Zehra EKİNCİ

DEVELOPMENT OF EVAGREEN REAL-TIME PCR ASSAY FOR THE MOLECULAR IDENTIFICATION OF BROWN ROT DISEASE CAUSING AGENT *R. SOLANACEARUM*

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SAMPLE SPINE

DEVELOPMENT OF EVAGREEN REAL-TIME PCR ASSAY FOR THE MOLECULAR IDENTIFICATION OF POTATO BROWN ROT DISEASE CAUSING AGENT *R. SOLANACEARUM*

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the Graduate School of Sciences and Engineering

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APPROVAL PAGE

This is to certify that I have read this thesis written by Zehra EKİNCİ and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics and Bioengineering.

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December 2014

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M.S. Thesis – Genetics and Bioengineering
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Thesis Supervisor: Assoc. Prof. Mustafa Fatih ABASIYANIK

ABSTRACT

Ralstonia solanacearum is a soil-borne and water-borne gram-negative bacterium which causes bacterial wilt disease on more than 200 plant species from 50 botanical families including ornamental plants and essential crops such as potato, tomato, eggplant, pepper, tobacco and banana. Bacterial wilt is considered one of the most destructive bacterial plant diseases due to its severe aggressiveness, worldwide geographic distribution, and unusual wide host range. *R. solanacearum* has been listed as a quarantine organism in Europe (OEPP/EPPO, 2004) and a Select Agent in the USA according to *the Agricultural Bioterrorism Protection Act of 2002*. Especially the strain phylotype IIB, sequevar 1 (historically known as; race 3 biovar 2), mainly infects potato, can survive at cooler climates and is considered as a serious threat to potato industry. Bacterial wilt of potato causes approximately $950 million losses in each year worldwide**.** To date, there have been several confirmed outbreaks of brown rot disease caused by *R. solanacearum* PIIB-1 in Turkey. We have developed a rapid, reliable and cost effective EvaGreen-based quantitative real-time PCR assay for *R. solanacearum*. This technique enables early and accurate detection of the bacteria that will be useful to prevent the disease dissemination by eliminating introduction of the quarantine pathogen into the country.

Keywords: *R. solanacearum*, EvaGreen dye, Real-time PCR, Quarantine.

PATATESTE KAHVERENGI ÇÜRÜKLÜK HASTALIĞINA SEBEP OLAN *R. SOLANACEARUM*'UN MOLEKÜLER TANISININ EVAGREEN GERÇEK-ZAMANLI PZR YÖNTEMİYLE GELİŞTİRİLMESİ

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ÖZ

Ralstonia solanacearum, bitkilerde bakteriyel solgunluk olarak bilinen hastalığa yol açan toprakta ve suda yaşayan bir bakteri türüdür. Yaklaşık 50 botanik aileye mensup 200 bitki türünü etkilemektedir. Oldukça geniş yelpazedeki konukçular arasında süs bitkilerinden tarımda önemli olan ürünlere (patates, domates, patlıcan, tütün ve muz gibi) kadar birçok bitki türü bulunmaktadır. Bakteriyel solgunluk agresifliği, geniş coğrafi dağılımı ve alışmışın dışında geniş konukçu aralığından dolayı en yıkıcı bitki hastalıkları arasında yer almaktadır. *R. solanacearum* birçok Avrupa ülkesinde karantina etmeni olarak listeye dahil edilmiştir (OEPP / EPPO, 2004). Amerika'da da 2002 biyoterörizm yasasına göre seçkin ajan sayılmıştır. Özellikle, soğuk iklimlerde dahi yaşamını sürdürebilen ve patateste kahverengi çürüklük hastalığına neden olan *R. solanacearum* PIIB-1(Şilotip IIB, sekvar 1 [eski adıyla; ırk 3 biyovar 2]) suşu, patates endüstrisi için büyük bir tehdit olarak görülmektedir. Sadece patatesteki kahverengi çürüklük hastalığı, dünya genelinde yılda yaklaşık 950 milyon dolar zarara yol açmaktadır. Şimdiye kadar, kahverengi çürüklük hastalığı ülkemizin farklı bölgelerinde de görülmüştür. Bu projede hızlı ve ekonomik teknik olan EvaGreen gerçek-zamanlı PZR yöntemi geliştirilmiştir. Bu tekniğin *R. solanacearum*'u erken ve kesin teşhis ederek, patojenin ülkeye girişini ve yayılımını önleyecek tedbirlerin alınmasında yardımcı olabileceği düşünülmektedir.

Anahtar Kelimeler: *R. solanacearum,* EvaGreen boya, Gerçek-zamanlı PZR, Karantina etmeni.

To my family

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SYMBOL/ABBREVIATION

BW	Bacterial wilt
C_T	Cycle threshold
ELISA	Enzyme-linked immunosorbent assay
EPPO	The European plant protection organization
EPS I	Extracellular polysaccharide I
EU	European Union
FAO	The food and agriculture organization of the United Nations
FISH	Flourescent *in situ* hybridization
HRP	Hypersensitive reaction and pathogenicity
IF	Immunofluorescence
ITS	Internal transcribed spacer
LAMP	Loop mediated isothermal amplification
MLSA	Multilocus sequence analysis
PC	Phenotypic conversion
PCR	Poymerase chain reaction
PIIB-1	Phylotype IIB sequevar 1
qPCR	Quantitative polymerase chain reaction
RSSC	*R. solanacearum* species complex
rRNA	Ribosomal RNA
SMSA	Semiselective medium of South Africa
Tm	Melting temperature
T3SS	Type III secretion system
UV	Ultraviolet
VBNC	Viable but non-culturable
YDC	Yeast extract-dextrose-calcium carbonate agar

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Potato (*Solanum tuberosum L.*) is a number one non-grain food product and the fourth most significant food crop in the world subsequent to wheat, maize, and rice. Approximately 376 million tons fresh tubers are produced from 19,3 million hectares with a yield of 1946.7 kg/da. In 2012, Turkey was ranked as 12th potato producing country in the world [1].

Table 1.1 Potato production in Turkey (FAO).

	Planted area (ha)	**Tonnes**	**Yield (kg/da)**
2010	140.663	4.548.085	3.233
2011	143.000	4.613.071	3.226
2012	172.090	4.795.122	2.786
2013	125.031	3.948.000	3.157

However, the potential production would be much higher since nearly a quarter of the yield is reduced by diseases. Beside the abiotic stresses such as poor soil conditions, low light intensity, drought, excessive rainfall, high temperature and high humidity, more than 40 pests including insects, nematodes, viruses, bacteria, and fungi have potential to cause diseases on potato crop [2].

Moreover, *R. solanacearum* is one of the most destructive plant pathogens which causes bacterial wilt (BW) disease on more than 200 plant species from 50 botanical families [3]. Especially the strain phylotype IIB, sequevar 1 (formerly known as race 3 biovar 2), mainly attacks on potato crop and other solanaceous crops (including tomato [*Solanum lycopersicum*] and eggplant [*Solanum melongena*]), significantly decreases the productivity and quality of the plant.

The specific name of the disease on potato crop is "potato brown rot". In addition to that, tomato (*S. lycopersicum*) and eggplant (*S. melongena*) are also important crops for Turkey, listed as fourth and fifth respectively in the annual list of the world's top 50 crop producing countries [1]. The global damage is estimated to be over $950 million per annum and at least 1.50 million hectares of potato crop are affected in about 80 countries [4].

