Recent advances in breeding for bacterial wilt (*Ralstonia solanacearum*) resistance in Tomato - review

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Tomato (*Solanum lycopersicum* L.), is the second most important vegetable in terms of total production and has worldwide commercial distribution. In India, it has been grown in an area of 634 thousand hectares with the production of 12433 thousand tons. In Karnataka it is grown in 48.3 thousand ha, producing about 1580 thousand tons (Source:NHB, 2010). The productivity of India and Karnataka remains 19.6 and 32.7 t/ha respectively. In tropics and sub tropical countries the productivity and quality of tomatoes are limited by a number of constraints including biotic stresses, such as debilitating diseases and insect pests and abiotic stresses, such as high temperature, high humidity, excessive rainfall, low light intensity, and poor soil conditions.

Of the major diseases of tomato and other solanaceous crops, bacterial wilt is considered as the most serious (Kelman, 1953). Bacterial wilt of tomato (*Solanum lycopersicon*) caused by *Ralstonia solanacearum* (Smith) has provided many enigmas for scientists working on tomato and other crop species. Although it is difficult to estimate total economic losses that causes directly or indirectly by bacterial wilt, it ranks one of the most important plant disease in the entire world (Gnanamanickam, 2006). The disease is endemic in tropics, subtropics and warm humid regions of the world. It is especially devastating during the warm wet months in the tropics and subtropics and causes incalculable losses to many hosts (Yang, 1979). The yield loss due to this disease is up to 90.62 per cent (Dharmatti *et al.*, 2009). In general, losses depend on local climates, soil types, cropping practices, the choice of crop and plant cultivar, and the virulent characteristics of the *R. solanacearum* local strains.

What has happened in these years with respect to crop production ?

- ✓ Increased production
- ✓ More intensive cultivation
- ✓ More off-season production
- ✓ More disease problems
- ✓ Bacterial wilt are major problems: Foliar disease, Begamo virus
- ✓ Hybrid penetration higher than 75%

Due to intensive as well as extensive cultivation and loss of natural variability in the genotypes, the disease became a prior concern.

Ralstonia solanacearum is a soil born and has complex taxonomic properties

with a great degree of diversity at all levels, including physiological, phenotypic, genotypic and host range (Genin and Boucher, 2004). Various strategies have been developed for controlling the disease, like application of chemicals, cultural practice and biological control but none of them have proved as effective as cultivation of a resistant variety/hybrid.

Taxonomy and diversity of *R. solanacearum*

The pathogen is soil-borne and has an extremely wide host range that can infect over 300 plant species, belonging to over 30 botanical families (Hayward, 1991). Members of the family of *Solanaceae* such as potato, tomato, eggplant, chili and tobacco are the major host crops for this pathogen (Jones *et al.*, 1991). *R. solanacearum* has complex taxonomic properties with a great degree of diversity at all levels, including physiological, phenotypic, genotypic, and host range (Genin and Boucher, 2004). Traditionally the bacterium has been subdivided into six biovars on the basis of carbohydrate catabolism and five races, designated by host range (Schaad *et al*., 2001). The determination of DNA–DNA homologies of isolates of *R. solanacearum* has shown that the relatedness between isolates of this species is often less than the limit of >70% which has been considered a threshold level within a species (Castillo and Greenberg, 2007).

Recently the bacterium has been classified into four phylotypes and 23 sequeres based on phylogenetic analysis of 16S-23S ITS but still there is no general consensus about sub-classification of *R. solanacearum* species (Fegan and Prior, 2005). Therefore, sub-specific classification studies, which categorize this polymorphism, are still valuable and needed to give sufficient information for prediction in the context of epidemiology and control of the bacterial wilt disease. In most parts of South-East Asia, the bacterial wilt pathogen has been isolated and characterized unambiguously and most of strains originated from this part of the world South-East Asia are placed in phylotype IV (Horita and Tsuchiya, 2001; Melanie *et al*., 2007). But still little is known about the genetic diversity of this pathogen (Khakvar *et al.*, 2011).

Thus, the species is divided into four phylotypes corresponding to four broad genetic groups, each of them related to a geographic origin.

I. Phylotype I contains all strains belonging to biovars 3, 4 and 5, isolated primarily from Asia.
II. Phylotype II includes biovar 1 and 2 strains, and 2T (a subgroup of biovar 2 for tropical areas) isolated from America, all race 3 strains pathogenic to potato and the race 2 banana pathogen.
III. Phylotype III comprises strains belonging to biovars 1 and 2 T from Africa and surrounding islands.
IV. Phylotype IV is more heterogeneous, with biovar 1, 2 and 2T strains from Indonesia, strains isolated in Australia and Japan, and also *R. syzygii* and the blood disease bacterium (BDB).

The diverse classification schemes proposed for *R. solanacearum* reflect the great phenotypic and genotypic variation

within the species, which has led to the term "*R. solanacearum* species complex", defined as "a cluster of closely related isolates whose individual members may represent more than one species".

AVRDC also conducted research determine the variability, biochemical characteristics, and pathogenic specialization of *P. solanacearum* from 1974 to 1977. More than 100 strains were collected from various hosts and different locations in Taiwan and categorized into groups according to colony morphology, physiological characteris-tics, and virulence or pathogenic specificity to a set of tomato differentials. Strains on TTC medium (2, 3, 5 triphenyl tetrazolium chloride) were classified into two types;

Type I strains (90% of the strains) were almost all pathogenic and their colony morphology was irregular, milky, and fluidal with a red center.

Type II strains were non- or mild pathogens with smooth colony margins and even, convex surfaces with a red to orange colored center.

Carbohydrate utilization tests using Hayward's basal medium supplemented with various carbohydrates indicated that the strains belonged to biotype III (Hayward, 1964) or based on the classification of Buddenhagen and Delman(1964) all strains from tomato were race 1.

Phenotypic characteristics of the pathogen

The small rod shaped single cell with rounded ends, with an average size of 0.5 to 0.7 by 1.5 to 2.5 µm. It is a Gram-negative bacterium. The bacterium has an oxidative metabolism and is generally considered a strict aerobe. However, it slow down the growth when cells are not in direct contact with the air. *R. solanacearum* produces poly-β-hydroxybutyrate granules as cell energetic reserve.

Symptomatology of the disease

There are several external and internal symptoms characterizing the bacterial wilt disease. The most frequent external symptoms of the infected plants are sudden wilting, stunting and yellowing of the foliage. The most frequent internal symptoms are progressive discoloration of the vascular tissue, mainly the xylem, at early stages of infection and portions of the pith and cortex, as disease develops, complete necrosis occurs. Slimy viscous ooze typically appears on transverse-sectioned stems at the points corresponding to the vascular bundles. As a result, collapse and death of the plant take place because of the degradation of occluded xylem vessels and the destruction of surrounding tissues. The disease can be confirmed by doing ooze out test.

Mitigation and disease management

Crop rotation is not a viable control tactic because the bacterium is indigenous to soils and can persist indefinitely in infested fields (Chellemi *et al.*, 1994). Soil fumigation does not provide acceptable levels of season-long disease control (Enfinger *et al.*, 1979). Developing resistant cultivar is the most logical solution for suppressing bacterial wilt epidemics. Although the emphasis of bacterial wilt research is on developing resistant cultivars, a cultivar having resistance to *R. solanacerum* and good horticultural

characteristics has been a challenge from many years.

Pathogenicity determinants

Ralstonia solanacearum possesses diverse genes involved in colonization and wilting of host plants, such as those coding for lytic enzymes and EPS, hypersensitive reaction and pathogenicity (*hrp*) genes, structural genes encoding effector proteins injected by a type III secretion system (T3SS) from the bacterium into the plant cell, genes coding for factors implicated in cell adherence and others.

