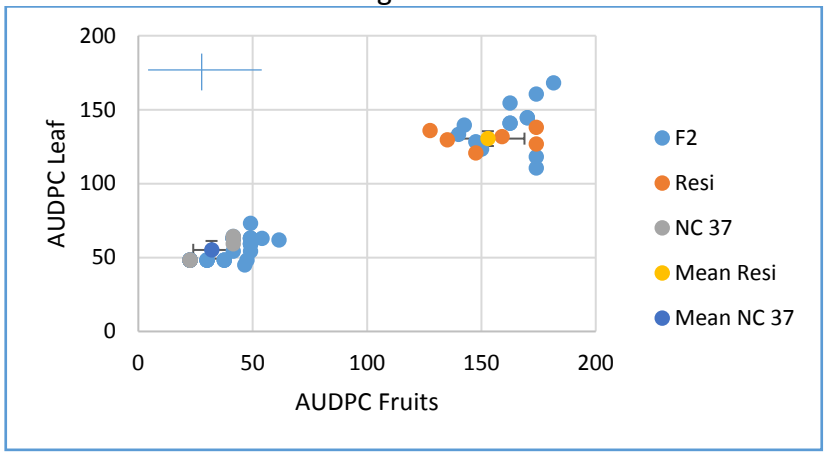


Fig. 3.2.3

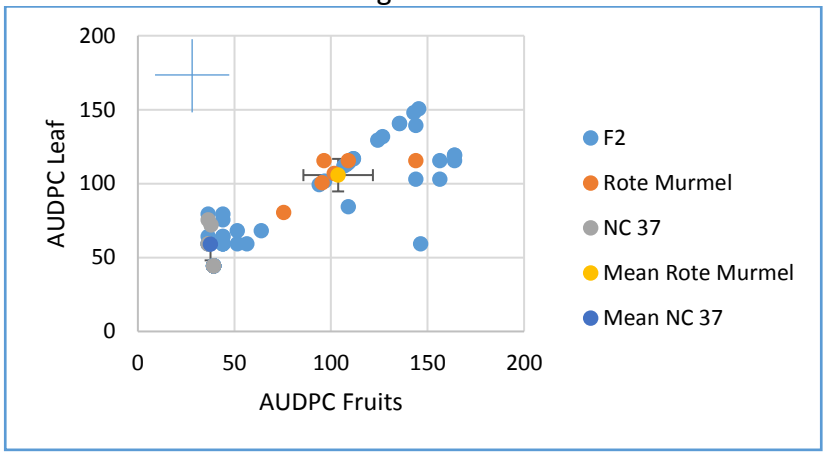


Fig. 3.2.4

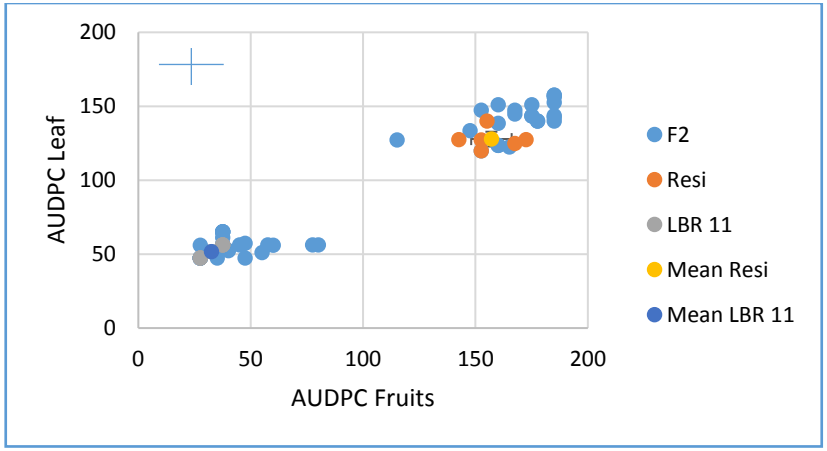
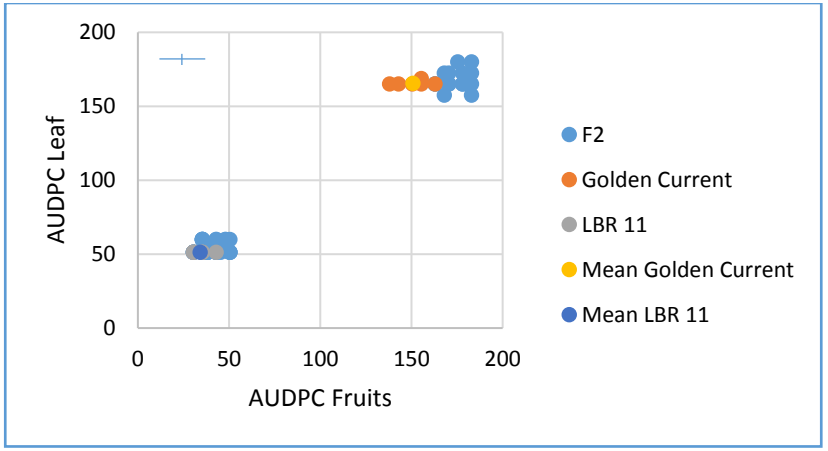


Fig. 3.2.5



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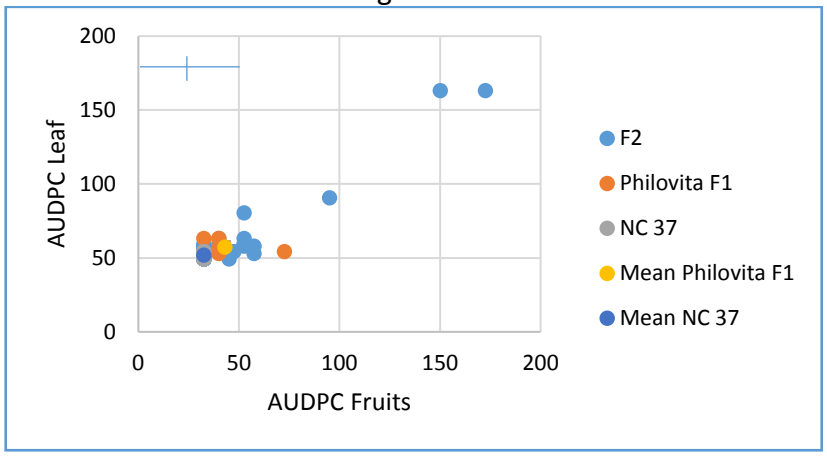


Fig. 3.2.7

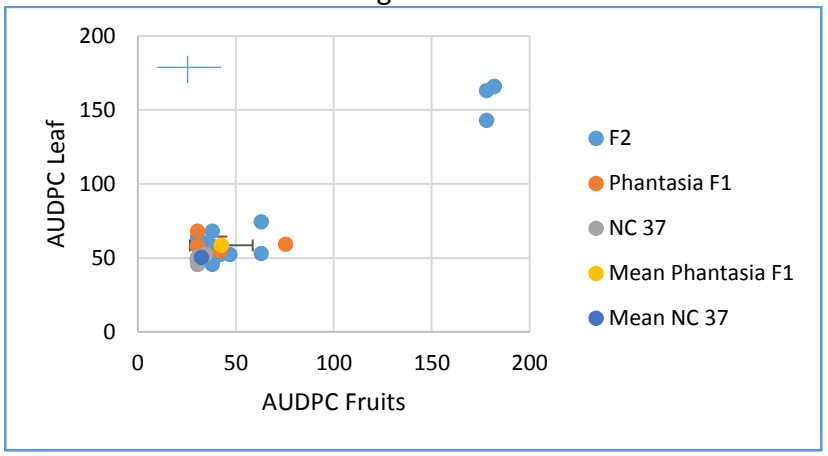
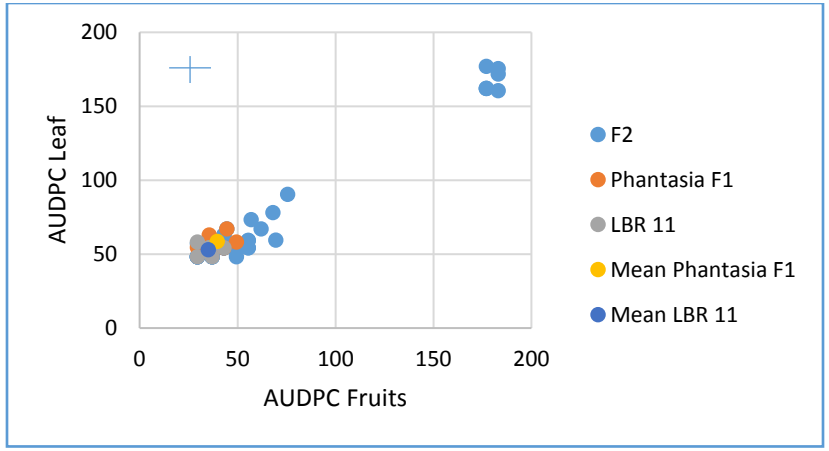


Fig. 3.2.8



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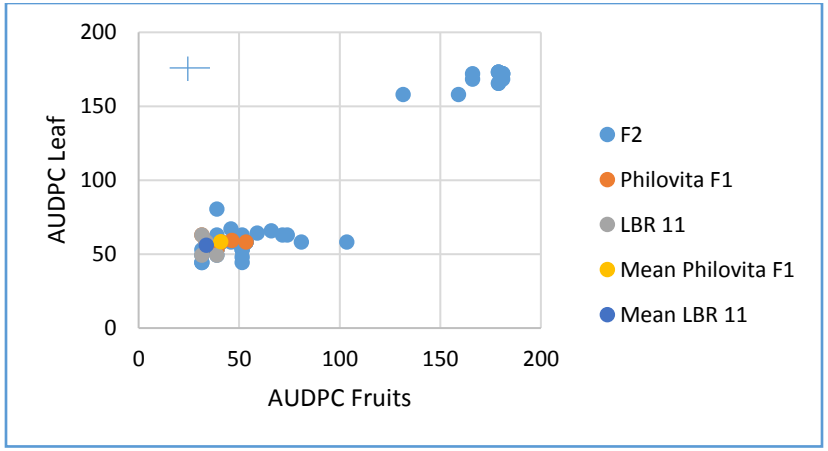