R. solanacearum is difficult to control, and preventive measures are necessary to avoid the spread of the disease through different ways of contamination such propagative plant material, irrigation water, soil, etc. Therefore, those precautions minimize the loss the pathogen causes each year. In this situation, fast and precise methods for detection and analysis of the quarantine pathogen are necessary to apply treatments, carry out agronomic measures or eradication practices. Highly specific, sensitive and reliable detection methods are required for the prevention measures since the phytopathogen can be latent in asymptomatic infections, and/or widely dispersed and present in low numbers, and/or in some particular physiological states in a propagative plant material and in other reservoirs [5, 6].

In recent years, many protocols based on molecular approaches are now routinely performed worldwide. Particularly, real-time PCR system, enables to detect and monitor the target organism simultaneously, has many advantages over other conventional molecular techniques by rendering high throughput, faster and more precise detection. Chemical detection systems are important when carrying out a real-time PCR assay. EvaGreen dye is one of the fluorescent reporter molecules used in this work. In this thesis project, we designed EvaGreen dye based real-time PCR assay for the specific detection of potato brown rot causing agent *R. solanacearum.*

1.2 LITERATURE REVIEW OF RALSTONIA SOLANACEARUM

R. solanacearum is a causal organism of the bacterial wilt disease. It is a gram-negative rod-shaped, non-spore-forming bacterium with an average size of 0.5 to 0.7 by 1.5 to 2.5 μm. It has one to several polar flagella which provide swimming motility. The bacterium has an oxidative metabolism and is generally defined as a strict aerobe. However, under some circumstances, a limited slow growth has been observed on cells that are not directly contact with the air [7]. *R. solanacearum* generates Poly-β-hydroxybutyrate granules as energetic reserves of the cell [6, 8].

The optimal growth temperature for *R. solanacearum* strains living in tropical areas is 35°C, whereas for the strains present at higher regions in the tropical and in subtropical and moderate climates, the optimal growth temperature is 27°C; no bacterial growth has been reported at 40°C or 4°C. So, the approximate minimal and maximal growth temperature values are 8-10°C and 37-39°C correspondingly. Concerning pH requirements, *R. solanacearum* grows in alkaline conditions and is inhibited in acid media. 1% NaCl liquid media does not prevent the bacteria to grow but, little or no growth has been observed in 2% NaCl [7, 9].

Typically, there are two morphological types of *R. solanacearum* colonies on agar plate: fluidal (mucoid) and afluidal (non-mucoid) [7, 9]. The mucoid substance is produced by the accumulation of extracellular polysaccharides (EPS) which gives an irregular shape on the surfaces of colonies, often with characteristic whorls in the centre. All *R. solanacearum* colonies are non fluorescent, but they can produce a diffusible brown pigment on some media [10].

Under certain circumstances, a spontaneous change from fluidal to afluidal morphology is observed in *R. solanacearum* colonies which is associated with a great reduction in disease-inducing capacity of the bacterial cells. This phenomenon occurs in most *R. solanacearum* strains and is defined as “Phenotypic Conversion (PC)” [11, 12]. PC-type variants can be easily seen in a prolonged bacterial culture on agar plates. It has been also reported that the organism undergoes PC in a non-aerated liquid medium with glucose and an organic source of nitrogen [13, 14].

1.2.1 The Current Taxonomic Classification

The bacterium which causes BW in potato, tomato and eggplant was firstly described by E.F. Smith in 1896 [12]. The pathogen has been described under the following names: *Bacillus solanacearum, Burkholderia solanacearum, Pseudomonas solanacearum, Ralstonia solanacearum* (see Table 1.2) [15].

Table 1.2 The present taxonomic hierarchy of *R. solanacearum*.

Ralstonia solanacearum	
Domain	Bacteria
Phylum	Proteobacteria
Class	Betaproteobacteria
Order	Burkholderiales
Family	Burkholderiaceae
Genus	Ralstonia
Species	Solanacearum

R. solanacearum strains significantly vary within the group in terms of genetic characteristics, host range, physiological, and serological levels. Due to that, they are considered as "species complex". A species complex is defined as a heterogeneous group of linked but genetically divergent strains. The sequence similarity between *R. solanacearum* genomes is usually below the 70% threshold level, common within bacterial species, has been shown by DNA-DNA hybridization studies [16]. The term "species complex" was first used by Gillings and Fahy [17] to explain the high genetic variation between isolates, and the concept of the *R. solanacearum* species complex (RSSC) was extended by Taghavi et al. [18] who added two closely associated species from Indonesia, *Ralstonia syzygii* (a pathogen of clove trees) and the causal organism of blood disease of banana, known as the BDB. Significant pathogenic variability has been observed in host range and aggressiveness between RSSC strains

attributed to their high competence for genetic exchange in planta [19]. However, the genetic source for this difference is unknown.

Some classification systems have been suggested to explain this intra-specific variability. For the past four decades, race and biovar systems have been commonly used to distinguish *R. solanacearum* strains. Traditionally, *R. solanacearum* strains were subdivided into five races based on variations in host range (see Table 1.3) [20].

Table 1.3 Races of *R. solanacearum* [6, 16].

Strains	Host
Race 1	tobacco, several other solanaceous crops in other plant families
Race 2	musaceous species including *Heliconia spp*. and triploid banana
Race 3	mostly attack potato
Race 4	mainly virulent on ginger
Race 5	mulberry tree

R. solanacearum strains were subdivided into five biovars based on different capabilities to utilize and oxidize several disaccharides such cellobiose, lactose, and maltose; and hexose achohols such dulcitol, mannitol, and sorbitol (see Table 1.4) [21].

Table 1.4 Biovars of *R. solanacearum* [6, 16].

	Disaccharides			Hexose Alcohols		
	Cellobiose	**Lactose**	**Maltose**	**Dulcitol**	**Mannitol**	**Sorbitol**
Biovar 1	-	-	-	-	-	-
Biovar 2	+	+	+	-	-	-
Biovar 3	+	+	+	+	+	+
Biovar 4	-	-	-	+	+	+
Biovar 5	+	+	+	-	+	-

Later, the biovar 2 was further subdivided into two groups: biovar 2-T (also called as biovar N2) and biovar 2-A (the original biovar 2 strain). The new group of *R. solanacearum* isolates (biovar 2-T) from the Amazon basin are able to use ribose and trehalose [22]. Although there is no link between biovars and races in general, it has been examined that biovar 2-A almost always matches up to race 3; and biovar 5 linked to race 5. However, this old classification system is poorly defined and is regarded as unsatisfactory and not taxonomically useful. Since there is a great variation in some groups (such race 1) and the tests are insufficient to determine the race of an isolate.

Advancements in molecular biology tools allow scientists to describe the intra-subspecific variation and relationships between species at different levels. A hierarchical classification for *R. solanacearum* was proposed by Fegan and Prior [16] based on 16S–23S ITS region and endoglucanase gene analysis. Four phylotypes were determined which belong to beta subgroup of proteobacteria. The phylotypes indicate the geographical origins and hereditary relations of the strains based on the analysis of 16S–23S ITS region. Phylotype I strain arose from Asia (Asiaticum) consists of all strains belonging to biovar 3,4,5; phylotype II strain arose from Americas (Americanum) contains strains belonging to 1,2 and 2T, phylotype III strain arose from Africa (Africanum) and surrounding islands includes biovar 1,2 and phylotype IV arose from Indonesia (Indonesian) includes strains biovar 1,2 and 2T [17, 32]. Phylotypes are subdivided into sequevars based on nucleotide variation (less than 1%) in the endoglucanase (egl) locus [23, 24]. According to this classification system, each strains has a specific phylotype and sequevar. RSSC has been recently examined by a multilocus sequence analysis (MLSA) which redefined and subgrouped phylotypes into five lineages. Namely, there are 5 phylotypes (I, IIA, IIB, III, IV), and eight clades grouped as followings: phylotype I and III associated with clades 1 and 6 respectively, phylotype IIA includes clades 2 and 3, phylotype IIB includes clades 4 and 5, phylotype IV includes clade 7, 8 and 9. Each clade contains one or more *egl*-based sequevars (see Table 1.5) [25].