Hydrolytic enzymes

Phytopathogenic bacteria have often developed enzymes to hydrolyze plant cell wall components to obtain nutrients and energy, which are further involved in early stages of the infective process, favouring the entry and advance of the pathogenic agent in host tissues. *R. solanacearum* produces several plant cell wall-degrading enzymes, secreted via the type two secretion system (T2SS). These include one β-1,4-cellobiohydrolase (CbhA) and some pectinases whose activities have been identified respectively as one β-1,4-endoglucanase (Egl), one endopolygalacturonase (PehA), two exopolygalacturonases (PehB and PehC), and one pectin methyl esterase (Pme). *R. solanacearum* Egl is a 43-kDa protein that has proved to be involved in pathogenicity.Inactivation of *egl*, *pehA* or *pehB* genes revealed that each contribute to *R. solanacearum* virulence, and a deficient mutant lacking the six enzymes wilted host plants more slowly than the wild-type. Since pectin catabolism does not significantly contribute to bacterial fitness inside the plant, it seems that cellulase and pectinolytic activities are preferably required for host colonization than for bacterial nutrition. Thus, *R. solanacearum* hydrolytic enzymes are thought to be involved in pathogenicity in planta.

Exopolysaccharide

Several phytopathogenic bacterial species produce high amounts of EPSs either in pure culture or during *in planta* multiplication. Although usually related to pathogenicity, it is often difficult to know if the EPSs take active part in symptom production or if they indirectly favour infection. In *R. solanacearum*, it has been reported that all virulent wild-type strains (mucoid colonies) produce EPS, while EPS-deficient mutants (non-mucoid colonies) are avirulent. *R. solanacearum* EPS appears to be highly heterogeneous, since it has a varying composition among strains. The main virulence factor is an acidic, high molecular mass extracellular polysaccharide (EPS I), a long (>106 Da) polymer with a trimeric repeat unit of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose. EPS I is more than 90% of the total *R. solanacearum* EPS produced, and approximately 85% appears as a released, cell-free slime, whereas 15% has a cell surface-bound capsular form. In studies carried out with EPS I-deficient mutants, it was found that EPS I caused wilting in infected plants. *In planta*, EPS would probably act by occluding xylem vessels, interfering directly with normal fluid movement of the plant, or by breaking the vessels due to hydrostatic overpressure. On the other hand, EPS I might also favour stem colonization by the pathogen, since EPS I-

deficient mutants were shown to multiply more slowly, and colonized poorly the stem of infected plants. In that sense, EPS I would be contributing to minimizing or avoiding the recognition of bacterial surface structures such as pili and/or lipopolysaccharide by plant defence mechanisms. As EPS-deficient mutants can infect and multiply to some extent *in planta* without inducing wilting symptoms, EPS might take part mainly in late stages of the process, modulating disease severity rather than the infective ability of the bacterium. In *R. solanacearum*, EPS is thought to be the main factor accounting for the virulence of the pathogen.

Hrp genes

In *R. solanacearum*, the *hrp* genes control induction of both, disease development and the hypersensitive reaction (HR). Therefore, *hrp* mutants are unable to induce symptoms in susceptible host plants and a HR in resistant plants or non-hosts. HR is a plant defense mechanism preventing the spread of pathogen infection to other parts of the plant. It is associated with plant resistance and characterized by a rapid and programmed plant cell death localized in the region surrounding an infection. It can be visualized as necrotic areas in the plant tissues affected. *R. solanacearum hrp* mutants do not seem to be involved in the infection process, since most of them could be isolated from the stems of infected plants but, they showed an impaired ability to multiply *in planta*, not observed when cultured on minimum media, which may indicate a possible role of *hrp* genes in diverting certain plant metabolites from the plant to the bacteria.

The *hrp* genes are clustered on the megaplasmid, and encode components of a T3SS and effector proteins. In all *hrp* clusters, conserved genes (*hrc* genes) might be forming the core of the T3SS. T3SSs have an important role in pathogenesis, since they are thought to secrete effector proteins translocated inside host cells, and accessory proteins supporting the translocation called translocators. Effector proteins would act in the invasive stages of the infection by either inhibiting plant defenses or inducing nutrient release from the host cell. In some cases effectors can elicit HR due to recognition by specific plant resistance genes. These effectors are then referred to as avirulence (Avr) proteins, since they would be hindering pathogenicity on the host having a corresponding resistance gene. T3SS includes extracellular appendages as the Hrp pili in plant pathogens, believed to function either in the attachment to plant cells and/or as conduits for protein translocation, since they might penetrate the plant cell wall. *In vitro*, Hrp pili-deficient mutants were impaired in secretion of effectors and accessory proteins. *R. solanacearum* produces Hrp-dependent pili, in addition to the polar fimbriae which were independent on the expression of the *hrp* genes; both types of pili are located at the same pole of the bacterium. *R. solanacearum* Hrp pili are mainly composed of the HrpY protein, essential for T3 protein secretion but not for attachment to plant cells. Two proteins secreted via the T3SS, PopF1 and PopF2, were identified as translocators, with PopF1 playing a more important role in virulence and HR elicitation than PopF2. Among the effector proteins, *R. solanacearum* T3SS secretes PopA, PopB, PopC and PopP1 under control of the transcriptional regulator HrpB.

PopA produces a HR-like response when infiltrated into plant tissue at high concentration, and may allow nutrient acquisition *in planta* and/or the delivery of other effector proteins into plant cells. PopB has a nuclear localization signal which enables it to be transported to the plant cell nucleus. PopC contains sequences analogous to those of some plant resistance gene products. PopP1 acts as an *avr* determinant towards resistant plants. PopA, PopB, PopC or PopP1- deficient mutants showed normal virulence in different host plants, probably due to functional redundancy. Five candidate effector proteins were shown to be translocated into host cells by the *R. solanacearum* T3SS, and 48 novel *hrpB*-regulated genes have been identified, with half of them encoding novel classes of probable effector proteins with no counterparts in other bacterial species. It is estimated that *R. solanacearum* exports large repertoires of pathogenicity effectors through the T2SS and the T3SS.

Lipopolysaccharide (LPS) and lectins

The recognition between *R. solanacearum* and the host has long been thought to implicate an interaction between *R.solanacearum* LPS and plant lectins, so involving LPS in the pathogenicity of the bacterium. Bacterial LPS is a component of the outer membrane and has three parts: the lipid A, the oligosaccharide core and the O-specific antigen. The core structure is composed of rhamnose, glucose, heptose, and 2-ketodeoxy-octonate,whereas the O-specific antigen is a chain of repeating rhamnose, N-acetylglucosamine, and xylose in a ratio of 4:1:1. Presence or absence of the O-specific antigen differentiated respectively between smooth and rough LPSs, which were respectively negative and positive HR-inducers. However, a specific interaction between *R. solanacearum* rough LPS and a plant cell wall receptor was not enough to iniciate the HR, although many of the mutations in the LPS also affected virulence. In *R. solanacearum*, smooth LPS apparently is required to prevent agglutination by certain plant lectins. *R.solanacearum* LPS and EPS are somehow related, since a gene cluster was found to be required for the biosynthesis of both cell surface components. Two genes encoding lectins have been characterised in *R. solanacearum*, presumably with a function in adhesion to plant surfaces, which is important for *R. solanacearum* pathogenicity during the early stages of infection. In fact, it was found that these lectins bind L-fucose and interact with the plant xyloglucan polysaccharide belonging to the hemicellulose fraction of plant primary cell walls.

Directed motility to the host

In the environment, *R. solanacearum* senses specific stimuli and moves towards plants by swimming motility to find more favourable conditions. *R. solanacearum* was actively attracted by chemotaxis to diverse amino acids and organic acids, and specially to host root exudates, whereas those from a non-host were less attractive. Furthermore, the ability of the pathogen to locate and interact with the host was dependent on aerotaxis or energy taxis, already described for *R. solanacearum*. Thus, several aerotaxis-deficient mutants were impaired in either localizing on host roots or moving up an oxygen gradient. Swimming motility, chemotaxis and aerotaxis seem to have a role in the early stages of host invasion.