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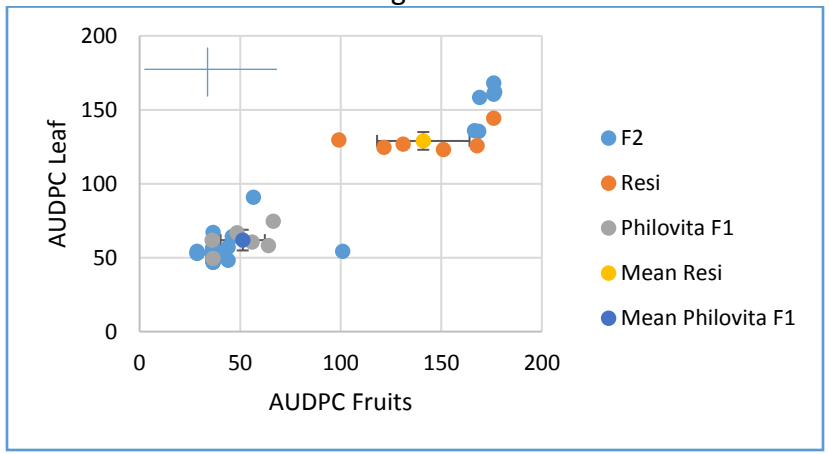


Fig. 3.2.11

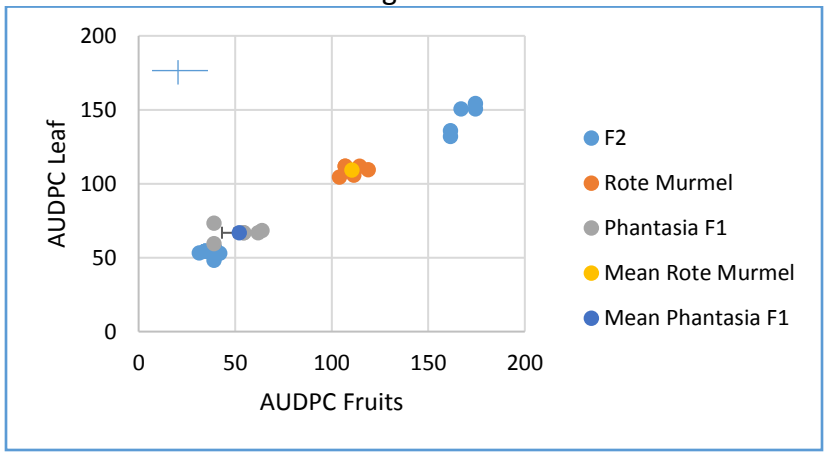


Fig. 3.2.12

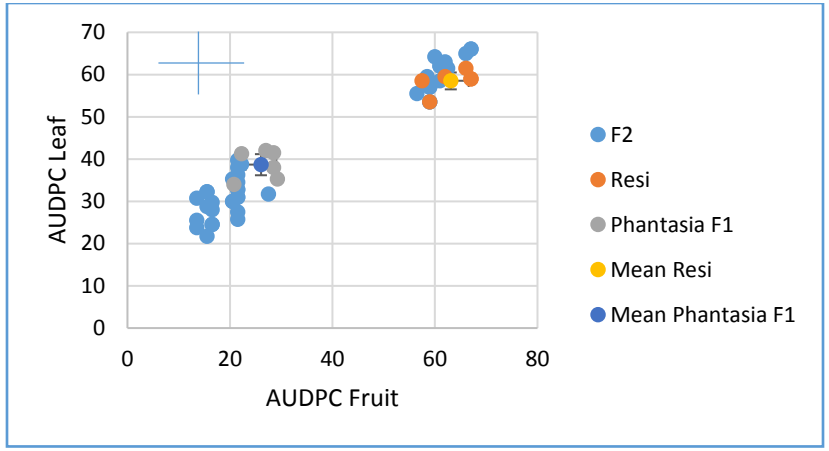


Fig. 3.2.13

[+]A 95% confidence interval of both the parents, from the mean value of each parent, is shown as error bars on the scatter plots.
[++] LSD_{05} of each parent was calculated. The mean of these LSD is shown as the LSD_{05} of each F_2 individuals in the figure.
[+++]Each figure represents 52 F_2 individuals and 6 parents each.