Table 1.5 Taxonomical Classification of *R. solanacearum* [16, 25, 26]. Same colored clades and sequevars are linked each other. The clades with white colored background include grey and white colored sequevar numbers within each separate phylogenetic groups. ([a]) indicates that the clades of the isolates are unknown.

RALSTONIA SOLANACEARUM

Phylotype	I			IIA		IIB		III			IV				
Clade	1			2 & 3		4 & 5		6			7	8 & 9			
Sequevar	10	12	13	6	24	1	3	19	20		9	11			
	14	15	16	35	36	4	4NPB	21	22		8	10			
	17	18	31	38	39	25	26	23	29						
	34	44	45	41	50	27	28[a]	42	43						
	46	47	48	52	7		51	44	49						
				40[a]											
Race	1	4	5	1	2	3		Not Determined			1	3	BDB	*R. syzygii*	
Biovar	3	4	5	2T	1	2A		2T	1	2A	2T	1	2A		

1.2.2 The Genome

Till now, several genomes of *R. solanacearum* strains with very different host ranges have been sequenced. The first sequenced strain of *R. solanacearum* was GMI 1000 [27] (classified as phylotype I; race 1, biovar 3) isolated from a tomato plant with symptoms [28]. GMI 1000 has a wide host range. The genome of that strain is 5.8 Mb in size and has G+C rich sequences (average value of 67%) with a coding potential for around 5,120 proteins. It was found to be organized in two independently replicating circular replicons: a 3.7 Mb chromosome and a 2.1 Mb megaplasmid respectively (see Figure 1.1). The larger replicon, also referred to “the chromosome”, includes the genes for the basic mechanisms required for cell functioning. The smaller replicon, megaplasmid, -with a features of plasmid-borne ori loci- encodes many crucial genes such hrp (hypersensitive response and pathogenicity) genes, flagellum biosynthesis genes, and most of the genes in exopolysaccaride pathway which are very important for pathogenicity [29, 30] .

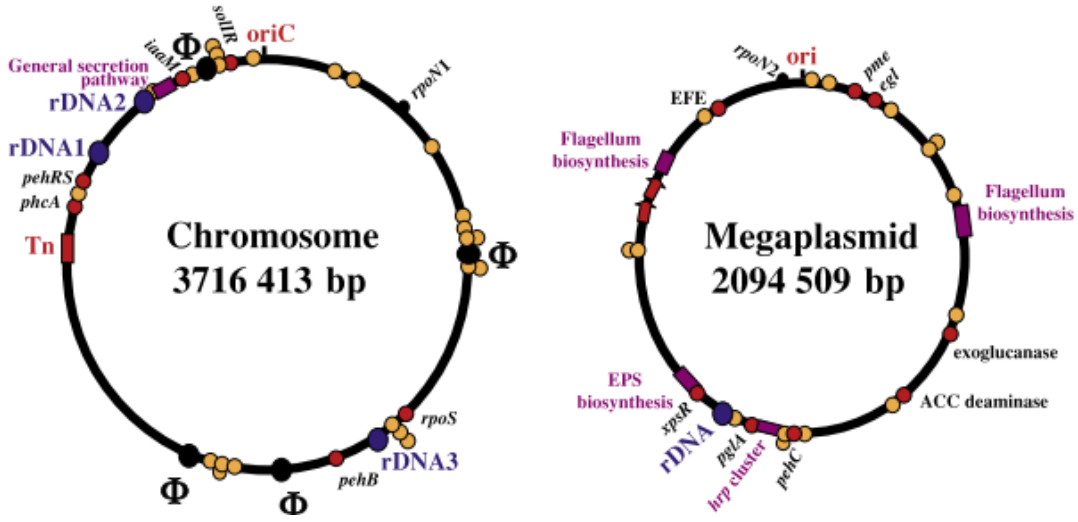

Figure 1.1 Genome of *R. solanacearum* strain GMI1000, adapted from Salanoubat et al. [27]. The Φ symbol: prophage sequences. Ori: origin of replication. rDNA: ribosomal DNA loci. Tn: a 44 kb conjugative transposon. rpoS: encoding sigma factor S; EFE: ethylene-forming-enzyme; Orange circles: genes encoding candidate effector proteins potentially secreted by the Hrp type III secretion system; and gene clusters represents the synthesis of the general secretion pathway and the bacterial flagellum.

Moreover, the draft genome sequences of race 2 biovar 1 strain Molk2 only infecting banana (specific to this host) and two *R. solanacearum* race 3 biovar 2 strains infecting potato: 1609 and UW551 have been established [31, 32]. A comparative analysis of different *R. solanacearum* genome sequences enables scientists to increase their knowledge about genetic and phenotypic relationships between the strains. Although the strains of *R. solanacearum* basically have two replicons in each cell, variations in genome affect the overall pathogen's behavior towards host plants. That also gives the answers of why the closely related strains are actually different from each other.

For instance, GMI1000 genome has a few protein-encoding genes which are absent in UW551. The genome of Molk 2 lacks approx. 30% of the genome of GMI 1000 which has been revealed from the study in which about 100 kb from GMI 1000 genome was compared with the Molk 2 genome [33]. It was found that 66 contiguous genes present in strain UW551 were absent in IPO1609. When the genome of these two race3 biovar 2 strains were compared, a 77 kb region on the megaplasmid replicon was found to be particularly deleted in strain IPO1609 [34]. This deletion reduces the

virulence of this strain which particularly associates with their aggressivity towards host plants.

Namely, horizontal gene transfers, transposable or mobile genetic elements may be the important factors losing and altering the genetic material of *R. solanacearum* species complex which provides flexibility to generate great genomic diversity.

1.2.3 Quarantine Status of *Ralstonia solanacearum* in European Union

Bacterial wilt disease caused by the bacterium *R. solanacearum* is a serious disease listed in the EU plant health legislation (Annex IAII of Directive 2000/29/EC). Especially, the strain belongs to phylotype IIB, sequevar 1 (PIIB-1) (known as race 3 biovar 2) is considered a serious quarantine pest by the European Plant Protection Organization (EPPO). Since, the pathogen causes diseases on plants in cooler climates besides in tropical, subtropical and warm temperate regions. The strain mainly infects potato (*Solanum tuberosum*) and causes brown rot disease. It can also infect tomatoes (*Lycopersicon spp.*) and other solanaceous plants. Due to that, the pest free areas (PFA's) where the potatoes should be planted according to FAO standards have been proposed by the Commission Directive 98/503/EC [35, 36]. In addition, Turkey is also a member of EPPO.