Resistant mechanism

- Secondary metabolism of polyphenols in resistant plants prevents the bacteria movement near to the plant system by acting as a repellent.
- Steroidal glycoalkaloid like α tomatine will be produced in higher concentration in resistant plants, compared to susceptible plants.
- Inhibitor extracts, tyloses and gums in resistant plants acts like a filters, there by preventing bacteria movement inside the plant system.

Types of resistance

1. **Based on existence**

   a. Performed b. Induced

2. **Based on type of host resistance**

   a. Immune b. Tolerance c. Resistance

3. **Based on genetic control**

   a. Oligogenic b. Polygenic

4. **Based on mechanism of resistance**

   a. Mechanical b. Biochemical

   c. Phytoalexins d. Phytotoxins

   e. Hypersensitive

5. **Based on degree and range of resistance**

   a. Horizontal and

   b. Vertical resistance

Steps in breeding for resistance

1. Source of resistance: The first requirement of any breeding procedure is to find a stable and cross compatible source of resistance. Such sources may be present in existing or old varieties, in wild forms of the same species, in closely related species or even in different genera.

2. Inheritance of resistance: It is not necessary for the plant breeder to understand exactly how resistance is inherited before a successful breeding programme can be carried out. Indeed details of the genetics of resistance are known in only a few cases. It is however, useful to know whether it is controlled by one gene (monogenically), a few (oligogenically) or many genes (polygenically) and whether cytoplasmic inheritance involved.

3. Methods of testing for resistance: In breeding for resistance to a disease, plant populations must be exposed to the disease in such a way that resistant and susceptible plants can be readily distinguished from each other. Selection for resistance to some disease can be carried out effectively in natural field epidemics.

4. The assessment of resistance: Differentiation between very resistant and very susceptible plants will be easy provided that the inoculation has been carried out correctly and that the environmental conditions are suitable for disease occurrence.

5. Selecting for resistance: Although it is relatively easy to select for resistance to disease, there may be many complicating factors. For instance, certain host plant genotypes may be highly resistance to some variants of a pathogen but very susceptible to others. It is therefore, desirable to test host genotypes against a wide range of variants of a pathogen before selection.

6. Production of resistant varieties: Breeding for resistance to this disease does not differ fundamentally from breeding for any other character.

Bacterial wilt resistant varieties/hybrids released by different institutes :

1. IIHR : Arka Abha, Arka Abhijit, Arka Alok, Arka Shreshta, Arka Ananya (ToLCV + BW), Arka Rakshak (ToLCV+BW+Early blight), Arka Samrat (ToLCV+BW+Early blight)

2. **UAS, Dharwad :** Megha
3. **KAU :** Anaga, Shakti, Mukti and Vellayani Viiji
4. **Orissa Agriculture University and Technology :** Utkal Pallavi (BT-1) , Utkal Kumari (BT-10), Utkal Deepti (BT-2) etc.

Factors affecting the host-resistance :

1. Environmental influences : Temperature, P^H , Rainfall
2. Variability among *P. solanacearum* strains: Strains and Pathogenesis

The optimum temperature for the growth and multiplication of pathogen is 32 -35^{o}C. However it can also survive from a minimum temperature of 10^{o}C to maximum of 39^{o}C.

Table : 1. Case study with reference to effect of temperature:

Cultivars	Disease reaction	Days for 50% or more plants to show wilting symptoms		
		26^0 C	30^0 C	32^0 C
KL 1	S	10 (S)	6 (S)	4 (S)
Kewalo	R	19 (MS)	7 (S)	4 (S)
VC 9	R	-- (MR)	12 (MS)	6 (S)
VC 11	R	-- (MR)	12 (MS)	5 (S)
VC 8	R	-- (R)	-- (MR)	14 (MS)
VC 48	**R**	**-- (MR)**	**-- (MR)**	**…. (MR)**

In this study, Mew and Ho (1976) reported the effect of soil temperature on resistance of tomato cultivars to bacterial wilt. They found that bacterial wilt resistance in the tomato was greatly influenced by soil temperatures, and confirmed the breakdown of resistance in some cultivars such as VC9 and VC11 as the temperature increases, because this temperature favours the multiplication of pathogen. However, resistance of one of the cultivars was not affected by soil temperature. The data thus suggest that there are two types of bacterial wilt resistance: one dependent on, and one independent of soil temperature. They are also reported that air temperatures have

less influence than soil temperatures on the development of wilt symptoms, but at high soil temperatures, the development of wilt symptoms was promoted when the air temperatures also were high.

Temperate *R. solanacearum* strain UW551 breaks the BW resistance of H7996 tomato

Ralstonia solanacearum strains GMI1000 and UW551 were both highly virulent on susceptible tomato cv. Bonny Best (fig.1). All inoculated plants were dead by 8 dpi and the strains had indistinguishable disease progress curves. In contrast, tomato breeding line H7996, a widely-used source of BW disease resistance, was quite resistant to tropical strain GMI1000; only 12% of the plants were dead by 14 dpi (Fig.1). However, H7996 was susceptible to *R. solanacearum* UW551, a typical sequevar 1 (Race 3 biovar 2) strain that causes losses in temperate zones and tropical highlands. UW551 killed about 80% of H7996 plants within 14 dpi. The virulence of strains GMI1000 and UW551 was significantly different (P,0.001) on the resistant tomato plants.

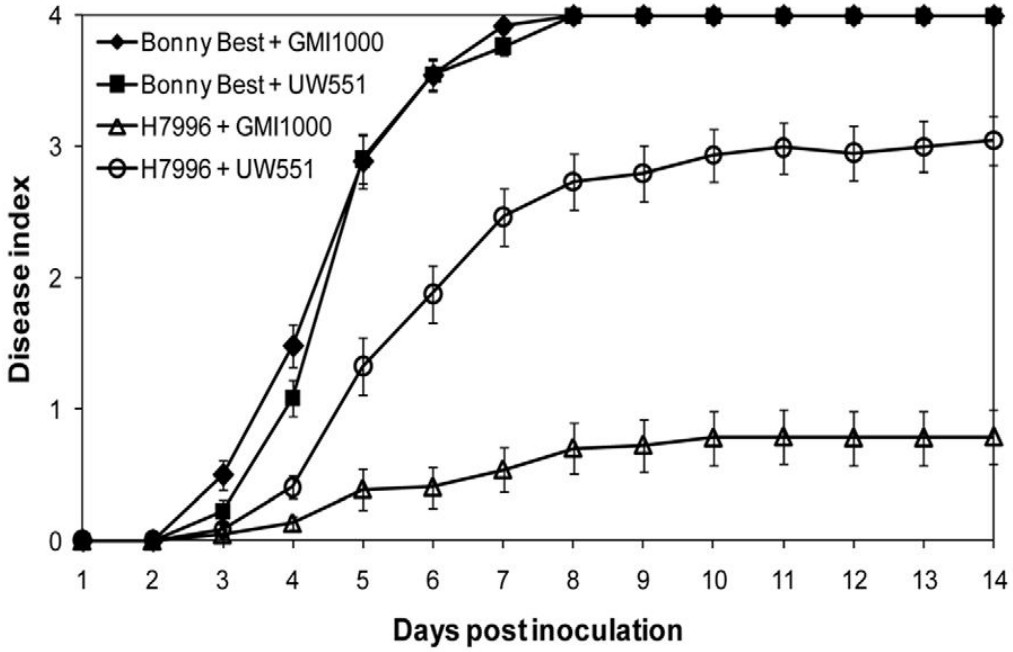


Figure 1. Virulence of *Ralstonia solanacearum* strains GMI1000 and UW551 on resistant and susceptible tomato plants

Table : 2.Case study on inoculom concentration

Inoculum concentration (cfu/ml)	Per cent plant mortality after inoculation (in days)						
	1	2	3	4	5	6	7
10	0	0	0	20	60	80	100
10^2	0	0	0	20	80	100	-
10^3	0	0	0	20	60	80	100
10^4	0	0	20	40	60	100	-
10^5	**0**	**0**	**40**	**100**	-	-	-
10^8	0	0	40	100	-	-	-
2×10^9	0	20	60	100	-	-	-
3×10^9	0	20	60	100	-	-	-
Control	0	0	0	0	0	0	0

Kishan and Chand reported effect of *Pseudomonas solanacearum* concentration on bacterial wilt of tomato in 1990. According to them, the mortality was 100 per cent within 7 days of inoculation in all the inoculum concentrations. The concentrations of $2x10^9$ and $3x10^9$ cfu/ml gave 20 per cent mortality on 2nd day and 100 per cent on 4th day. Plant mortality was only 20 per cent on 4th day in 10, 10^2 and 10^3 and on 3rd day in 104 cfu/ml but was 100 per cent on 6th (103 and 104 cfu/ml) and 7th (10 and 10^3 cfu/ml) day. The four highest concentrations (10^5 – $3x10^9$ cfu/ml) induced 40-6- per cent plant mortality on 3rd day and 100 per cent on 4th day. They concluded that 10^5 cfu/ml can be used for inducing disease in water culture.