Fig. 3.2 Scatter plots of fruit infection vs leaf infection in the year 2016

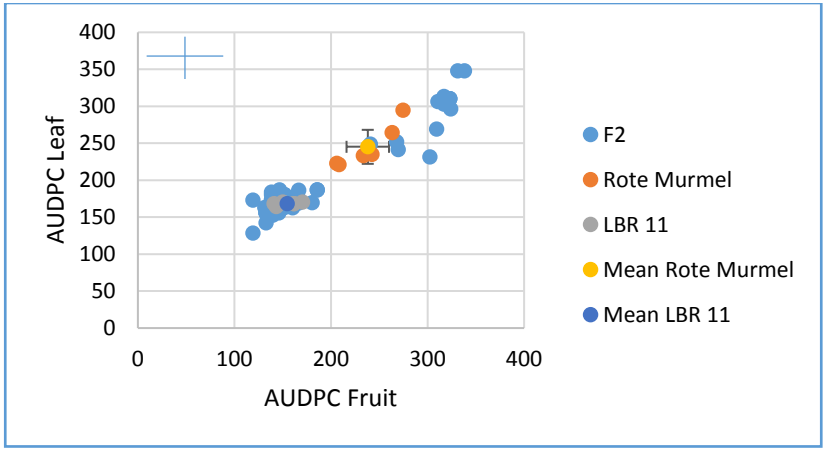


Fig. 3.3.1

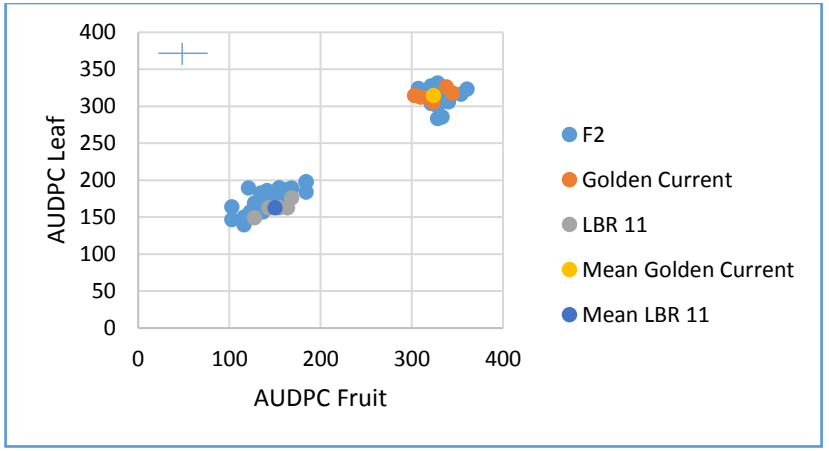


Fig. 3.3.2

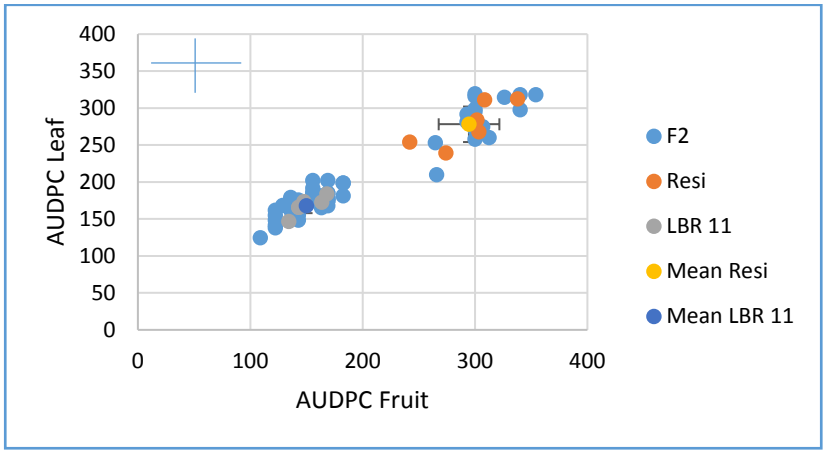


Fig. 3.3.3

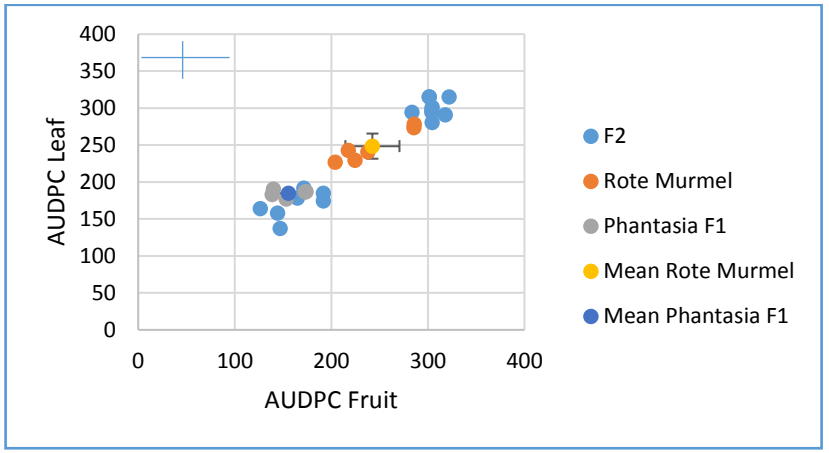


Fig. 3.3.4

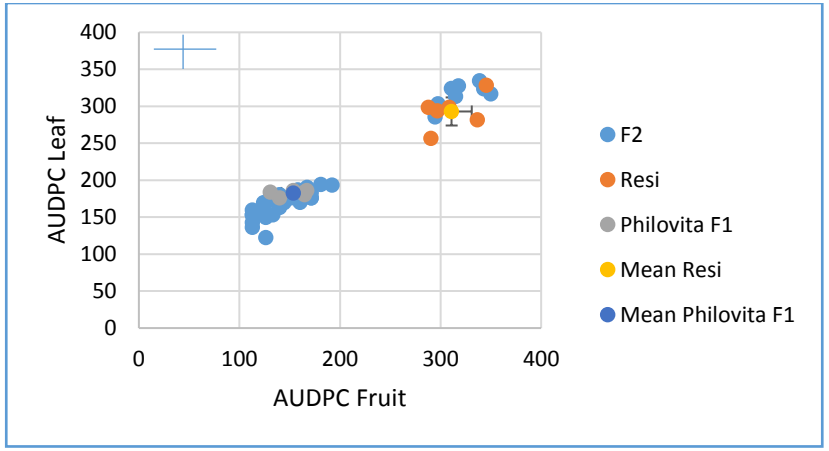


Fig. 3.3.5

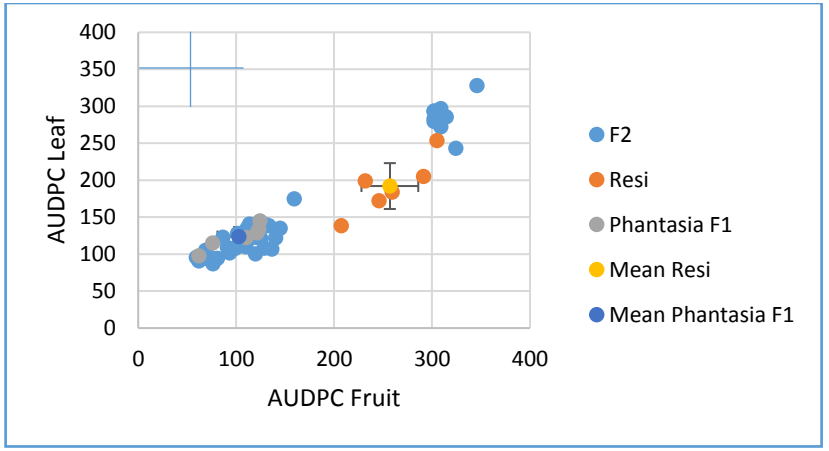


Fig. 3.3.6

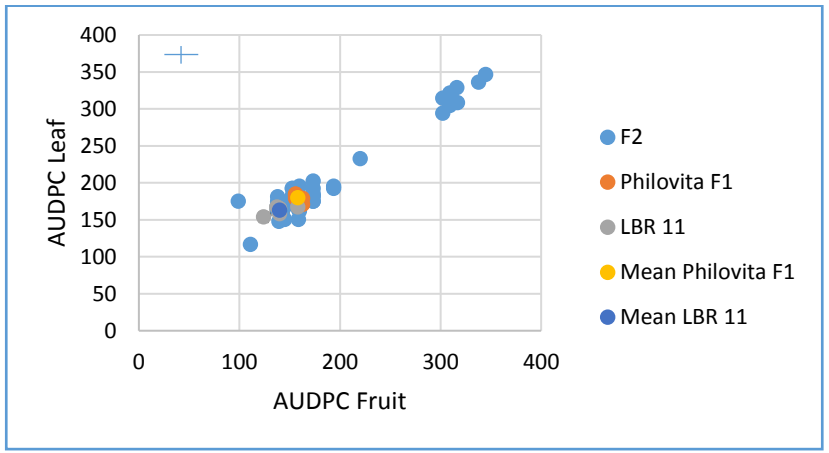


Fig. 3.3.7

[+]A 95% confidence interval of both the parents, from the mean value of each parents, is shown as error bars on the scatter plots.
[++] LSD_{05} of each parent was calculated. The mean of these LSD is shown as the LSD_{05} of each F_2 individuals in the figure.
[+++]Each figure represents 52 F_2 individuals and 6 parents each.

Fig. 3.3: Scatter plots of fruit infection vs leaf infection in 2017

3.4 Discussion

A high level of infection is necessary for the successful phenotyping of populations under field conditions. The 'free growing' method is an ideal method to enhance the infection levels. The plants were grown without any pruning which led to the formation of small tomato bushes of 1.5 - 2 meter in diameter. This helps the tomato plants to keep micro climate inside the bush which is humid and less hot when compared to the atmosphere. This is suitable for the spread of late blight disease and was very well visible at the initial stages of the infection period. Also, this system is suitable for screening of large number of plants with low man /woman power.

We scored both leaf and fruit infections because under the free growing conditions different genotypes show different pattern of bush formation. Some genotypes tended to spread side wise (more side shoots) while some formed small bush. This may create a bias in fruit infection under low disease pressure. Though leaf and fruit infections are correlating, scoring of both traits was done to increase the accuracy and which helped us to select superior lines for practical breeding purpose.

3.4.1 Performance of parents and ANOVA result

The ANOVA results showed that the parental genotypes were significantly different for both leaf and fruit resistance. The effect of year indicated the influence of changes in disease pressure and difference in duration between scoring dates. This can be expected under field conditions for a crop like tomato (Bernousi *et al*. 2011).

3.4.2 Performance of F_2 population

The disease pressure at the location Ballenhausen was high in 2016 when compared to Reinshof and which resulted in cutting edge distinction between the most resistant F_2 individuals. The crosses Rote Murmel x Phantasia F_1 and Resi x Philovita F_1, located at Reinshof, experienced a less disease pressure. In fact, it created a difficulty to identify the most resistant plants among the resistant F_2 group. Considering the fact that the other parents (Rote Murmel

and Philovita F_1) are coming from the same groups as Resi and Phantasia F_1, which showed transgression, all the three crosses were decided to repeat in the following year to concrete the theory of pyramiding of resistance genes. The year 2017 had very high disease pressure which helped in a precise phenotyping of individuals. All the three above mentioned crosses showed signs of positive transgression. F_2 individuals which are more resistant than the better parent, beyond the 95% confidence interval and LSD, shows the signs of possible gene pyramiding.

The populations belonging to the group OOTP x Exotic showed different segregation patterns in 2016 under less disease pressure. But all the 3 crosses with LBR 11 were repeated in 2017 and all showed the same trend in 2016 which may be a result of less infection pressure and also due to the differences in bush formation. Especially in cross with Rote Murmel, many F_2 individuals were more spread like Rote Murmel and which made the fruits to get exposed to late blight more than other individuals in a less infected condition, whereas in 2017 under heavy infection pressure, all the crosses might have received equal disease pressure.

All the crossed in the group Commercial x Exotic showed the same pattern. They have formed two clear groups. All the parents were in the resistant group while a number of F_2 individuals were susceptible. The segregation of F_2 population resulted from the parents of same groups found to be following a similar pattern.

3.4.3 Genetic background of the parent genotypes

Each genotype groups were compared with other groups to check whether same group carries same source of resistance. The segregation pattern of every group genotypes were cross checked with genotypes from other groups. Exotic genotypes LBR 11 and NC 37 were compared with the segregating populations resulted from cross with commercial hybrids and OOTP genotypes. LBR 11 and NC 37 were showing the same pattern of segregation in all the populations. This helps us to conclude that both LBR 11 and NC 37 carry the same source of LB resistance.

Commercial hybrids, Philovita F_1 and Phantasia F_1 are also following the same pattern with respective crosses from other groups. Which implies that both genotypes carries same source of LB resistance.

3.4.4 Correlation between leaf and fruit infection

Pearson correlation coefficient was calculated to confirm the positive relationship between leaf and fruit infection. A strong positive correlation (>0.70) was shown by most populations except a few. The populations which showed moderate positive (0.50 - 0.70) are mainly the crosses with exotic genotypes. Many of the F_2 individuals resulted from exotic genotypes showed bush formation in the field, like the exotic parents, this phenomenon resulted a bias in fruit infection by hiding the fruits inside the bush from late blight spore distribution especially in the year 2016 with less disease pressure. The year 2017 showed a strong positive correlation in all the crosses.

3.5 Conclusion

Late blight resistance breeding is a very challenging research subject for plant breeders across the world. The rapid evolution potential of the pathogen makes it very difficult to control (Raffaele *et al*., 2010). Comprehensive approaches are necessary to overcome the challenges raised by the pathogen.

The resistance provided by the OOTP genotypes were found to be novel though it was less resistant in comparison with the commercial and exotic genotypes. Resistance in OOTP in combination with other groups found to be promising, especially with the commercial genotypes. The phenomena of positive transgression are a sign of pyramiding of different resistant sources and has a potential in late blight resistance breeding.

4. Identification of QTL associated with late blight field resistance in a tomato F_2 population

4.1 Introduction

Late blight resistance breeding is the one of the flagship programmes among tomato breeders around the globe. Its rapid spreading and evolving capacity of *P. infestans* (Drenth *et al.* 1994) keeps it under the lime light. Three major resistance genes, viz., Ph-1, Ph-2 and Ph-3, have been identified in wild tomato species *S. pimpinellifolium* (Foolad *et al*. 2008). The dominant Ph-1 gene was first identified in early 1950s and is located at the distal end of Chromosome 7 (Peirce 1971). Ph-1 is no more resistant and used in breeding (Mutschler *et al.* 2006; Foolad *et al.* 2014). Ph-2 and Ph-3 are partially resistant genes found on chromosome 10 and 9 respectively. Ph-2 in combination with Ph-3 gives a stronger resistance (Wagner 2012; Nowicki *et al.* 2013). Ph-3 is the most effective, commercially available, resistant gene against *P. infestans.* The resistance conferred by Ph-3 has been reported broken in Brazil (Chunwongse *et al.* 2002; Miranda *et al.* 2010). The high evolutionary potential of *P. infestans* (Raffaele *et al.* 2010) and limitation of chemical control measures under organic cultivation invite the necessity to search for new resistance sources.

4.2 Materials and Methods

4.2.1 Plant Materials

The F_2 population of 180 plants derived from Resi x Phantasia F_1 was selected for mapping. The population showed transgression in field trial 2016. F_2 plants derived from 2 different F_1 plants, named Family1 and Family 2, were used for mapping. Each family consisted of 90 plants.

4.2.3 Experimental setup

Please refer to section 3.2.4 for Reinshof and Ballenhausen.

Seeding was done on April 5th to facilitate cloning of individual plants for the locations Ballenhausen and Westen. Potting was done on April 25th.

Westen and Ballenhausen were included to spread the risk of phenotyping due to low infection. The field was located at Westen (52.840427, 9.293076), North Germany. The field was cultivated with Potatoes in the previous year.

4.2.2 Cloning

The main shoot of each plant was cut with an average length of 12-15 centimetres from the apex. The leaves, except newly developed and side shoots were removed. The cuttings were planted pots of in 500 ml volume in Bio-Topferde (HAWITA GRUPPE GmbH, Germany) potting mixture. The cuttings were made and planted on May 26th. These cuttings were used as planting material at Westen. The mother-plants were left with two healthy side shoots. The longest and strongest among the remaining two side shots were used as planting material for Ballenhausen. Side shoots were cut and potted in in pots of 500 ml volume on June 8th. On average, shoot lengths were adjusted to 8-10 centimetres. Newly planted cuttings were always kept under shade to avoid moisture loss and eventual death. The main stem left with one remaining side shoot was used as the planting material for Reinshof.

4.2.3 Experimental design

Please refer to section 3.2.3 for Reinshof

Westen and Ballenhausen

Westen was the site cloned mapping population (Resi x Phantasia F_1). A population of 180 F_2 individuals were spread across 6 rows, each row was formed of 31 F_2 plants and 4 of each parent. The trial dimension was kept 1.5 meters within and between the rows. The trial at Ballenhausen was abandoned at a later stage due to a very low infection level, hence no details are provided here.

Westen was the first location where planting took place in the season on June 21st, followed by Reinshof on June 26th and finally Ballenhausen on July 10th.

4.2.4 Trial maintenance

Please refer to section 3.2.4 for Reinshof. There were no weed control measures taken at Westen due to the uniform distribution of weeds.

4.2.5 Phenotyping

Please refer to section 3.2.5

4.2.6 Statistical analysis

Correlation coefficient between leaf and fruit infection within and across the locations and Analysis of Variance (ANOVA) were done using 'R' (R 3.3.2 for Macbook).