1.2.4 Geographic Distribution

R. solanacearum is present in six of the seven continents [16]. It was thought that the bacterium has emerged from south America and Indonesia which dates back to the time of the geological division of the continents, however the origin of this pathogen is not exactly known [37]. Over the last decades, there has been increase in spread of the brown rot disease over the European countries. There have been infested yields arisen from such countries: Spain, France, Belgium, Sweden, the Netherlands and the United Kingdom [38]. The spread of the pathogenic bacteria from such contaminated soil areas into residues or surface water is facilitated by overwintering in non-host plants such *Solanum dulcamara* (bittersweet) and *Solanum nigrum* (Black nightshade). In the USA, the organism has accidently been introduced by ornamental plants which is considered a threat posed to the potato industry [39, 40]. The introduction of *R. solanacearum* into

several EU countries appears to occur via the latently infected potatoes which are imported from regions where the disease is widespread [41].

According to EPPO reports, there have been several confirmed outbreaks of brown rot disease caused by *R. solanacearum* PIIB-1 in potato and tomato crops in Turkey. The potato brown rot was first detected in potato fields in the district of Nevşehir in 1995. Surveys were conducted and showed that 5 farms were found infected. In 2006, the disease was detected again in one ware potato field near Altınova (a district of Balıkesir, Marmara region). Delimiting surveys were conducted and showed that approximately 163 ha were infected at Altınova. In summer 2006, severe wilt symptoms were observed on tomato fields at Barakova (a district of Çanakkale, Aegean region) [38]. Recently, an outbreak has occurred in Alpu (a district of Eskişehir Province) (unpublished data).

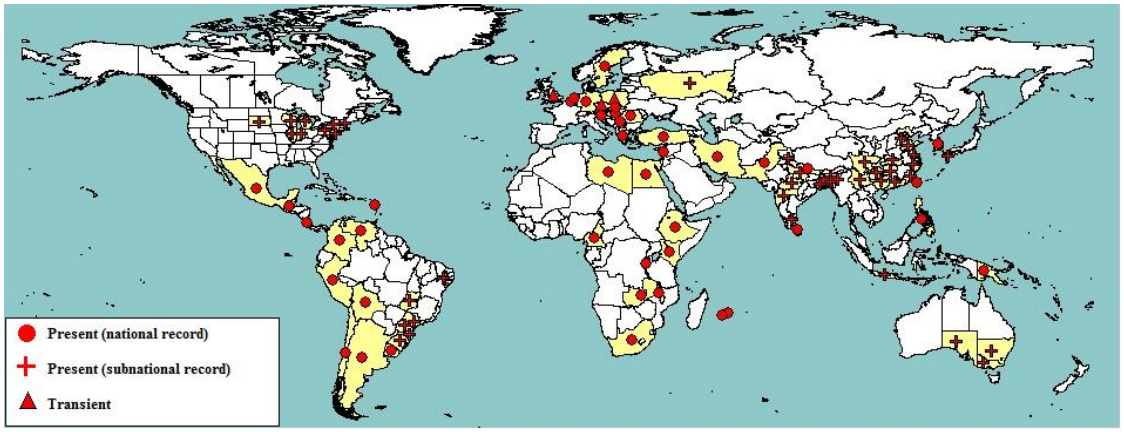

Figure 1.2 Geographical distribution of *R. solanacearum* (race 3 biovar 2) (adapted from PQR [38]).

Figure 1.3 Distribution of *R. solanacearum* (race 3 biovar 2) in Turkey according to EPPO pest reports [38] and the recent information from the laboratories of bacteriology department / Directorate of İstanbul Agricultural Quarantine.

1.2.5 Pathogenicity

The invasion pattern of *R. solanacearum* is different from most phytopathogens: The pathogen enters plants through secondary root emergence or root wounds. It has been also reported that insects carry the strains infecting banana through aerial transmission. *R. solanacearum* colonizes in intercellular spaces of the root cortex and vascular parenchyma. Then, it spreads through the vascular system by disrupting cell walls. The bacterial cell density rapidly increases in xylem vessels (i.e. usually > 10^{10} cells/cm of stem in tomato) which results in wilting and plant death [1, 42-44]. Later, the organism goes back to the soil and lives as a saprophyte until it finds to infect new host plant (see Figure 1.4). Lacking a 'true' host plant, the bacterium is able to live in water courses or in the soil (in clay or heavy soils with favorable moisture and temperature) for several years that are significant ways for pathogen dissemination [10]. The long-term survival also depends on the factors: the ability to metabolize lignin derivatives of plant cell debris or colonize in weeds or plants which are asymptomatic carriers of the organism. For example, *R. solanacearum* can survive in water courses by staying in roots of the *Solanum dulcamara* (bittersweet) plants without showing the wilting symptoms [45]. *R. solanacearum* can survive in an extended nutrient shortage. It has been reported that the bacterium preserved virulent capacity and wilted host plants even after 132 days of incubation in ultrapure water [44, 46].

Many studies reveals that pretty large genome size (5.7 Mb) of the bacterium reinforces itself genetically to respond well to the diverse abiotic stresses [27].