Table:3. Case study on effect of nematodes on resistance

Deberdt *et al.* reported effect of nematode on bacterial wilt severity in tomatoes at two different temperature regimes in 1999.

Cultivar	Plant status	Unfavorable to wilt (22-27 ^{0}C) a		Favorable to wilt (27-32 ^{0}C) b	
		RS	RKN	RS	RKN
Floradel	Healthy	24	16	17	4
	Infected	2	6	0	5
	Wilted	4	8	13	21
	Mean score	2.33 ± 0.52 c	3.93 ± 0.63 d	4.47 ±0.74 b	7.27 ± 0.53 c
Caraibo	Healthy	29	30	25	16
	Infected	1	0	0	2
	Wilted	0	0	5	12
	Mean score	1.13 ± 0.13 a	1.0 ± 0.00 a	2.33 ± 0.55 a	4.47 ± 0.71 b

They arranged Floradel and Caraibo plants ramdomly in a growth chamber and subjected to two sets of environmental conditions: each with 12-h photoperiod and 70-90% RH, at fevourable (27-32^{0}C) and unfevourable (22-270C) temeratures for wilt development. They noticed wilt symptoms over a period beginning 10 days after bacterial inoculation, regardless of the treatment x cultivar combination. Disease symptoms increased greatly on susceptible Floradel between days 10 and 25 at 27-32^{0}C, compared with the resistant cultivar Caraibo at these temperatures. At 22-270C, R.solanacearum was not pathogenic on Caraibo and only weakly pathogenic on Floredel. In all treatment x temperature combinations, Floradel had the higher bacterial wilt index. At 22"- 27"C, secondary infection with RKN significantly increased the mean wilt score on Floradel. Bacterial wilt indexs were low on the resistant cultivar at these temperatures and no statistical differences were recorded following secondary infection with either species of nematode. At 27-32"CY RKN significantly increased the mean disease score on Floradel compared with infection by *R. solanacearum* alone. A similar pattern but with a lower disease incidence was observed on resistant Caraïbo.

They concluded with saying that, High temperatures reduce the expression of resistance, even in a cultivar selected for heat tolerance, such as Caraïbo. Unfortunately, genetic resistance to *R. solanacearum* is often diminished as a result of nematode infection. It has a synergistic effect. It is generally concluded that nematodes provide wounds through which the bacteria may enter and also release metabolites useful for bacterial growth.

Why there is need of breding repeatdly ?

- ✓ Instability in resistance.
- ✓ Genetic variability in the strains.
- ✓ Lines bread in 1984, generally yield about 5 to 15 t/ha, the more

advanced genotypes tend to yield above 20 t/ha, or in some cases even as high as 35 t/ha (Opena *et al.*, 1990).

- ✓ Hanson *et al.* (1996) Mean survival of AVRDC entries bred in the 1980s (59.4%) was significantly greater than mean survival of AVRDC lines bred in the 1970s (45.7%).

Expression of defence mechanisms in tomato:

Tomato plants responded to *R. solanacearum* infection by up regulating marker genes for the salicylic acid (SA) and ethylene (ET) defence pathways. Quantitative RT-PCR gene expression analysis in susceptible and resistant tomato plants infected with *R. solanacearum* revealed little or no activation of the jasmonic acid (JA) pathway marker genes Pin-2 and LoxA. However, both PR-1b and Osm, which are ET-induced, and GluA and PR-1a, which are regulated by the SA pathway, were expressed at significantly higher levels in plants with pathogen cell densities 36108 CFU/g, relative to water-inoculated controls (Fig.2).

Resistant tomato plants activated the SA and ET defense pathways more rapidly than a susceptible cultivar BW-resistant H7996 responded to large populations of both *R.solanacearum* strains by increasing expression of genes in the ET and SA signaling pathways by two to three orders of magnitude (Fig. 2). Defence genes in H7996 were noticeably induced even at lower pathogen cell densities (16107 CFU of GMI1000/gm stem and 36108 CFU of UW551/gm stem). In contrast, susceptible cv. Bonny Best had no detectable defense response to 16107 CFU/gm. This result is consistent with the general observation that disease-resistant plants have faster and stronger defence responses.

Screening methods by various inoculation Techniques

Inoculation of cotyledons through the use of wounding done by needle puncture or carborundum to introduce the pathogen; that technique is recognized to be very effective in different conditions. Stem Inoculation simple and commonly used the inoculation is achieved through the injection of bacterial suspension with a fine needle hypodermic syringe into the vascular tissue of the stem. The technique has been proven effective.

Inoculation through petioles: The technique consists to wounding the petiole at it's attachment point to the stem and applyng inoculum. The method is not always successful under dry conditions since the inoculum drop can dry before getting inside the plant.

Leaf Inoculation: The inoculum is sprayed on the needle wounded leaves or applied onto leaves directly.

Root Inoculation through infested soils: The method is an effective inoculation technique. Bacterial suspensions (50ml of $8x10^{6}$ $cfu.ml^{-1}$) of inoculum solution are used to water seedlings during transplanting. Winstead and Kelman, (1952) have used the root injury technique to inoculate tomato and tobacco plants in green house by applying bacteria suspension on cut lateral roots of 4-6 weeks old plants. Inoculation by dipping the roots in bacterial suspension: The method consisting in dipping plant root

in bacterial suspension before transplanting can be used in field and in green house. The method was explained by Klement *et al.,* (1990). Wounding induced through cutting 1-2cm of root before transplanting will increase the probability of disease occurrence. The technique is effective however the incubation time is longer (one month).