4.2.7 Genotyping

Leaf sample harvest for DNA isolation

90 F_2 individuals were used for the extraction of DNA from each family at Reinshof. Leaf samples were collected directly from the field. From the youngest completely opened leaf of each plant were selected as a source of leaf tissue. 10 leaf disks were collected, using a leaf puncture, into one of the wells of a 96-well-plate. The leaf puncture was cleaned with 70% ethanol after collecting leaf samples from every plant. Two previously decided wells were left empty for using standards by TraitGenetics. The wells were filled according to a pre-designed plate layout. After filling all the wells, the plate was kept open in a thermocol box filled with desiccant material for removing excess moisture in the wells. Once all the wells were found moisture free, the strip-caps were put on to close the wells and the plate was sent to TraitGenetics for carrying out the further steps.

Genotyping and Molecular Map construction

The genotyping was done using the SolCAP 10K array provided by Illumina which contains 7720 SNP markers. A linkage map was constructed using JoinMap®5. Maps were constructed separately for both families. Out of the 7720 markers, 2067 markers were polymorphic in family 1 and 1805 markers were polymorphic in family 2. A combined map was created by combining both the maps and the resulting map had 2236 markers. In the combined map, 602 markers out

of 2236 were not polymorphic in either of the families. The non-polymorphic regions of the map were treated as missing points.

A framework map was constructed by selecting the most informative markers, means with minimum missing values, at about 10cM distance. The distances between the markers were recalculated by re-mapping using JoinMap®5. The resulting maps of family 1, family 2 and the combined map had 101, 93 and 108 markers, respectively.

4.2.8 Quantitative Trait Loci (QTL) Mapping

QTL mapping was done in R studio, an open-source integrated development environment for R programming language, using the package 'R/qtl' (Broman *et al*. 2003). Composite Interval Mapping (CIM) was performed to detect QTL, using the *cim* function. The whole genome was scanned at steps of 1 cM by using the *cim* function. A genome wide LOD significance threshold (5%) was estimated by 1000 permutations (Churchill and Doerge, 1994). Three markers were allowed as co-factor with a 10 cM window size. The conversion of recombination frequency to genetic distance was done using the Kosambi map function (Kosambi 1944). The *fitqtl* function was used to obtain effects and interactions in CIM. Regions were considered as candidate QTL if the LOD score exceeded the LOD threshold. Left & right flanking markers and the closest marker to the QTL were obtained by the *find.flanking* function.

4.2.9 Calculation of QTL confidence interval

A 99% confidence interval was calculated by the 2 unit down method. A horizontal line was drawn 2 units down from the LOD peak of each QTL. The gap between the interception points of the line with LOD curve has been considered as 99% confidence interval for the respective QTL.

4.3 Results

4.3.1 Performance of parents at two locations

The parents, Resi and Phantasia F_1, expressed different levels of resistance at Reinshof. Resi was resistant at the beginning of the season and the resistance was broken periodically.

Phantasia F_1 exhibited strong resistance for both leaf and fruit infection. The resistance was broken towards the end of the season (Fig. 4.1). Westen was a different in case of disease pressure. The resistance of Resi was broken at the initial days of infection. Phantasia F_1 was comparatively better than Resi in resistance, but it showed an entirely different scenario at Westen. The resistance was found broken soon after the infection started (Fig. 4.1).

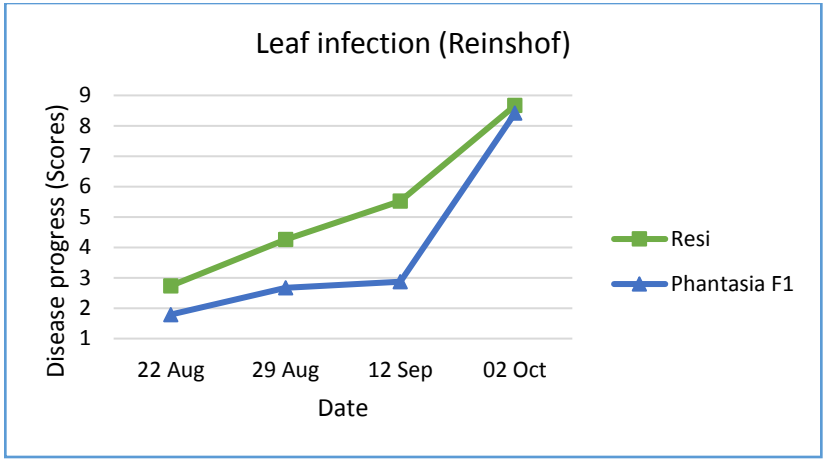
4.3.2 Analysis of Variance of parents

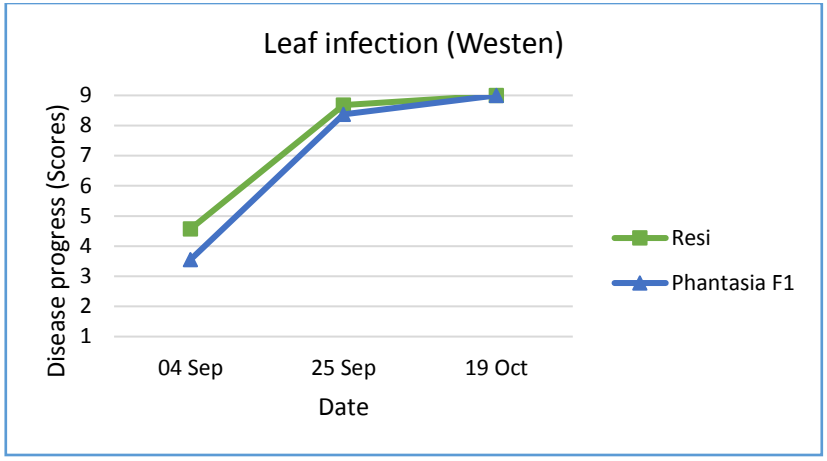
The two parent genotypes were found to be significantly different for leaf and fruit infection for both the locations (Table 4.1).

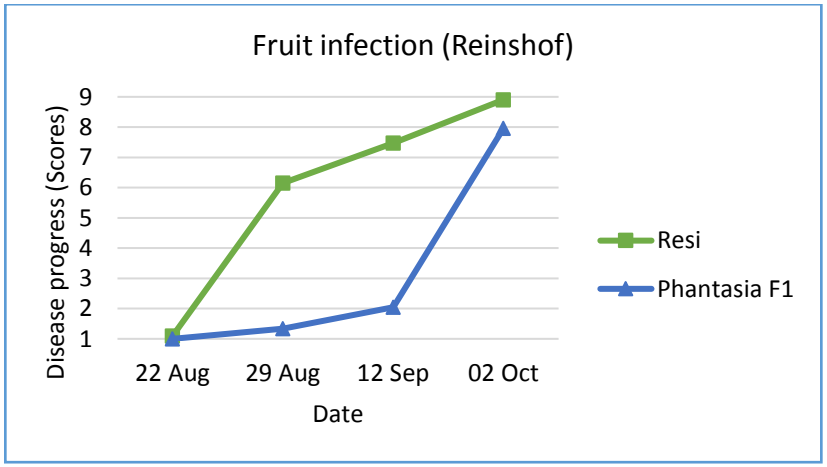
Table 4.1 Analysis of variance of leaf and fruit infection of parents for Reinshof and Westen.

Sources of variations	***Degree of freedom***	***Sum of Squares***	***Mean Square***	***F value***	***Pr(>F)***	***Least significant difference (5%)***
Genotype (Leaf)	1	9636	9636	61.70	4.68e-07 ***	11.79
Residuals	17	2655	156			
Genotype (Fruit)	1	33456	33456	37.29	1.16e-05 ***	28.26
Residuals	17	15252	897			

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1







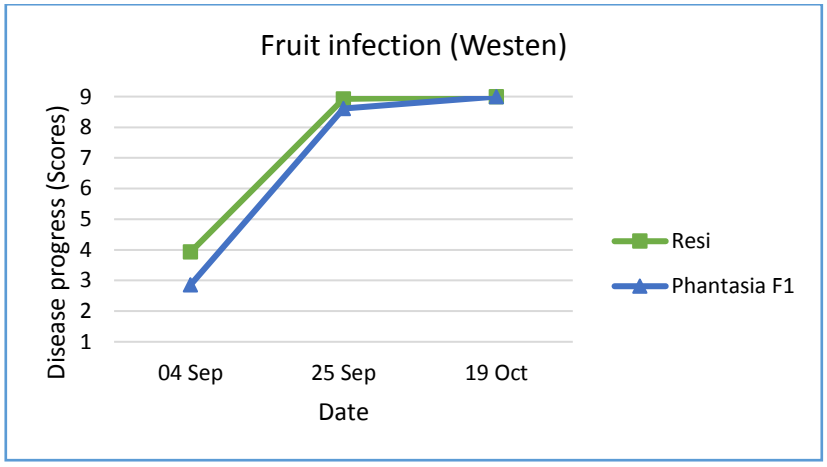


Fig. 4.1 progress in leaf & fruit infection across location (parents) 2017

4.3.3 Correlation between leaf and fruit infections of F_2 individuals

The Pearson correlation coefficients between leaf and fruit infection within the locations were 0.85 and 0.95 for Reinshof and Westen, respectively. Between locations correlations ranged from 0.67 to 0.69 (Table 4.2).

Table 4.2 Pearson correlation coefficient for leaf & fruit resistance within & across locations of 184 F_2 plants

	Fruit Reinshof *(P< 0.05)*	***Leaf Westen*** *(P< 0.05)*	***Fruit Westen*** *(P< 0.05)*
Leaf Reinshof	0.85	0.67	0.67
Fruit Reinshof		0.67	0.69
Leaf Westen			0.95

4.3.4 Analysis of Variance of F_2 individuals

The ANOVA results suggest that there is significance difference between F_2 individuals at both locations for both leaf and fruit infection (Table 4.3).