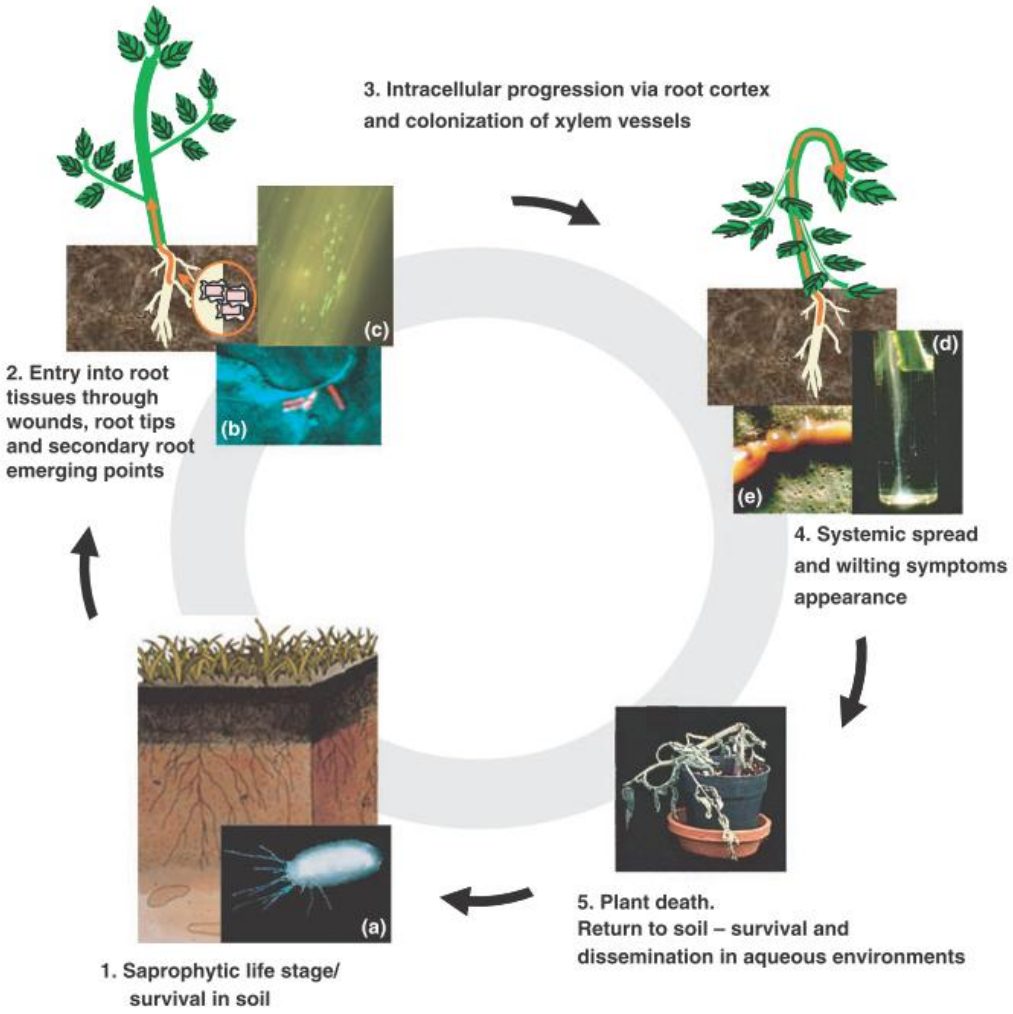

Figure 1.4 Infectious cycle of the pathogen (adapted from Genin, S. [44]). (a) wild-type strain GMI1000 under Transmission electron microscopy. (b) bacteria (in red) attached to plant cell surfaces examined under confocal microscope. (c) Green fluorescent protein-expressing R. solanacearum demonstrated on the surface of a tomato root. (d) Bacterial ooze exudes from an infected tomato stem in water. (e) cross-cut section of stem vessels releases bacterial exopolysaccharide matrix.

Under non-favourable environmental conditions, *R. solanacearum* can survive by changing itself into resistant forms which are:

a) Viable but non-culturable forms
b) Starved cells
c) PC-type variants
d) Biofilms

1.2.5.1 Viable But Non-Culturable Forms

In the VBNC state, the cells are metabolically active (alive), but they cannot grow on the solid media. Namely, the pathogen is appeared to be a dormant cell as some non-sporulating bacteria show the same physiological state. It is a kind of survival strategy of the organism for adverse environmental conditions such as temperature shifts, starvation, drought, osmotic stress, and exposure to the heavy metal copper [47]. Grey et al. showed that the cells become VBNC in the presence of certain amount of cupric sulfate in liquid microcosms [48]. In pure water, prolonged storage of the bacterial cells at 4°C caused VBNC state and their wilting ability was also gradually reduced [46]. *R. solanacearum* also moves into VBNC state at 4°C in soil [49]. In environmental water, *R. solanacearum* VBNC state was induced under extensive oligotrophic conditions. In addition, the quantity of cells at VBNC state increases when the plant undergoes extensive necrosis due to infection [48, 50].

1.2.5.2 Starved Cells

Another physiological state observed in these pathogenic bacteria is the starvation-survival response. Unlike the VBNC and those of active growth, it is a survival strategy for energy-deficient systems. Namely, bacterial populations with this state become non-growing but culturable [51]. In environmental water microcosms, *R. solanacearum* will be in this state at permissive temperatures. Starved cells are able to be pathogenic in the water microcosms for several years [50, 52].

1.2.5.3 PC-Type Variants

It is considered that PC-type variants (already described in section 1.2) may possess some selective advantages over the wild type. Under oxidative stress, it has higher motility which favors aerotaxis. The switch from pathogenic form to PC especially occurs when the bacteria move from host to non-host environments. It was suggested that PC-type variants might be particularly adapted to tolerate harsh conditions. A reversible switch from the PC to the wild type has also been observed in planta which is shown as the confirmation for the existence of this phenotypic conversion/reversion cycle [12, 50, 53].

1.2.5.4 Biofilms Produced by R. solanacearum

Even though, how *R. solanacearum* can form biofilm and the factors involved in this process are not well-known, biofilm-like aggregations have been observed on different strains in contact with abiotic and biotic surfaces. The pathogen produced biofilm-like structures on the external part of tomato seedling roots. Biofilms were also examined on PVC wells at the liquid-air interface. In addition, aerotaxis-deficient mutants produced biofilms excessively on abiotic surfaces. It was deduced that the cell might protect itself from toxic oxygen levels at the liquid-air interface via producing protective thicker biofilms. So, regulation of biofilm formation is linked to survival mechanism of the pathogen [50, 54].

1.2.6 The Virulence Factors

R. solanacearum has various factors involved in colonization and wilting of host plants through the specialized systems of networks and regulatory cascades. In a response to various environments, the bacteria dramatically change its physiological state by comprehensive shifts in gene expression associated with the primary network that regulates virulence and pathogenicity genes. Those are the cores of PC system (see Figure 1.5) [55]. The main virulence factors are the followings:

a) Extracellular polysaccharide I
b) The type III secretion system and effector proteins
c) Type II secretion system and cell-wall-degrading enzymes
d) Motility

1.2.6.1 Extracellular Polysaccharide I

The heterogeneous polymer of N-acetylated extracellular polysaccharide I (EPS I) is one of the significant virulence factors which is produced by the phytopathogen. EPS I-deficient mutants are almost not virulent and unable to colonize efficiently in plant xylem vessels [56]. The observations revealed that EPS I directly induces plant droop by physically blocking the vascular system and thereby interferes water movement. It has been considered that EPS might suppress plant antimicrobial defenses by covering surface properties of the bacterium that could be recognized by plant [42, 57]

1.2.6.2 The Type III Secretion System and Effector Proteins

The Type III Secretion System (T3SS) plays an important role in pathogenesis of numerous phytopathogens and animals. The hrp gene cluster, stretching over a 23-kb region on the mega plasmid, encodes the T3SS in *R. solanacearum*. In major groups of gram-negative bacteria, hrp genes are essential causes of pathogenesis on suitable hosts and responsible for the initiation of the hypersensitive response (HR) on resistant plants. Approximately, 70–80 type III effectors are generated by *R. solanacearum*. The effector proteins are significant virulence factors and are injected by T3SS into the plant cell. Therefore, both the T3SS and the effectors contribute greatly to development of the disease [58].

1.2.6.3 Type II Secretion System and Cell-Wall-Degrading Enzymes

A number of cell-wall-degrading Enzymes, containing three polygalacturonases (PehA , PehB and PehC), an endoglucanase (Egl), a pectin methylesterase (Pme), and a cellobiohydrolase (CbhA) are secreted by *R. solanacearum*. It has been shown that Egl, PehA, PehB and CbhA contribute to virulence through Type II Secretion System. [59].

1.2.6.4 Motility

R. solanacearum has type IV pili-driven twitching motility and flagella-driven swimming motility that are significant for its ability to cause the disease and ecological fitness. The bacterial mutants lacking either motility or tactics showed low virulence when they were applied to tomato plants on a soil-drench but displayed normal virulence when directly injected into plant xylem. That demonstrates directed motility and the swimming motility are necessary factors for *R. solanacearum* virulence in the early stage of host colonization and invasion [60].

Namely, a complex regulatory network of signal transduction pathway inside the pathogen regulates these major virulence factors which sense the different environmental signals or quorum sensing molecules (hydroxy palmitic acid methyl ester; 3-OH PAME). The system has a transcriptional regulator PhcA activated by cell density and crowding. The major virulence factors such EPS1 and some exoenzymes are produced by high levels of this transcriptional regulator. The quorum sensing molecule 3-OH PAME, linked with the cell density and confinement, controls the levels

of PhcA. When a great number of cells are around, the concenration of 3-OH PAME increases which then triggers the PhcA activation (see Figure 1.5) [55].