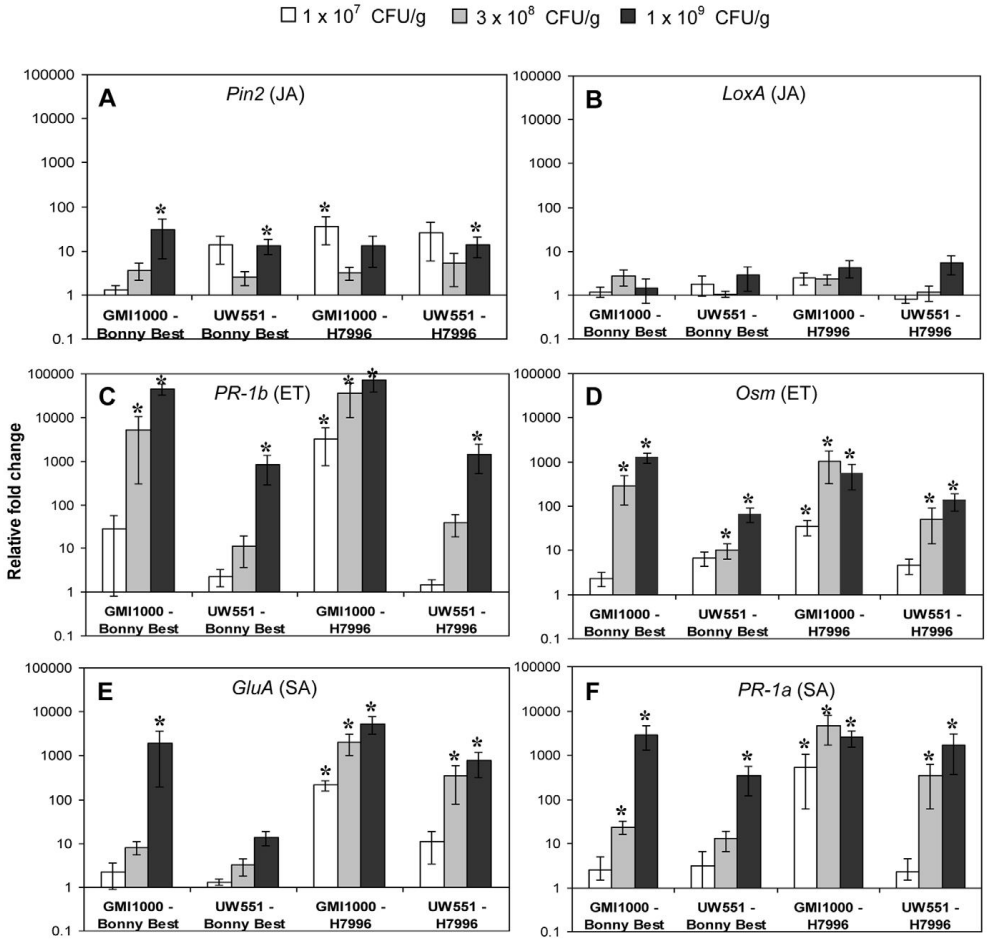


Fig. 2: Expression of tomato defense genes following soil-soak inoculation with *R. solanacearum* strains GMI1000 or UW551 in susceptible cultivar Bonny Best or horizontally resistant line H7996. Reported by Milling *et al.,* 2011.

Winstead and Kleman in 1952 found that younger plants respond faster to inoculation than oldest plant. The latent period between inoculation and symptom's appearance going over two weeks some time is generally longer than most of the known bacterial disease; the situation is certainly due to the cause of the wilt depending more to the number of the bacteria present in the xylem rather than the toxicity produced by the bacteria.

Drench inoculation

Clipping of leave

Root dip method

Table : 4. Case study on indirect screening of resistant line

Grimault *et al.* reported bacterial counts and stem colonization by *R. solanacearum* in symptomless tomato plants differing in resistance in 1996.

Cultivar	Status	Bread in	Collar [a]			Mid stem [b]		
			Density (log cfu/g)	Colonization frequency (%)		Density (log cfu/g)	Colonization frequency (%)	
Floradel	S	US	8.28	1/1	100 %	7.02	1/1	100 %
FMTT 3	R	Taiwan	7.61 ±0.16	6/6	100 %	4.78± 0.94	6/6	100 %
CLN 657	R	Taiwan	6.91 ±0.22	9/9	100 %	4.19± 1.12	2/9	22%
Caracoli	MR	Antilles	7.13 ± 0.11 [a]	32/32	100 %	4.01± 0.46	15/32	47%
PT 4165	MR	Taiwan	7.32 ±0.04 [a]	35/35	100 %	5.13 ±0.34	20/35	57%
CRA 90-30	R	Antilles	6.66 ±0.17 [a]	35/35	100 %	3.66± 0.43	16/35	45%
Calinago	R	Antilles	6.94 ±0.10 [a]	35/35	100 %	4.24± 0.47	16/35	45%
Caraibo	R	Antilles	6.90 ±0.11 [a]	35/35	100 %	4.66± 0.54	10/35	28%
CRA 66	R	Antilles	5.51 ±0.28 [b]	25/35	71%	2.35	1/35	2%
Hawai 7996	R	US	5.50 ±0.29 [b]	26/35	74%		0/35	0%

This table shows that *P.solanacerum* populations were higher at collar than at midstem region regardless of the cultivar. Population of *P.solanacerum* collected from the stems of cultivars Floradel, FMTT3 and CLN 657 were not included in the analysis of variance testy because of the lower number of symptomless plants. They

concluded with, population size of bacteria at the collar or the midstem may not provide an accurate way of classifying cultivars for wilt resistance. In contrast, the frequency of colonization of stem significantly differed among cultivars, and was correlated with the level of resistance to wilt.

Advances with respect to screening methods are

- ✓ Inoculating seeds directly to rapidly identify resistant source (AVRDC).

  Recently, a laboratory test has been developed to evaluate the feasibility of inoculating seeds to rapidly identify resistant sources (AVRDC, 1990). These tests have shown differences in certain parameters measured like vigor and wieght, and seedling length. More tests are currently evaluating different isolates and determining better ways to increase the potential use of this method for initial mass screening.
- ✓ Tissue culture (Toyoda *et al.,* 1989).

Toyoda reported that leaf explants-derived callus tissues which were resistant to toxic substances, derived from *P. solanacearum,* in the culture filtrate were selected in vitro and regenerated into plants. These plants expressed resistance to *P. solanacearum* at the early infection stage by suppressing or delaying the growth of the inoculated bacteria. Complete resistnace was obtained in self-pollinatd progeny of regenerations derived from non-selected callus tissues. These plants showed high resistance when inoculated with the virulent strain used in the experiment, and were also resistant when planted in a field infested with a different strain of the pathogen. Even though it is more economic, the standard protocol are yet to be established.

- ✓ **EPS triggered a strong oxidative burst in resistant plants** (Milling *et al.,* 2011)

To determine if the defense-associated gene expression patterns we observed in response to wild-type and EPS-deficient *R. solanacearum* cells correlated with biochemical indicators of active plant defenses, we used the fluorescent dye dihydrorhodamine123 to assess tomato stem levels of ROS, a common element of plant antimicrobial defenses. This qualitative dye revealed that infection by wild-type *R. solanacearum* UW551 triggered a strong oxidative burst in the vascular bundles of both resistant and susceptible tomato plants (Fig.3). In contrast, H7996 plants infected with 10^4 to 10^5 CFU/g of UW551DepsB accumulated noticeably less ROS than did H7996 stems carrying similar populations of wild-type *R. solanacearum* (Fig. 3). No such response was observed in cv. Bonny Best, where stems containing 10^4 to 10^5 CFU/g of UW551DepsB had ROS levels indistinguishable from those in stems infected by the wild-type strain (Fig. 3). These differences in ROS accumulation triggered by wild-type and EPS-deficient bacteria were also seen in tomato leaves, indicating that this phenomenon is not unique to stem tissue (Fig. 3).

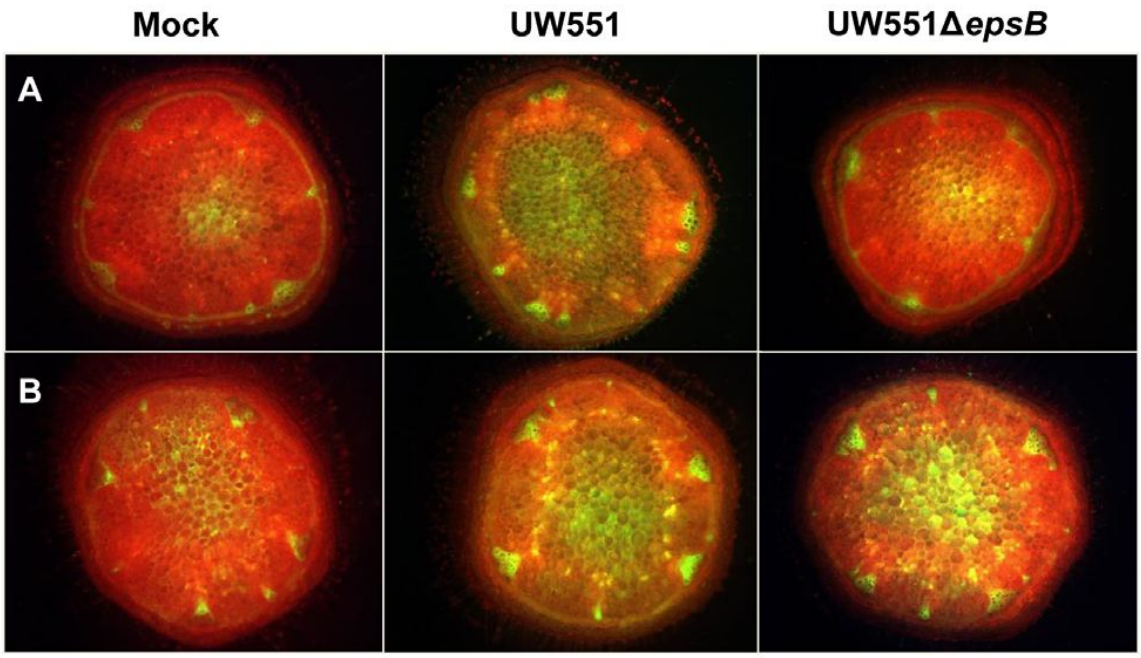


Fig. 3. Accumulation of reactive oxygen species (ROS) in tomato stem tissue

Sources of resistance :