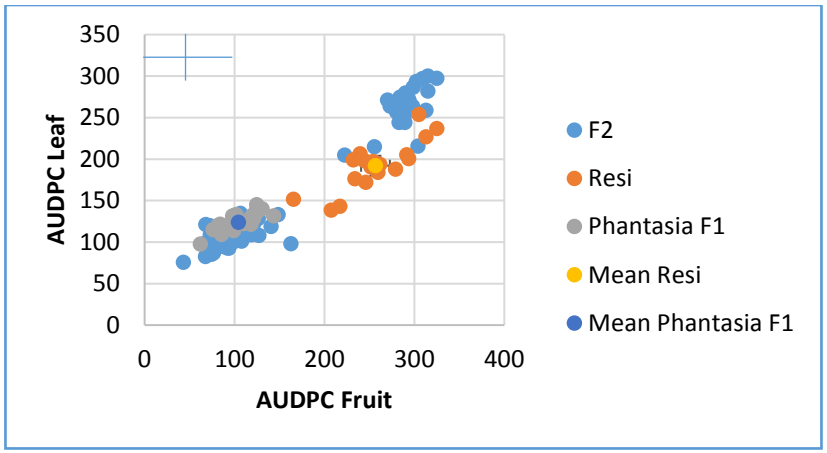
Table 4.3 Analysis of variance; leaf and fruit infection of F_2 individuals across the locations

Sources of variations	***Degree of freedom***	***Sum of Squares***	***Mean Square***	***F value***	***Pr(>F)***	***Least significant difference (5%)***
Genotype (Leaf)	179	904932	5055	3.851	< 2e-16 ***	71.45
Residuals	179	234978	1313			
Genotype (Fruit)	179	1370407	7656	4.009	< 2e-16 ***	86.23
Residuals	179	341837	1910			

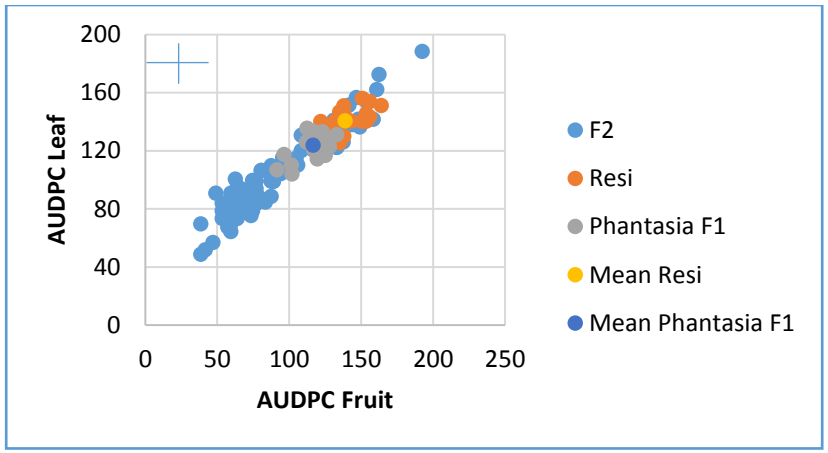
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.3.5 Performance of the mapping population

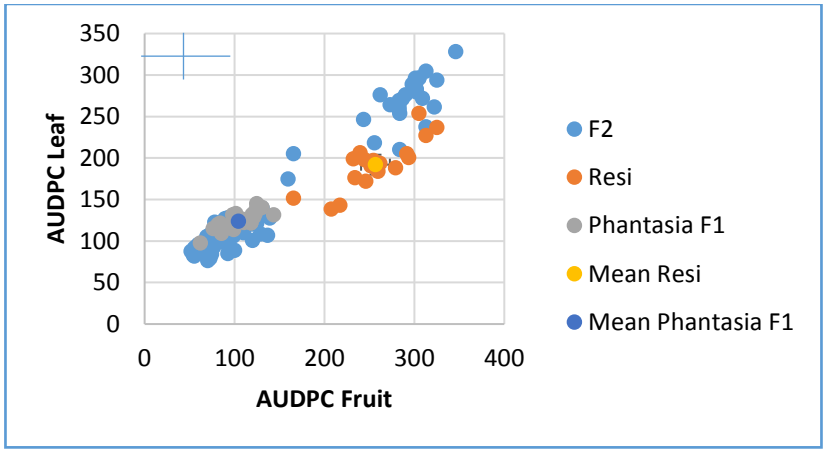
The F_2 individuals segregated into two groups at Reinshof, resistant and susceptible. Some of the F_2 individuals in the resistant group has shown higher level of resistance than the most resistant parent. At Westen, the F_2 individuals have shown continues pattern rather than two groups. Many F_2 individuals were showed increased resistance than the better parent.



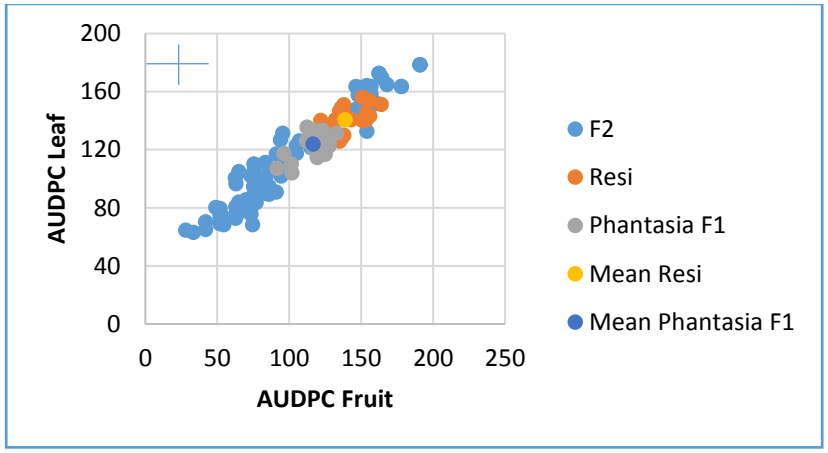
Family 1, Reinshof



Family 1, Westen



Family 2, Reinshof



Family 2, Westen

*A 95% confidence interval of both the parents, from the mean value of each parents, were shown as error bars on the scatter plots.
** LSD05 of each parents were calculated. The mean of these LSDs were shown as the LSD05 of each F_2 individuals in the graph.

Fig. 4.2 XY scatter plots of fruit and leaf infections of family 1 & 2 for the locations Reinshof and Westen.

4.3.6 Quantitative Trait Loci (QTL) analysis

QTL analysis; Family 1

1 QTL associated with leaf resistance were found on chromosome 9 (Table 4.4) It was significant at 5% probability. QTL on chromosome 9 was stable across the locations. The QTL on chromosome 9 found to be a major QTL accounts for a total of 72.7 & 72.5% of phenotypic variation at Reinshof and Westen, respectively.

The QTL for fruit resistance was also found in the chromosomes 9. The QTL on chromosome 9 was stable across the locations and accounted 48.3% & 51.7% phenotypic variation at Reinshof and Westen respectively. (Table 4.4).

QTL analysis; Family 2

There was only 1 QTL detected in family 2 for both the traits and across the locations. QTL was found on chromosomes 9. The QTL was responsible for a phenotypic variation of 77.2% & 79.3% in leaf resistance at Reinshof and Westen respectively. Meanwhile, it contributed 63.9% & 66.7% variations for fruit resistance (Table 4.5).

QTL analysis; combined map

Only one QTL was found in the combined map for the trait leaf resistance on chromosome 9. The QTL on chromosome 9 was present at both the locations. It was responsible for a phenotypic variation of 75.0% and 76.0% at Reinshof and Westen, respectively.

For fruit resistance, only 1 QTL was found on chromosome 9. The QTL on chromosome 9 was significant at 5% probability and stable across the locations. It contributed 56.0% and 59.4% phenotypic variation at Reinshof and Westen respectively (Table 4.6).

Table 4.4 QTL mapped in family 1 for the traits leaf and fruit resistances

Chromosome	***Position***	***Additive effect*** [+]	***Dominance effect*** [++]	***LOD***	***R^2***	***TR^2***
Leaf resistance; Reinshof						
Ch. 9	11.9	-75.5	-58.1	25.6	72.7	72.7
Leaf resistance; Westen						
Ch. 9	11.9	-94.1	-71.7	25.5	72.5	72.5
Fruit resistance; Reinshof						
Ch. 9	11.9	-27.9	-10.1	13.0	48.3	48.3
Fruit resistance; Westen						
Ch. 9	11.9	-33.7	-16.1	14.4	51.7	51.7

[+] Half of the difference between the two homozygotes.

[++] Difference between the heterozygote and the average of the two homozygotes.

Table 4.5 QTL mapped in family 2 for the traits leaf and fruit resistances

Chromosome	***Position***	***Additive effect*** [+]	***Dominance effect*** [++]	***LOD***	***R^2***	***TR^2***
Leaf resistance; Reinshof						
Ch. 9	12	-84.1	-58.6	28.6	77.2	77.2
Leaf resistance; Westen						
Ch. 9	12	-86.8	-66.9	30.4	79.3	79.3
Fruit resistance; Reinshof						
Ch. 9	12	-36.0	-17.4	19.7	63.9	63.9
Fruit resistance; Westen						
Ch. 9	12	-43.9	-24.0	21.2	66.7	66.7

[+] Half of the difference between the two homozygotes.

[++] Difference between the heterozygote and the average of the two homozygotes.

Table 4.6 QTL mapped in combined map for the traits leaf and fruit resistances

Chromosome	***Position***	***Additive effect*** [+]	***Dominance effect*** [++]	***LOD***	***R^2***	***TR^2***
Leaf resistance; Reinshof						
Ch. 9	11.9	-80.0	-58.7	54.3	75.0	75.0
Leaf resistance; Westen						
Ch. 9	11.9	-98.1	-74.5	55.9	76.0	76.0
Fruit resistance; Reinshof						
Ch. 9	11.9	-32.2	-14.1	32.1	56.0	56.0
Fruit resistance; Westen						
Ch. 9	11.9	-39.1	-20.4	35.3	59.4	59.4

[+] Half of the difference between the two homozygotes.

[++] Difference between the heterozygote and the average of the two homozygotes.