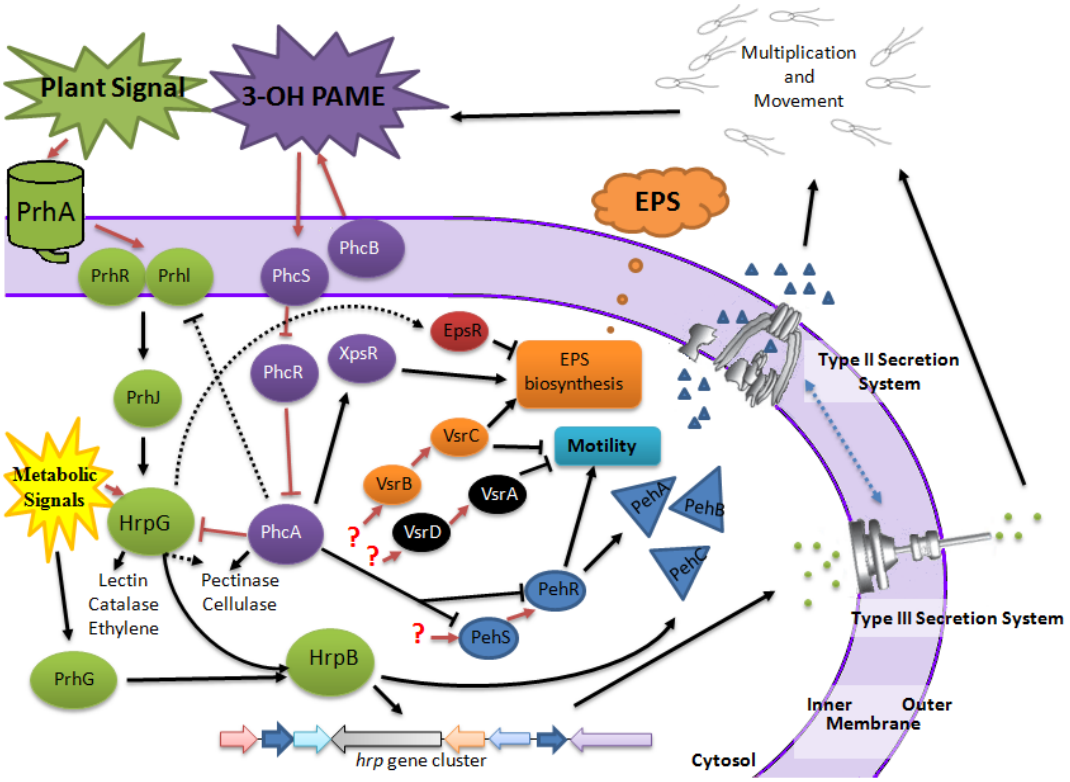

Figure 1.5 Major regulatory pathways of *R. solanacearum* virulence genes [61, 62]. Arrows and T-bars represent activation or repression, respectively. Black lines indicate control at the transcriptional level and red lines indicate post-transcriptional effects. Full lines show major effects and dotted lines show slight transcriptional influences. Blue dotted lines with two arrow heads show interaction. EPS, exopolysaccharide; 3-OH PAME, 3-hydroxy palmitic acid methyl ester.

1.3 ECONOMIC IMPORTANCE

Bacterial wilt disease in crop fields of tomato, potato, tobacco, banana, groundnut, and ginger plants have caused adverse economic effects in many parts of the world. Due to lack of enough documented information about its impact on subsistence farming, specifically on potato and planting of wilt-susceptible crops, it is difficult to make accurate predictions on the economic loss. The deficits are commonly due to climates influencing over small geographical areas, soil types, farming practices, the choice of

plant cultivar and crop, and the virulent features of the *R. solanacearum* local strains . Losess are generally increased in zones where the pathogen has a quarantine status (i.e under the implementation of EU Directives) owing to the use of strict measures, Particularly, economic costs of BW to the potato industry in the world have been predicted 950 million US dollars [63].

1.4 CONTROL METHODS

It is difficult to remove *R. solanacearum* from the soil. Due to that, diverse intervention strategies have been developed to manage and suppress the disease. The most significant and applicable methods are:

1.4.1 Resistant Cultivars

Till now, breeding for disease resistant plants is the most efficient method to manage the bacterial wilt disease. Thus, choice of resistance cultivars (genotype) after each generation is proposed in breeding programs [64]. For example, hot pepper accessions and sweet pepper (*Capsicum annuum L.*) are two resistant cultivars used to control BW in Japan. It has been demonstrated that resistant tomato plants suppress the bacterial multiplication in xylem by limiting the *R. solanacearum* movement from the primary xylem to other xylem tissues [65]. In addition, the disease prevention can be achieved by planting the crops to the pest-free fields [35, 36].

Aside from the ethical discussions, biotechnological approaches for disease resistance seem to be alternative to the conventional ones. Recently, many researches has been carried out on many effective antimicrobial proteins, peptides from plants, such as thionins , lectins, enzyme inhibitors, disease-related proteins [66] and lysozymes which naturally exists in insects [67], plants [68], animals [69], and humans [70]. Namely, Recombinant DNA technology enables the use of genes from insects, animals, fungi, and other plants for plant resistance which is a promising strategy to control bacterial diseases.

1.4.2 Cultural Practices

Crop rotation, incorporating or intercropping of green manure crops such as mung bean (*Vigna radiata*) and sunn hemp (*Crotalaria juncea*) are the control strategies for BW, prior to producing a susceptible crop [71]. Incidence of bacterial wilt can be reduced by planting maize with crop rotation or intercropping [72].It is suggested that brown rot disease is notably reduced by using non-susceptible crops and crop rotation for 5-7 years. But, it is difficult to apply this kind of control measures, since *R. solanacearum* strains can attack wide range of hosts [7].

1.4.3 Chemical Methods

Chemical treatments are not so efficient for controlling BW since the pathogen can be inside the xylem or stay at depth in the soil. Moreover this type of method is not economic and practicable to apply in the field. Even though some chemicals have positive effect on controlling the disease to some extent, many reports has suggested that there is no effective chemical control available for BW [7, 71, 73].

Moreover, volatile essential oil compounds generated by various plant species take part in host defense mechanisms against phytopathogens. For example, Pradhanang et al. [74] suggested that lemongrass oil, palmarosa, and thymol are able to suppress *R. solanacearum* populations in soil but further studies are reqired to be done.

1.4.4 Biological Control

Biological control is the use of other viable organisms which totally or partially inhibit or destruct the pathogen population. It is an environmentally safe and ideal approach for sustainable agriculture. Thereby, the less dependency on chemical input yields more protection for environmental quality [75]. One of the examples for biocontrol agents is the use of bacteriophages against the disease. It has been shown that bacterium significantly suppressed the population dynamics of *R. solanacearum* [76]. Another bio-control agent to control the pathogen is the use of plant growth promoting bacteria (PGPR) strains. PGPR could reduce BW in different levels and enhance the tomato crop production [77].

1.5 CURRENT DIAGNOSTIC METHODS FOR BROWN ROT

R. solanacearum is one of the most important plant pathogens causing disease in potato worldwide. The organism is difficult to detect in soil or in infected tubers because it grows very slowly in contrast to other bacteria and present in low numbers. It is easily overgrown on agar media by antagonistic competitors [78]. Detection and identification of *R. solanacearum* from either symptomatic or asymptomatic plants and from water or soil samples are possible with several microbiological and molecular methods [6].