1. *Lycopersicon pimpinellifolium* : polygenic in nature (recessive genes)
2. *Lycopersicon esculentum* var. cerasiforme, : a small-fruited primitive tomato, Presence of epistasis.

L. cerasiforme ***L. pimpinellifolium*** ***L. peruvianum***

In tomato, problem associated with the utilization of these resistance sources for bacterial wilt resistance breeding is that presence of linkage drag between small fruited character and bacterial wilt resistance. This linkage drag can be broken down by marker assisted background selection in advanced generations.

Genetics of disease resistance : The first study on genetics of disease resistance was that by Biffen in 1905. In majority disease resistance studies reported presence of oligogenic or polygenic inheritance.

Gene-for-gene relationship

The gene-for-gene relationship between a host and its pathogen was postulated by Flor in 1956. It has been found that for every resistance gene present in the host, the pathogen has a gene for virulence. Susceptible reaction would result only when the pathogen is able to match all the resistance genes present in the host with appropriate virulence genes. If one or more resistance genes are not matched by the pathogen with the appropriate virulence genes, resistance reaction is the result. In most of the pathogens, virulence is recessive to a virulence.

Opena *et al*. (1994), Mohamed *et al.* (1997) and Dharmatti *et al.* (2009) reported that genetic combination between the two resistance sources can lead to higher levels of resistance. The proportion of wilt resistant F1's was generally higher when both the parents were resistant when compared to resistant x susceptible crosses.

Acosta (1964) reported that high degree of bacterial wilt resistance is difficult to incorporate in a line, selections should be continuously indexed for resistance with utilization of heterosis (Susceptible x Resistant).

Table 5: Some of studies are listed below

Resistance governed by	Reported by	Year of report
Multiple recessive genes	Villareal and Sen-Husiung Lai	1978
Two independent genes	Tikoo *et al.*	1983
Polygenically.	Ferrer	1984
Oligogenes	Danesh et al.	1994
Duplicate form of epistasis.	Musa *et al*	1997
Oligogenic or polygenic	Oliveira *et al.*	1999
Non-additive gene & presence of epistasis	Venkataramreddy	2001
Polygenic	Wang-Jaw Fen *et al.*	2002
Additive, A x A interaction	Sharma *et al.*	2005
Single recessive gene	Thakur *et al.*	2004
Additive, dominance & AxD interaction	Sharma and Verma	2004

Table 6: HETEROSIS BREEDING: as reported by different workers

Positive Heterosis	Gururaj Kulkarni	2005
Positive Heterosis	Janaki *et al.*	2006
Negative Heterosis	Dharmatti *et al.*	2006
Positive Heterosis	Sadhankumar *et al.*	2007
Dominance, A x D, D x D	Jaiprakashnarayan e*t al.*	2007

Tomato Breeding for Bacterial Wilt Resistance –Issues

- ✓ No immunity
- ✓ Ubiquitous Pathogen –many races, strains etc
- ✓ Profound Environmental Effects –temperature, moisture, soil types, interactions
- ✓ No Reliable, Repeatable Seedling Test
- ✓ Limited Genetic Information
- ✓ Association of Resistance With Small Fruit
- ✓ Flavor??

Table: 5. Case study on number of backcrosses to recover adequate resistance

Opena and co-workers reported number of backcrosses to recover adequate resistance in 1994.

Cross	Generation	Mean survival rate (%)	Standard deviation	Selectable progenies (%)
CLN 690	BC_1	55.4	16.9	25
	BC_2	77.5	6.5	30
	BC_3	72.3	7.3	25
CLN 735	BC_1	71.4	11.3	15
	BC_2	65.8	11.3	20
	BC_3	70.8	13.4	25

Selection limit theoretically set at minimum of (m+1 s) or higher for purposes of comparison, corresponding to minimum of 85% and 91% survival rate for CLN 690 and CLN 735, respectively.

If it is assumed that only progenies whose BW reading are equal to or greater than m+1 s are to be selected, a selectable fraction of 30 % is already attainable in CLN 690 by BC2 generation. In contrast, there is a steady increase in selectable proportions with progressive backcrossing in CLN735. In the BC2, this fraction is already 20% of the population, a figure may be considered already workable in the breeding program. Finally they concluded that two backcrosses may be indeed be sufficient to recover good levels of BW resistance in backcross programme.

Single seed descent from elite selections (SSDES)- A selection method to improve resistance to bacterial wilt in tomato

Rajan and Peter,in the year 1987, evaluated various selection methods for bacterial wilt resistance breeding and they reported that, Of the four methods of selection, *Viz*, mass, pure line, single seed descent from elite selections (SSDES) and bulk, employed in tomato improvement, the SSDES method was found superior in breeding for bacterial wilt resistance. The level of resistance to bacterial wilt increased from 77.87 to 90.14% under SSDES method.

Among various methods of selection single seed descent method found superior for improvement of resistance among progenies after third generation, compared to other methods and this is attributed mainly to maintenance of large population, so in terms maintenance of large variation and rapid advancement of generation.

Table 6. Evaluation for resistance to bacterial wilt in progenies developed through three methods of selection

Method of selection	Generation	Total parents	Plants wilted			
			Juvenile stage	Adult stage	Total	%
Mass selection	**I**	**750**	**27**	**66**	**93**	**12.49**
	II	750	15	100	115	15.33
	III	750	20	62	82	10.93
Pure line selection	I	750	38	79	117	15.60
	II	750	3	103	106	14.13
	III	750	31	60	91	12.31
Single seed descent	I	750	46	93	139	18.53
	II	750	16	110	126	16.80
	III	**750**	**14**	**60**	**74**	**9.85**
Bulk	I	100	6	20	26	26.00
	II	100	12	14	26	26.00
	III	100	2	14	16	16.00
Base population		2377	171	286	457	22.13

Recent advances in breeding are

1. Marker assisted breeding
2. Transformation studies
3. Mutation breeding
4. Somatic hybrids

1. **Marker assisted selection**

Marker assisted selection (MAS) is indirect selection process where a trait of interest is selected, not based on the trait itself, but on a marker linked to it. For example if MAS is being used to select individuals with a disease, the level of disease is not quantified but rather a marker allele which is linked with disease is used to determine disease presence. The assumption is that linked allele associates with the gene and/or quantitative trait locus (QTL) of interest. MAS can be useful for traits that are difficult to measure, exhibit low heritability, and/or are expressed late in development.

Quantitative traits refer to phenotypes (characteristics) that vary in degree and can be attributed to polygenic effects, i.e., product of two or more genes, and their environment. Quantitative trait loci (QTLs) are stretches of DNA containing or linked to the genes that underlie a quantitative trait. Mapping regions of the genome that contain genes involved in specifying a quantitative trait is done using molecular tags such as AFLP or, more commonly SNPs . This is an early step in identifying and sequencing the actual genes underlying trait variation.