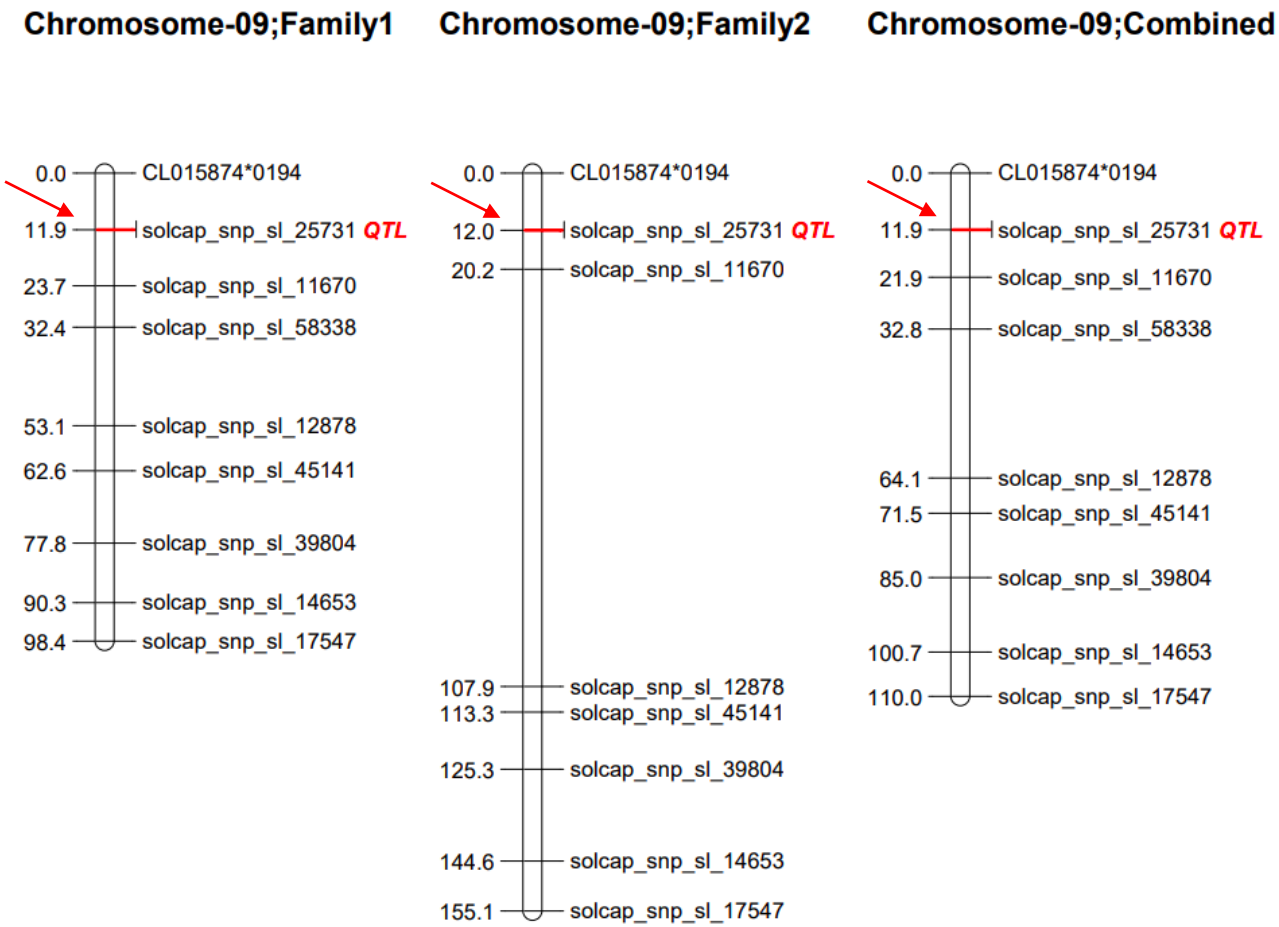
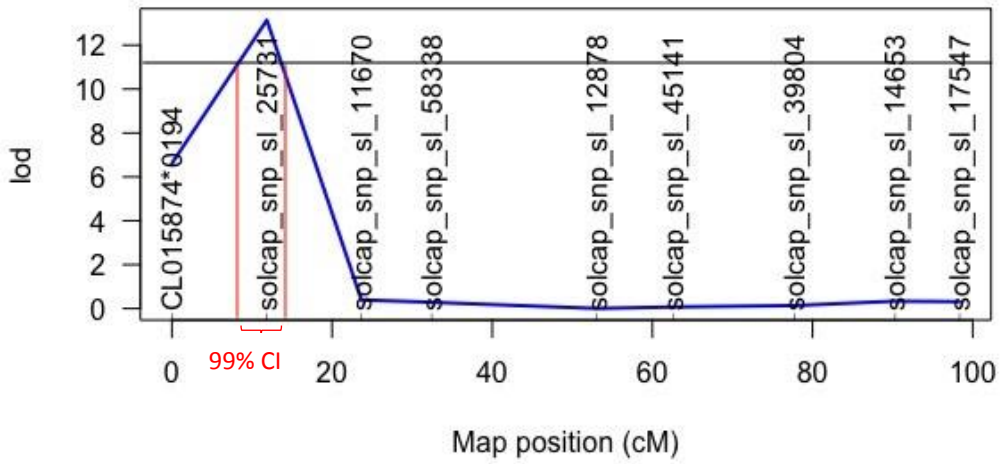


Fig. 4.3 Genetic map showing the position of QTL on frame work maps of chromosome 09 on family 1, family 2 and combined map.



*The 99% confidence interval has been calculated by drawing a horizontal line 2 unit down the LOD peak of QTL. The distance between the interception points of the horizontal line with the LOD curve has been considered as confidence interval.

Fig. 4.4 The 99% confidence interval of QTL

4.4 Discussion

4.4.1 Performance of parents

Both the parents exhibited a different level of resistance against late blight at Reinshof. Resi was the less resistant parent. Resi showed medium level of resistance at the initial days of infection. The resistance was broken periodically while the conditions became more favourable for disease. Phantasia F_1 showed very good resistance at the initial days. The resistance was broken very slowly when compared to Resi.

Both the parents showed a different response at Westen. Both the parents showed disease symptoms on the first day of scoring. Resi was more infected on the first day of scoring while Phantasia F_1 was comparatively less infected. But the resistance of both the parents were broken on the second date of scoring. The ANOVA results suggest that both the parents are significantly different in their resistance to late blight. This was clearly evident for both traits; leaf and fruit resistance.

4.4.2 Performance of F_2 individuals

The F_2 individuals showed a clear segregation for late blight resistance for both the traits at Reinshof. The segregation pattern at Westen was rather continues than two clear groups. ANOVA results suggests all the individuals are significantly different for both leaf and fruit resistances.

The correlation between leaf and fruit resistance were positively correlating with and between the locations. Which suggests that the performance of individual plants were almost same regardless location and difference in disease pressure.

4.4.3 QTL analysis

QTL analysis was done separately for both the families, since 26.9% of the polymorphic markers were not polymorphic in both the families. Though, a combined map was crated and non-polymorphic regions we treated as missing points. This approach was used to look for a QTL not shown in individual analysis.

A major QTL on chromosome 09 was found in both families and combined analysis across the locations for both leaf and fruit infection. The phenotypic variation caused by this QTL ranged from 48.3% to 79.3%. Which means that this QTL played the pivotal role in the late blight resistance. The QTL has been mapped to chromosome 09 where the late blight resistant gene Ph-3 has been reported (Chunwongse *et al.* 1998, Chunwongse *et al*. 2002). The QTL was located between the markers *CL015874*0194* and *solcap_snp_sl_25731* at 11.9 or 12 cM.

The additive and dominant effect of the major QTL found on chromosome 09 is negative. Since the major resistance is contributed by the parent Phantasia F_1, we have to assume that the QTL is originated from Phantasia F_1. The high evolutionary potential of *P. infestans* (Raffaele *et al.* 2010) suggests that the necessity to look for resistant QTL /genes.

4.5 Conclusion

Late blight resistance is a major challenge in outdoor tomato cultivation. The resistance provided by commercially available genes are already broken by many pathogen strains. Also, these genes provide only race specific resistance. The genetic background of resistance provided by the parents (Resi and Phantasia F_1) are unknown. The QTL found on chromosome 09 was responsible for the major phenotypic variations for both the traits and was stable across the locations. Though no other QTL were found in this analysis, especially when the parent Resi is medium resistant to late blight, it is required to do the QTL analysis with a larger population to find additional QTL.

5. Screening of late blight field resistance and validation of known genes in tomato

5.1 Introduction

Tomato (*Lycopersicon esculentum* Mill.*)* is the most widely grown vegetable food crop in the world. The total production of tomatoes exceeded 177 million tonnes (FAOSTAT 2016). Tomato late blight (LB) caused by *Phytophthora infestans* (Mont.) de Bary is a major cause of crop as well as economic loss around the globe in temperate and humid environments (Foolad et al. 2008 and Nowicki et al. 2013). The asexual and sexual life cycles of *P. infestans* and its capacity to rapidly overcome plant resistance genes make it difficult to control (Foolad et al. 2008; Nowicki et al. 2012). The very common asexual reproduction can produce thousands of zoospores in a short span of days (Sullenberger et al., 2018). Sexual reproduction results in oospore formation (Judelson, 1997) and leads to the emergence of new races which could overcome host resistance (Drenth et al-, 1994). This fact led scientists to describe *P. infestans* as a pathogen with "high evolutionary potential" (Raffaele et al. 2010b). According to the reports, the resistance imparted by the Ph-3 gene has been overcome by new *P. infestans* isolates (Chunwongse et al., 2002; Miranda et al., 2010). These facts create an urge to search for novel sources of resistances.

The two sets of genotypes used in this study, Diversity set and TGRC, UC Davis germplasm (TGRC set) contain commercially available resistant genes and a number of genotypes with unknown sources of resistance. The performance of these genotypes functions as real time indicator of infection pressure and as a sensor for newly introduced more virulent strains when compared to previous years.

5.2 Materials and Methods

5.2.1 Plant material

Diversity set

Diversity set is a collection of 17 genotypes known for its resistance against late blight (table 5.1). This includes genotypes, carrying well known late blight resistance genes, such as New Yorker (Ph-1), Mecline (Ph-2), Mountain Magic (Ph-2 & Ph-3) and genotypes showing different levels of resistance with unknown sources. The susceptible genotype Zuckertraube was used as a check.

TGRC set

22 *Lycopersicon* accessions were received from TGRC, UC Davis. 17 were selected (table 5.2), 15 were late blight resistant and 2 were early blight resistant, for screening and to understand the performance of known resistance genes such as Ph-1, Ph-2 & Ph-3 under the test conditions. Apart from Mecline, a second accession having Ph-2 (LA3152) and 3 accessions with Ph-3 genes (LA1269, LA4285, and LA4286) were also available for screening. Zuckertraube and Philovita F1 were used as susceptible and resistant checks respectively.

5.2.2 Experimental setup

The trial was conducted at Reinshof in 2016 and 2017. For detailed description, see section 3.2.3

Field trials were conducted at two locations in Germany, in 2016. Reinshof (51.503985, 9.923220), experimental farm of Georg-August-University Göttingen, south of Göttingen, Central Germany.

In 2016, sowing took place on May 10th and June 8th, respectively in multi-pot trays QP 96 (Hermann Meyer KG, Germany). Tray wells were evenly filled with Bio Kräutererde (HAWITA GRUPPE GmbH, Germany) substrate. Every tray well was supplemented with 2-3 seeds and kept in greenhouse (Day Night, 16:8 h and 22^{o}C (day) & 18^{o}C night)). A week after germination, extra plants from each well were removed. The seedlings, for Reinshof, were potted on June 22nd in plastic pots of 500 ml volume and a potting mixture Bio-Topferde (HAWITA GRUPPE GmbH, Germany) was used. Potting of Ballenhausen batch took place on June 23rd. The potted plants were moved to a polyhouse and a distance of single pot was maintained between every pot. The field planting took place on June 29th at Reinshof and on July 15th at Ballenhausen.