1.5.1 Presumptive Diagnosis Based on Symptoms and Morphological Examination

The most typical external symptoms of the disease are wilting, stunting and yellowing of leaves. These can occur at any stage of growth of susceptible host, although the total wilting and collapse of the plant is more common in young plants. Internal symptoms are based on the color change of the vascular bundles to brown or yellow indicating early stages of the disease. As the disease progresses, most of the plant seems to fade and discoloration of tissues occurs from dark brown to black. In advanced stages, the degradation of occluded xylem vessels, combined with extensive invasion and destruction of surrounding tissues (phloem, cortex and epidermis) cause the collapse and death of the plant [6].

1.5.1.1 Symptoms on Potato Plant

Wilting of foliages towards the top of the plant at high temperatures during the day with revival at night is the early sign of the infection in the field. The green appearance are only seen in initial stages of wilting foliages but soon after yellow and brown necrosis occurs. The plant also develops epinasty. Rapid irretrievable wilting is observed on one shoot or entire plant which leads to the collapse and death of the plant. Bacterial exudes, a brown and milky ooze, appear on the surface of the vascular tissue of crosswise cut stems from wilted plants or it can be discharged by squeezing [6].

1.5.1.2 Symptoms on Potato Tuber

When a crosswise cut near to the heel (stolon end) or lengthwise cut over the stolon end is performed on potato tubers, the vascular ring appears glassy yellow to

light brown from which a pale creamy exudates come out spontaneously after several minutes (see Figure 1.6). The vascular discoloration is the sign of the early stage of infection which later turns more distinct brown and necrosis reaches out into the parenchymatous tissue. In advanced stages, soil particles may adhere to sides of the potato where the infection oozes out of the heel end and the eyes. Internal collapse of vascular tissues may appear as reddish-brown and slightly hollow lesions on the skin. In the advanced stages of the disease, fungal and bacterial soft rots generally emerge as secondary infections [6].

Figure 1.6 Potato tubers infected by *R. solanacearum* / The Department of Bacteriology - Directorate of İstanbul Agricultural Quarantine.

1.5.1.3 Symptoms on Tomato

Drooping youngest foliages are the first visible sign of the disease. Soil temperature at around 25 °C with saturated humidity is sufficient to support growth of *R. solanacearum* which causes epinasty and wilting of one side or of the entire plant and then, total plant collapse is seen in the next few days. Less wilting develops at a soil temperature below 21 °C, but a lot of adventitious roots may grow on the stem. Watersoaked streaks can be examined from the base of the stem which indicate necrosis in the vascular system. White or yellowish bacterial ooze in discoloured brown vascular tissues releases from transverse cut of stem [6].

1.5.1.4 Symptoms on Other Hosts

Solanum dulcamara and *Solanum nigrum* plants are the weed hosts of *R. solanacearum* usually remain asymptomatic, unless soil temperatures exceed 25 °C or inoculum levels become very high. For example; *S. nigrum* growing beside BW of potato or of tomato plants. The wilting symptoms in weed hosts are similar as described for tomato. Light brown discoloration of internal vascular tissues may be observed on underwater roots and stems (base and parts) of non-wilting *S. dulcamara* plants when cut transversely. Bacterial ooze may occur from the crosswise cut of stem even in the absence of wilting symptoms when placed vertically in water [6].

When basal portions of stem cuts of symptomatic plants is taken and placed vertically in a translucent container with clear water. After several minutes the onset of bacterial exudates is observed in the form of milky threads, as indicative of growth of the pathogen in the vascular bundles of the plant (bacterial flow test) (see Figure 1.4 / section d). During sampling, it is also important to differentiate the symptoms of brown rot in potato caused by *R. solanacearum* and symptoms of ring rot caused by *Clavibacter michiganensis subsp. sepedonicus* . The use of Potassium hydroxide (3%) provides the differential response against bacterial exudates, due to the different cell wall compositions of these pathogens which allow the discrimination (KOH test) [6].

Other methods based on observation of the poly-hydroxybutyrate granules serve as reserve material present in the cells of *R. solanacearum* , can be used as a technique for the presumptive diagnosis. These procedures require to perform special color stains by using an optical immersion microscope [6, 8].

Note that none of these methods allow the specific identification of *R. solanacearum*, but are useful when performing sampling and can be used as auxiliary techniques for phytosanitary stations with few resources.

1.5.2 Isolation Methods

Isolation of *R. solanacearum* from symptomatic plant samples is usually easy, due to the high concentration of the bacteria in the plant tissues. However, latency in advanced stages of the infection or overgrowth of saprophytic bacteria as competitors makes the diagnosis more difficult. When it comes to soil and water samples, the

isolation of the bacteria is more complex, because when antagonistic bacteria and other microorganisms present in high numbers, the bacteria may enter in VBNC state. Countering these drawbacks, various ways for the selective isolation of the pathogen have been designed [78, 79], however, none of them are entirely satisfactory.

The semiselective medium South Africa (SMSA) was the most used medium due to its selectivity. It has the detection values between 10 to 10^2 CFU / ml from pure bacterial suspensions (see Figure 1.7) [80]. However, one must differentiate *R. solanacearum* from other bacteria that can grow in this medium. In addition to that, their designs are more complex and implementations are more expensive due to the use of specific antibiotics. The sensitivity of the selective isolation in solid culture medium can be improved if a prior enrichment step is performed in liquid semiselective medium [78-80] . The application of this technique increases the concentration of the pathogen in the sample which is useful for the techniques such as ELISA (Enzyme-Linked Immunosorbent Assay) and Polymerase Chain Reaction (PCR) [81, 82].

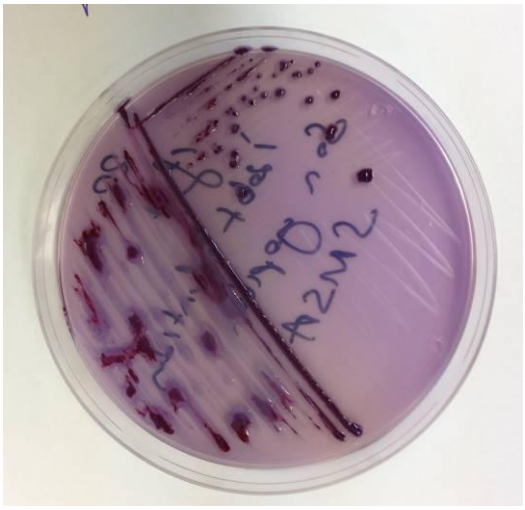

Figure 1.7 *Ralstonia solanacearum* on SMSA medium / The Department of Bacteriology - Directorate of İstanbul Agricultural Quarantine.

The immuno or immunocapture, is another technique, used as a detection method for plant pathogenic bacteria. It is based on the selective capture of target cells by using specific antibodies, in a sample containing a variety of microorganisms [83]. This

methodology allows to concentrate the pathogen of interest in the sample. Moreover, it eliminates the presence of antagonistic bacteria that may appear in isolation as well as decrease the inhibitory effect of some compounds for techniques such as PCR and ELISA [78]. The technique is recommended as an alternative to the use of selective media, due to the high percentage recovery of target cells from different samples and the short time used in isolation. Its cost is not so high and low detection values are obtained (10^4 CFU / ml) from pure bacterial suspensions. It allowes the detection of the pathogen in weed reservoir [83]. Peckham et al. [84] suggested that the method increases pathogen detection levels in water samples when used prior to PCR.