In plants QTL mapping is generally achieved using bi-parental cross populations; a cross between two parents which have a contrasting phenotype for the trait of interest are developed. Commonly used populations are recombinant inbred lines (RILs), doubled haploids (DH), back cross and F_2. Linkage between the phenotype and markers which have already been mapped is tested in these populations in order to determine the position of the QTL. Such techniques are based on linkage and are therefore referred to as "linkage mapping".

The gene of interest is directly related with production of protein(s) that produce certain phenotypes whereas markers should not influence the trait of interest but are genetically linked (and so go together during segregation of gametes due to the concomitant reduction in homologous recombination between the marker and gene of interest). In many traits, genes are discovered and can be directly assayed for their presence with a high level of confidence. However, if a gene is not isolated, markers help is taken to tag a gene of interest. In such case there may be some inaccurate (even false) positive results due to recombination between the marker of interest and gene (or QTL). A perfect marker would elicit no false positive results.

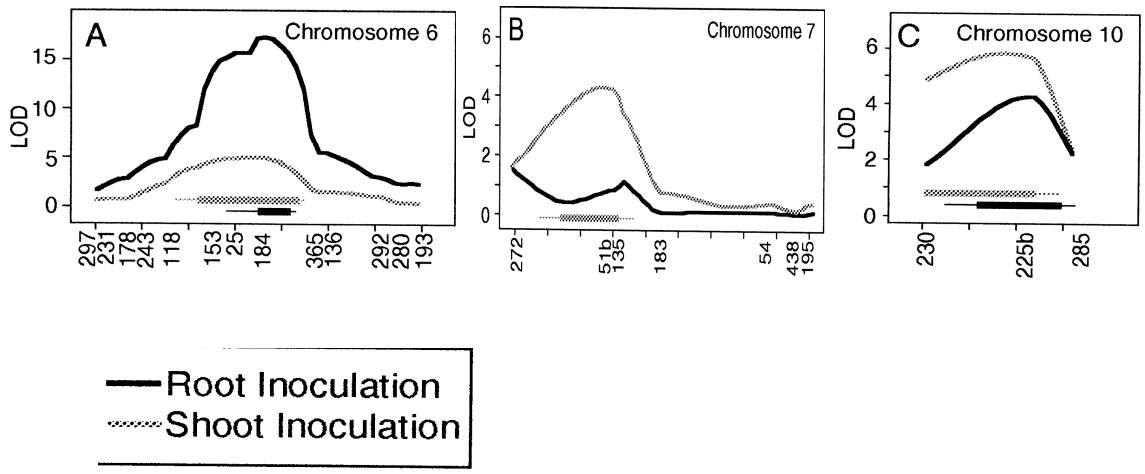
Generally the first step is to map the gene or quantitative trait locus (QTL) of interest first by using different techniques and then use this information for marker assisted selection. Generally, the markers to be used should be close to gene of interest (<5 recombination unit or cM) in order to ensure that only minor fraction of the selected individuals will be recombinants. Generally, not only a single marker but rather two markers are used in order to reduce the chances of an error due to homologous recombination. For example, if two flanking markers are used at same time with an interval between them of approximately 20cM, there is higher probability (99%) for recovery of the target gene.

Genetic dissection of oligogenic resistance to bacterial wilt in tomato

To study resistance to bacterial wilt (caused by *Pseudomonas solanacearum*) in tomato, Danesh *et al.* (1994) analyzed 71 F_2 individuals from a cross between a resistant and a susceptible parent with 79 DNA markers. F2 plants were inoculated by two methods: bacteria were injected into shoots of cuttings or poured into soil surrounding wounded roots. Disease responses were scored on a scale of 0 to 5. Statistical comparisons between DNA marker genotypes and disease phenotypes identified three genomic regions correlated with resistance. In plants inoculated through roots, genomic regions on chromosomes 6 and 10 were correlated with resistance. In plants inoculated through shoots, a region on chromosome 7 was significant, as were the regions on chromosomes 6 and 10. The relative impact of resistance loci on disease response differed between shoot and root inoculations. To confirm the existence of a partial resistance gene on chromosome 6, an F2 individual homozygous for the resistant parent's alleles on chromosomes 7 and 10, but heterozygous for markers on chromosome 6, was selfed. Analysis of the F_3 progeny confirmed that a partial resistance locus was located on chromosome 6, very close to CT184. The presence of a partial resistance locus on chromosome 10 was similarly confirmed by analysis of progeny of another F_2 plant chosen on the basis of its marker phenotype.

Table: 7. Regions of the genome associated with bacterial wilt disease reaction in the F_2 generation

Chrom-osome	Interval	Root inoculation			Shoot inoculation		
		LOD	Variation (%)	d/a	LOD	Variation (%)	d/a
6	CT 184_TG 365	17.29	77.3	0.82	4.99	30.2	1.19
7	TG51b_TG 135	--	--	--	4.27	24.4	-1.61
10	TG 230_TG 285	4.35	24.6	0.26	5.91	38.2	0.02



Inference: in root inoculation sequence present between markers CT 184 - TG 365 on chromosome 6 explained highest amount of variance (77.3 %) for bacterial wilt infestation with highest Log of Odds value. The Log value was 17.29 indicating probability of presence of the QTL for bacterial wilt resistance between these markers was $10^{17.29}$. Similarly for shoot inoculation sequence present between markers TG 230 - TG 285 on chromosome 10 explained highest amount of variance (38.2 %) for bacterial wilt infestation with highest Log of Odds value (4.35).

Table 8. Dissection of BW QTL

Strain	Race	Resistant source	QTL location on chromosome							Reference
			2	3	4	6	8	10	12	
RS145	ND	L285	+					+		Dara-Caros (1998)
UW364	ND	L285				+		+		Danesh *et al.* (1994)
Pss4	1	H7996				+	+		+	Wang *et al.* (2000)
T 519	1	H7996				+			+	Carmeille *et al.* (2006)
GMI 8217	3	H7996		+	+	+	+			Thoquet *et al.* (1996)
JT 516	3	H7996		+	+	+	+			Carmeille *et al.* (2006)

Inference: Most of the research workers reported that presence of QTL with resistance to bacterial wilt on chromosome six, hence this chromosome serves as a source of resistance for bacterial wilt resistance breeding.

Association mapping

Association mapping, also known as "linkage disequilibrium mapping", is a method of mapping quantitative trait loci (QTLs) that takes advantage of historic linkage disequilibrium to link phenotypes

(observable characteristics) to genotypes (the genetic constitution of organisms). Association mapping is based on the idea that traits that have entered a population only recently will still be linked to the surrounding genetic sequence of the original evolutionary ancestor, or in other words, will more often be found within a given haplotype, than outside of it. Association mapping thus asks if a particular genetic marker (most often a SNP) is more common in a particular phenotype than you would expect by chance. It is most often performed by scanning the entire genome for significant associations between a panel of SNPs (which, in many cases are spotted onto glass slides to create "SNP chips") and a particular phenotype. These associations must then be independently verified in order to show that they either a. contribute to the trait of interest directly, or b. are linked to/ in linkage disequilibrium with a quantitative trait locus (QTL) that contributes to the trait of interest. The advantage of association mapping is that it can map quantitative traits with high resolution in a way that is statistically very powerful. Association mapping, however, also requires extensive knowledge of SNPs within the genome of the organism of interest, and is therefore difficult to perform in species that have not been well studied or do not have well-annotated genomes.