In 2017, sowing took place on May 8th. Two weeks later, excess plants were removed and the plants were potted on May 31st. The field planting was carried out in two days, the replication 1 was planted on June 26th and replication 2 was planted on the next day. Plants were potted in Bio Kräutersubstrat (Klasmann-Deilmann GmbH, Germany).

5.2.3 Experimental design

The trial was designed as a randomized complete block design with three replications and one plant per replication. Two replications were accommodated in a single row and the third one was in another row.

5.2.4 Growing system

The field trials were in a free growing system. Here, unlike traditional single shoot system, the plants were grown without any pruning. Eventually, the plants grew as small bushes of about 1.5 m diameter.

5.2.5 Trial maintenance

In 2016, weeds between the rows, were controlled by tractor with rotavator a week after planting. It was followed by weeding using a front hoe two weeks later. Weeding within the row was done on August 3rd and 4th by hand hoe. In 2017, first weeding at Reinshof took place on July 24th using a tractor rotavator. It was followed by hand hoeing on August 3rd around the plants and a hand rotavator was used to remove weeds between the plants on the next day. Last weeding took place on September 8th by hand.

5.2.6 Phenotyping

The first scoring of LB started when at least half of the trial plants showed symptoms of infection. Scorings were done depending on the infection progress. The disease severity was scored according to table 3.3. The scorings were used to calculate the Area Under Disease Progress Curve (AUDPC) using the following equation (Kranz, 1996).

$$\text{AUDPC} = \sum_{i=0}^{n-1} \left(\frac{x_{i+1} + x_i}{2}\right)(t_{i+1} - t_i)$$

Where, x_i is the score at time i, t_i is the day of the ith observation, and n is the number of scores.

5.2.7 Statistical analysis

Correlation coefficient between leaf and fruit infection and Analysis of Variance (ANOVA) were done using 'R' (R 3.3.2 for Macbook).

Table 5.1: Diversity set; genotypes with late blight resistance gene and source

No.	*Genotype*	*Gene/s*	*Source*
1	Golden Currant	Unknown	Organic Outdoor Tomato Project
2	Resi	Unknown	Organic Outdoor Tomato Project
3	Philovita F1	Unknown	Commercial hybrid
4	Phantasia F1	Unknown	Commercial hybrid
5	L3707	Unknown	Section's germplasm
6	L3708	Unknown	Section's germplasm
7	LA1033	Unknown	Section's germplasm
8	NC-37	Unknown	Supplied by Yigal Cohen, Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel
9	LBR 11	Ph-2 & Ph-3 (Hanson *et al.*, 2016)	Donated by a private seed saver. Original accession from World Vegetable Center (Taiwan) was not available because of technical reasons.
10	New-Yorker	Ph-1 (Oyarzun *et al.* 1998)	Section's germplasm
11	Mecline	Ph-2 (Foolad *et al.* 2014)	Section's germplasm
12	Pieraline 2-6-14	Unknown	Section's germplasm
13	Cherry Bomb F1	Unknown	Commercial hybrid
14	Mountain Magic F1	Ph-2 & Ph3 (Gardner & Panthee, 2010)	Commercial hybrid
15	Crimson Crush F1	Ph-2 & Ph3 (Stroud, J. A. 2015)	Commercial hybrid
16	Primabella	Unknown	Organic Outdoor Tomato Project
17	Rote Murmel	Unknown	Organic Outdoor Tomato Project
18	Zuckertraube	None	Unknown

Table 5.2: TGRC set; Genotypes with species name and late blight resistant gene

No.	*Genotype*	*Species and cultivar*	*Gene/s*
1	LA2009	*L. esculentum* cv. New Yorker	Ph-1
2	LA3151	*L. esculentum* cv. Mecline	Ph-2
3	LA3152	*L. esculentum*	Ph-2
4	LA1269	*L. pimpinellifolium*	Ph-3
5	LA4285	*L. esculentum*	Ph-3
6	LA4286	*L. esculentum*	Ph-3
7	LA3145	*L. esculentum*	Unknown
8	LA3158	*L. pimpinellifolium*	Unknown
9	LA3159	*L. pimpinellifolium*	Unknown
10	LA3160	*L. pimpinellifolium*	Unknown
11	LA3161	*L. pimpinellifolium*	Unknown
12	LA3330	*L. pimpinellifolium*	Unknown
13	LA3331	*L. pimpinellifolium*	Unknown
14	LA3333	*L. esculentum* var. cerasiforme	Unknown
15	LA3845	*L. esculentum*	Unknown
16	LA3846	*L. esculentum*	Unknown
17	LA1033	*L. hirsutum*	Unknown
18	Zuckertraube	*L. esculentum*	None
19	Philovita F1	*L. esculentum*	Unknown

5.3 Results

5.3.1 Diversity set

The performance of genotypes to late blight infection varied drastically, genotypes showed complete susceptibility to strong resistance during the field season (Fig 5.1 & 5.2). Genotypes like New Yorker (Ph-1) and Mecline (Ph-2) were poor in resistance and were equal to

the susceptible check Zuckertraube. Genotypes such as L3707, L3708 showed very strong resistance and followed by NC-37 and LBR 11. These genotypes were the most resistant in the group. The performance of all the genotypes was consistent for both the years. LA1033 is *L. hirsutum* and fruit infections were not scored, it can create a bias due to the special growing habit and very small fruits.

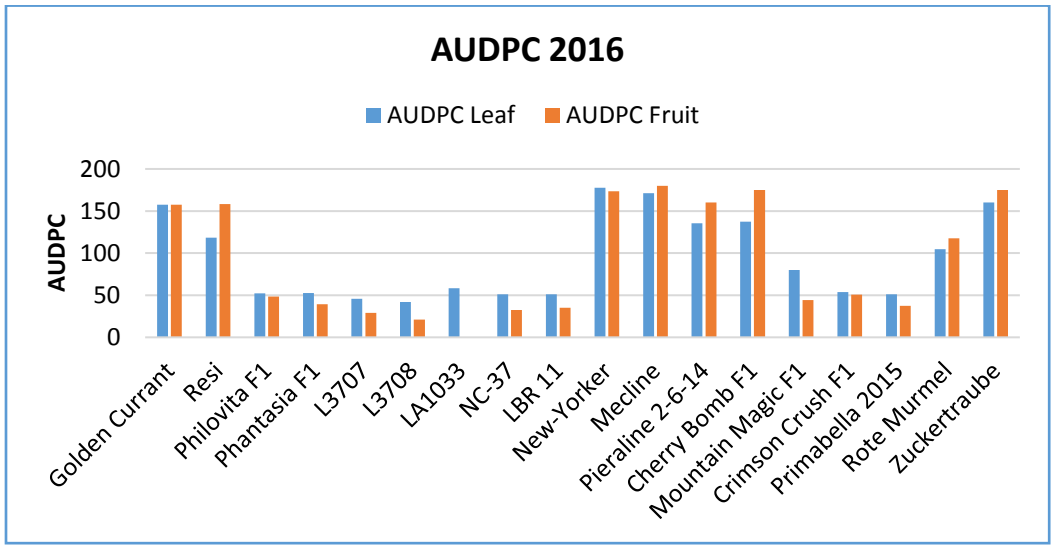


Fig. 5.1 Leaf and fruit AUDPC of Diversity set (2016)

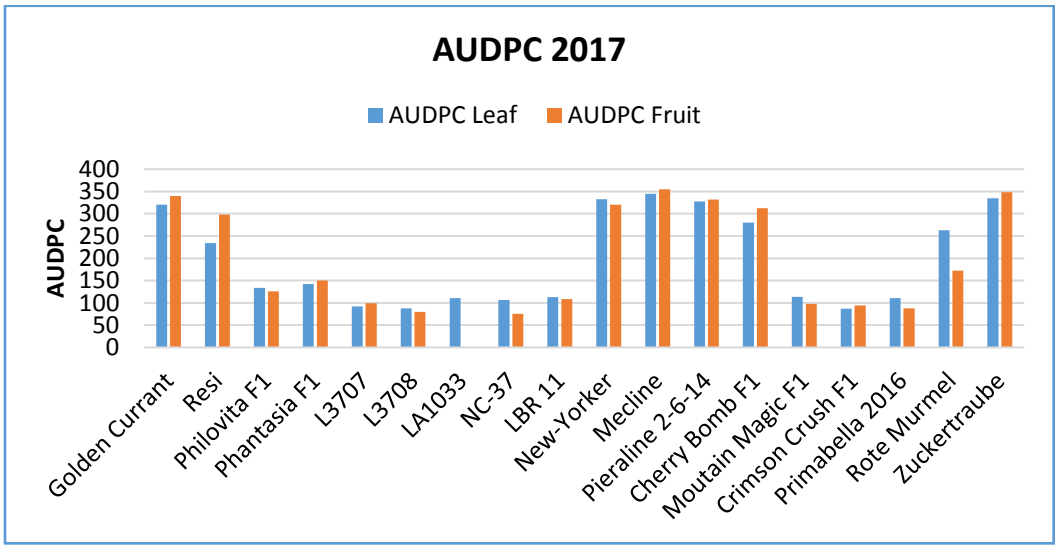


Fig. 5.2 Leaf and fruit AUDPC of Diversity set (2017)

Table 5.3: Pearson correlation coefficient between leaf and fruit infection with in and across year of Diversity set (2016 & 2017)

	Leaf 2016 ***(p< 0.001)***	***Fruit 2016*** ***(p< 0.001)***	***Leaf 2017*** ***(p< 0.001)***
Fruit 2016	0.87		
Leaf 2017	0.87	0.85	
Fruit 2017	0.79	0.88	0.91

All the genotypes showed significant and high (p< 0.001) correlation (0.79 – 0.91) between leaf and fruit resistance between the replication and across the years (Table 5.3). The ANOVA results showed that the genotypes were significantly different for leaf and fruit resistance (Table 5.4). The effect of the year indicated the influence of changes in disease pressure and difference in AUDPC calculation. This can be expected under field conditions for a crop like tomato (Bernousi et al. 2011).

Table 5.4: Analysis of variance of leaf and fruit infection showing interactions for Diversity set

Sources of variations	***Degree of freedom***	***Sum of Squares***	***Mean Square***	***F value***	***Pr(>F)***	***Least significant difference (5%)***
Genotype (leaf)	17	586630	34508	40.00	< 2e-16 ***	33.75
Year	1	280184	280184	324.78	< 2e-16 ***	
Genotype x Year	17	80758	4750	5.51	1.29e-07 ***	
Residuals	71	61252	863			
Genotype (fruit)	17	884201	52012	92.00	< 2e-16 ***	27.17
Year	1	257978	257978	456.32	< 2e-16 ***	
Genotype x Year	17	82110	4830	8.54	2.64e-11 ***	
Residuals	71	40139	565			

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

5.3.2 TGRC set

Most of the accessions were susceptible under the test condition and more or less equal to the susceptible check in performance. Genotypes like New Yorker and Mecline were susceptible as in the former group. While all the 3 accessions having Ph-3 gene (LA1269, LA4285 and LA4286) showed a very good resistance against late blight when compared to all other accessions in the trial, except LA1033 (Fig 5.3).

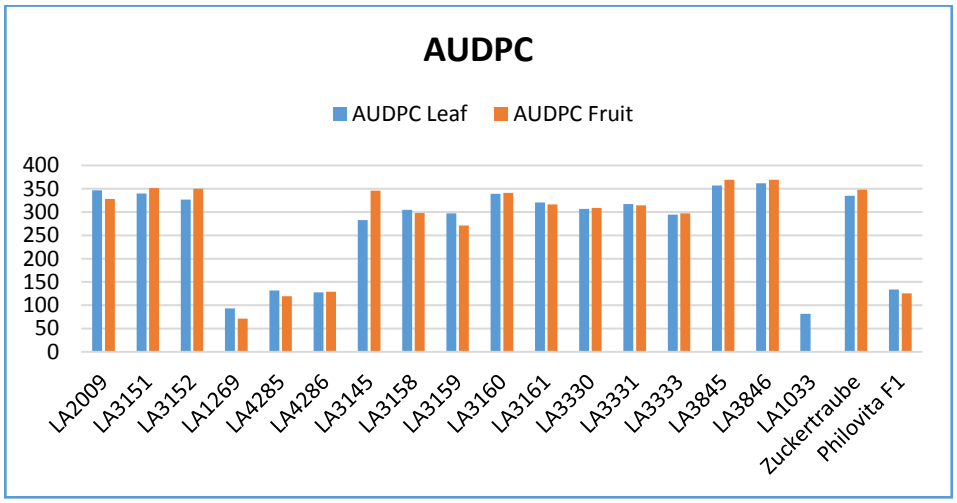


Fig. 5.3 Leaf and fruit AUDPC of TGRC set (2017)

The correlations between leaf and fruit infection were significant ($p < 0.001$). The ANOVA also showed that the accessions are significantly different for both leaf and fruit infection (Table 5.5).

Table 5.5: Analysis of variance of leaf and fruit infection showing interactions for TGRC set

Sources of variations	***Degree of freedom***	***Sum of Squares***	***Mean Square***	***F value***	***Pr(>F)***	***Least significant difference (5%)***
Genotype (leaf)	16	438350	27397	25.12	1.14e-13 ***	54.74
Residuals	32	34904	1091			
Genotype (fruit)	16	613950	38372	37.51	3.24e-16 ***	53.01
Residuals	32	32734	1023			

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

5.4 Discussion

The diversity set is a group of genotypes which is known for its resistance to late blight and the group is updated periodically according to the release of new late blight resistant genotypes by commercial companies or research institutions. For example, Cherry Bomb F_1 and Crimson Crush F_1 are the latest additions to the set.

In both years, all genotypes showed the same kind of performance in comparison with each other and to the susceptible check Zuckertraube. Also, a very high correlation between leaf and fruit infection shows that the genotypes were uniform for both the traits. Also, both the traits were showing strong correlation between years, which suggests that the genotypes were consistent over years. ANOVA suggests that the performance of genotypes was significantly different for late blight resistance.

The performance of TGRC set was also a mixed reaction in terms of resistance. Most of the accessions were susceptible to late blight, while 3 accessions having Ph-3 gene were showed a very high level of resistance over the infection period. Also, the high correlation between leaf and fruit within and between the replications shows the accessions were stable in terms of late blight resistance. The ANOVA results shows that the genotypes are significantly different for its level of late blight resistance.

The performance of New Yorker (Ph-1) was not surprising, since the resistance provided by Ph-1 gene was broken years back and completely susceptible now (Sullenberger et al., 2018). Though Ph-2 gene gave partial resistance >> when and where?? (Moreau et al., 1998), it was found to be susceptible under the test conditions. This was consistent for both the years for the trial of diversity set and same result was obtained in TGRC set trial. The accessions of New Yorker and Mecline used in TGRC set trial were different from that of diversity set. Which also forms the conclusion that Ph-1 and Ph-2 were susceptible under the test conditions. All the 3 accessions having Ph-3 gene (LA1269, LA4286, LA4286) expressed a very strong resistance against late blight. It was consistent in all the 3 replications.

Genotypes having both Ph-2 and Ph-3 genes; such as LBR 11 (Hanson et al, 2016), Mountain Magic F1 (Gardner & Panthee, 2010a) and Crimson Crush F1 (Stroud, J. A. 2015) also showed very similar response as the Ph-3 gene containing accessions from TGRC set, which may lead to the conclusion that the resistance showed by these genotypes should be a result of Ph-3 gene. NC-37 is very similar to LBR 11 in many phenotypic characters as well as late blight resistance. Meantime, LA1033 (*L. hirsutum*), also showed consistent and strong resistance against late blight.

The experiments showed Ph-3 is the only known resistance gene which is resistant in the research area and it transpires the necessity to continue the search for new late blight resistance sources. Apart from that, two accessions, LA3707 and LA3708, were found to be very resistant and promising for future research.

6. Summary

Summary

Tomato (*Lycopersicon esculentum*) is the most widely grown vegetable food crop in the world. Late blight caused by *Phytophthora infestans* (Mont.) de Bary is a major cause of crop loss in tomato around the globe. It spreads quickly and cause complete yield loss under favourable conditions. Late blight resistance breeding has been a hot subject among tomato breeders since many years.

The major objectives of this study are the evaluation of F2 populations for field resistance of late blight, identification of QTL for late blight field resistance through SNP genotyping and screening for potential new sources of resistance to late blight. The Diversity set had been used to study the disease progress, using genotypes with different levels of resistances, and TGRC set was used for the evaluation of known resistant genes under test conditions and to search for new sources of resistances.

The project started with 7 genotypes with different levels of late blight field resistances as parents for F2 populations. The parent genotypes were selected from three different groups, genotypes from Organic Outdoor Tomato Project (OOTP), commercial hybrids and exotic sources. Thirteen F2 populations derived from these 7 parents were used in the study. The F2 populations were divided into 3 groups, OOTP x Commercial, OOTP x Exotic, Commercial x Exotic.

In the year 2016, the 13 F2 populations were evaluated under field conditions. Twelve of them were planted at Reinshof and one at Ballenhausen. Out of the 13 populations, 6 populations had been selected for further evaluation in 2017 at Reinshof and the population, Resi x Phantasia F1, was selected as mapping populations in 2017.

The mapping population was divided into two families since they were resulted from two F1 plants. Each family had 90 F2 individuals and each individual was cloned (vegetative

propagation) and planted at three locations, Reinshof, Westen and Ballenhausen. The trial at Ballenhausen was abandoned at a later stage because of lack of infection.

Apart from the F2 populations, two trials were conducted at Reinshof, Diversity set and TGRC set. Diversity set was a group of 17 genotypes having different levels of late blight field resistance. Diversity set served as an indicator of disease progress and emergence of pathogen strain when compared to previous year. TGRC set also contained 17 genotypes received from Tomato Genetic Resource Center, University of California, Davis. Cultivars with known late blight resistant genes such as Ph-1, Ph-2 and Ph-3 were present in TGRC set. This trial was conducted to assess the performance of known resistant genes under trial conditions and to search for new resistant sources in the TGRC germplasm.

Parent genotypes were different in field resistance to late blight. Commercial and Exotic genotypes showed very good resistance while OOPT genotypes showed a medium level of resistance. The ANOVA result showed that the parent genotypes are significantly different for late blight resistance for leaf and fruit.

The population Resi x Phantasia F1 showed positive transgression and used as mapping population. The other two crosses from the same group (OOTP x Commercial) also repeated in 2017. The populations belonging to the group OOTP x Exotic showed different segregation patterns. All the 3 crosses with genotype LBR 11 were repeated in 2017. All the crosses in the group Commercial x Exotic showed same pattern with two clear groups. All the parents were in the resistant group while a number of F2 individuals were susceptible. The correlation coefficient of leaf and fruit resistance in F2 individuals ranged from 0.56 to 0.85 in 2016 and 0.70 to 0.94 in 2017 among the populations.

Both LBR 11 and NC 37 were showing same pattern of segregation in all the populations. This helps us to conclude that both LBR 11 and NC 37 carries the same sources of late blight resistance. Commercial hybrids, Philovita F1 and Phantasia F1 are also following the same

patter with respective crosses from other groups. Which means both the genotypes carries same source of late blight resistance.

SNP genotyping was done using SolCAP 10K array provided by Illumina which contains 7720 SNP markers. A linkage map constructed. QTL mapping was done separately for both the families and also for combined. Presence of a strong QTL on chromosome 9 was detected in both the families and combined analysis. The QTL was stable across the locations and also was present for both the traits. The phenotypic variation caused by the QTL ranged from 48.3% to 79.3%.

The genotypes in Diversity set showed same response in both the years, the disease progress in the genotypes showed same pattern. The susceptible genotypes were infected in the initial stage and resistant genotypes showed good level of field resistance. Correlation between leaf and fruit resistance were 0.87 and 0.91 in 2016 and 2017 respectively. The ANOVA result suggests that all the genotypes are significantly different for both leaf and fruit resistance.

The TGRC set was an estimation of performance of known resistant genes under the conditions, also search for new resistance sources. Ph-1 and Ph-2 were completely susceptible to late blight under field conditions. All the three genotypes with Ph-3 gene showed a very high level of resistance to late blight. Apart from these, *L. hirsutum* accession LA1033 showed high level of resistance to late blight. All the other accessions from TGRC were susceptible to late blight.

The resistance provided by the OOTP genotypes were found to be novel though it was less resistant in comparison with the commercial and exotic genotypes. Resistance in OOTP in combination with other groups found to be promising, especially with the commercial genotypes. The phenomenon of positive transgression is a sign of possible gene pooling and has a potential in practical late blight resistance breeding. The QTL found on chromosome 09 was responsible for the major phenotypic variations for both the traits and was stable across the locations. Though the effect of QTL on chromosome 08, chromosome 10 and chromosome 11

are rather small, the phenotypic data suggests that there are some other QTL affected the late blight resistance.

The trials diversity set and TGRC set showed Ph-3 is the only known resistant gene resistant under test condition and it transpires the necessity to continue the search for new late blight resistance sources. Apart from that, two accessions a.k.a. LA3707 and LA3708, were found to be very resistant and promising for future research.

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Appendix 4:

DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

   Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

   Göttingen, ...

   .....

   (Signature)

   .....

   (Name in block capitals)

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

   Göttingen,.....

   .....

   (Signature)

   .....

   (Name in block capitals)