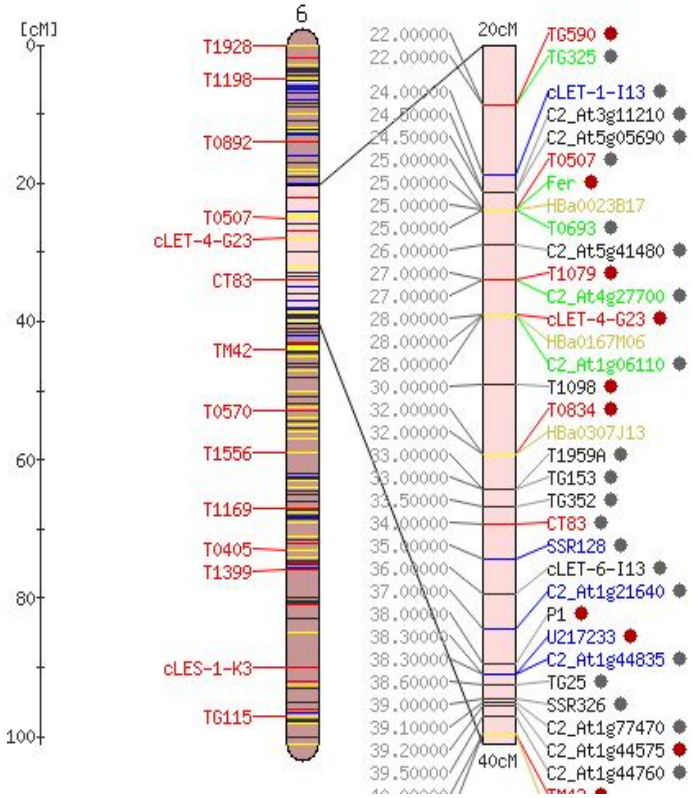
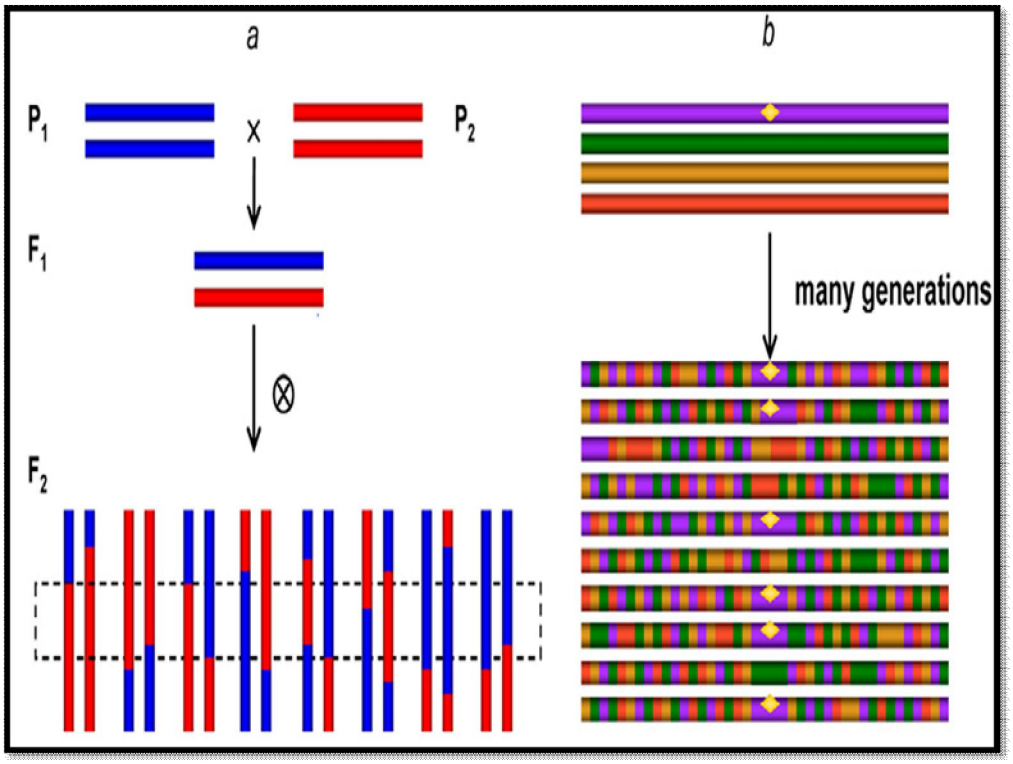


Fig 4. Candidate gene of QTL 6

Figure showing a candidate gene responsible for resistance on a chromosome six. Compare to random gene, candidate gene is one whose function is known.

QTL mapping Association mapping



Inference: Compare to QTL mapping, in association mapping populations like, lines or old cultivars are going to be used where pedigree is don't known. Since presence of large number of recombinations, high resolution map can be obtain in a small population. In order to get high resolution in QTL analysis, large mapping population required. Association mapping helps to study more number of alleles at a time where in QTL mapping only two alleles can be studied.

Advantages of association mapping:

- Much higher mapping resolution
- Greater allele number
- Broader reference population
- Do not required bi-parental mapping population

WRT Tomato

- Novel QTLs found
  - – QTL1 (SSR134): close to *Cf-9*
  - – QTL9 (SSR19): close to *Tm2*
  - – Need to be verified
- Site specific markers can be explored and used for MAS

Table 9. A comparison of association mapping and QTL mapping

Attribute	QTL mapping	Association mapping
Detection goal	Quantitative trait locus	Quantitative trait nucleotide
Experimental populations for detection	Defined pedigree,i.e., BC, F2, RI	Linkage disequilibrium experiments: unrelated individual
Marker discovery costs	Moderate	Moderate for few traits, high for many traits
Extent of inference	Pedigree specific	Species or subspecies wide

Transgenic studies : Hongbo Zhang *et al*. in 2004 reported tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum.*

Whenever plants are under stress, they produces large number of hormones by various pathway, like ethylene pathway which gives resistance to plants for this stresses. Ethylene responsive factors (ERFs) are important in regulating plant pathogen resistance, abiotic stress tolerance and plant development. Recent studies have greatly enlarged the ERF protein family and revealed more important roles of ERFs in plants. Here, Hongbo Zhang and his coworkers reported that, tomato ERF protein TSRF1, which is transcriptionally up-regulated by ethylene, salicylic acid, or *Ralstonia solanacearum* strain BJ1057 infection. Biochemical analysis indicates that TSRF1 specifically interacts in vitro with the GCC box, an element present in the promoters of many pathogenesis-related (PR) genes. Further investigation evidences that TSRF1 activates in vivo the expression of reporter b-glucuronidase gene controlled by GCC box. More importantly, overexpressing TSRF1 in tobacco and tomato constitutively activates the expression of PR genes, and subsequently enhancing transgenic plant resistance to the bacterial wilt caused by *Ralstonia solanacearum* strain BJ1057. Therefore our investigation not only extends the functions of ERF proteins in plant resistance to *R. solanacearum*, but also provides further clues to understanding the mechanism of host regulatory proteins in response to the infection of pathogens.

Zubeda *et al.,* 2010 used Agrobacterium strain EHA 101 containing a binary vector pTCL5, having hygromycin and beta-glucoronidase (GUS) gene in addition to Xa21 gene for transformation studies..

Mutation breeding (Herlihy *et al.,* 2005):

The mutants were assessed over a 5 week period after which time 21 plants were found to have an improved/partial resistance ranging from 10-80% over the control inoculated planted. These initial results indicate the role mutation breeding can play in creating genetic variation within tomato and also the value of an in vitro

screening step in assessing the mutant population. Somatic hybridization has been exploited in potato.

CONCLUSION

Resistance for bacterial wilt is greatly affected by environmental factors and the race and strain diversity of the pathogen, which makes it is necessary to search a horizontal source of resistance among local genotypes. To change the present scenario of bacterial wilt loss and in order to develop a bacterial wilt resistant varieties/hybrids, in a comparatively short span of time, marker assisted selection using resistance tightly linked markers, transgenics and somatic hybridization need to be exploited.

Future line of work :

1. The breeding lines with various combinations of genes could be developed and tested against arrays of bacterial pathogens and races to identify epistatic relationship that provide resistance to pathogens in any given production region.
2. Biovar 2 and 3 are also infected tomatoes in various regions but till now no high level resistance has been identified.
3. To test the *Solanum lycopersicoides* for resistance.
4. Developed markers can be used to pyramid identified QTLs into new cultivars.

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