1.5.3 Biological Methods

Pathogenicity tests are easily applied to *R. solanacearum*. However, since they depend on the specific relationship between host and pathogen, it is important to determine strains of the pathogens according to old or new classification systems, and then the host can be choosen to be used in the assay [20, 78]. Inoculation methods vary depending on types of the plants; small volumes of the pathogen are applied through wound or opening in axillary buds which are deliberately made via puncturing. In more natural methods, the bacteria are applied to the roots of plants. In the latter case, it was observed that the intentional injury of the roots improves the consistency of the infection, as it is an additional input gate for the bacteria, however, this alternative has the disadvantage that large inoculums for implementation are needed (see Figure 1.8) [6].

With optimal conditions of temperature, humidity and light, the detection values of various pathogenicity trials are between 10^3-10^5 CFU/ml and the results are obtained within 7 days. When the concentration of the pathogen is less than these values, the symptoms may not show up or be delayed more than 20 days. However, it is also possible to isolate the pathogen in asymptomatic plants between values of 10-10^2 CFU / ml [6].

Biological methods are of great importance for the diagnosis. They are used to confirm the pathogen's ability to cause disease in the host plant. Despite this, these techniques are laborious, expensive, limited for application in large scale, and too much time is required to obtain results [5].

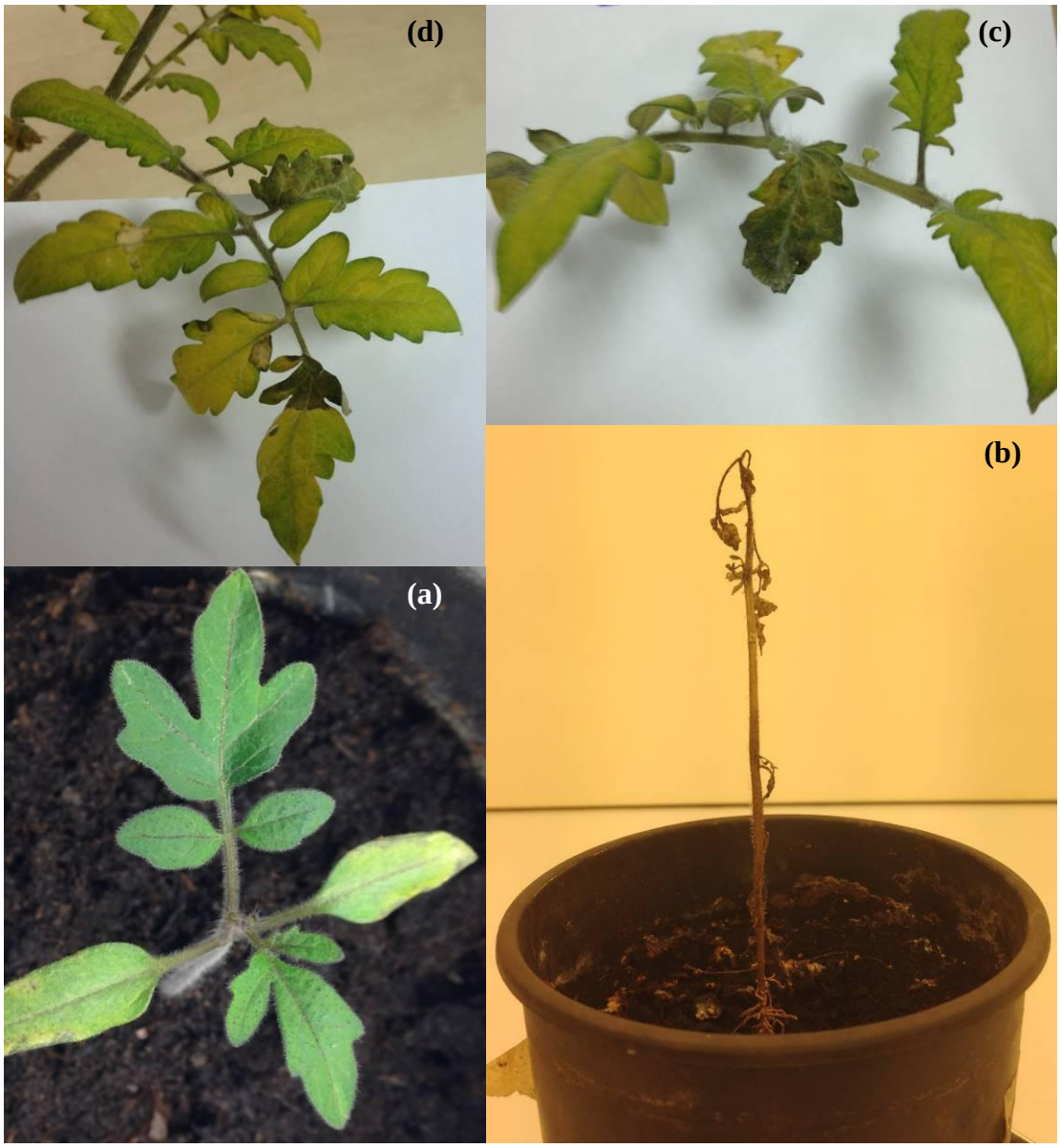

Figure 1.8 Infected tomato plants (pathogenicity test) / The Department of Bacteriology- Directorate of İstanbul Agricultural Quarantine. (a) The bacteria was suspended with healthy tomato seedlings overnight and then planted into pots and placed into incubation chamber (25°C room temperature and 75-80 % humidity). Later on plant showed the symptoms such dark discoloration of stem parts (b) Within a month, plant collapsed and died. (c) and (d): the isolate was directly injected into the healthy tomato plant and then placed into incubation chamber (25°C room temperature and 80 % humidity). After 5 days, the parts where the plant got injections showed wilting symptoms and yellow discoloration of leaves which indicated the presence of pathogenic form of *R. solanacearum*.

1.5.4 Serological Techniques

Serological tests are useful especially for isolation of the pathogen from plant material which sometimes fails with other techniques. These types of tests allow the detection of latent form of the disease. However, the use of polyclonal antibodies for serological detection of *R. solanacearum* may lead to cross-reaction with closely related species such as *Ralstonia picketii* [6] and *Ralstonia syzygii* [85, 86]. So, the positive results should be confirmed by secondary (cultural, pathogenicity, PCR, biological methods) tests. Besides, it is difficult to obtain a universal antibody, since most of the monoclonal antibodies are highly selective and do not react with all strains of *R. solanacearum* due to the large variability.

The indirect immunofluorescence (IF) was widely reported for the detection of *R. solanacearum* infections in potato tubers. The detection limits of the technique are between 10^3-10^4 CFU/ml, including live, dead cells and the VBNC state. The main advantages are that it is possible to observe the morphology of the bacteria, eliminate any false positive and allow the quantification of the bacterial population. A disadvantage of the technique is the frequent occurrence of cross-reactivity with bacteria that share antigens with cell wall morphology similar to *R. solanacearum*. Another limitation is the subjective interpretation of the staining of cell morphology , which depends on the experience of the observer [6].

ELISA is another technique for detecting *R. solanacearum* from different types of samples. The limitations are determined by factors such as the quality of the antibodies used, the mode of technique and the presence of inhibitors in the sample compounds. The use of monoclonal antibodies and selective enrichment prior to the application of the technique enable to counter these drawbacks. High detection values are obtained as 10^4-10^5 CFU/ml bacterial suspension from pure and even 1-10 CFU / ml from potato tuber extracts [81, 85, 87] .The technique is easy to perform. For this reason, it is continuously being used in routine diagnosis as an auxiliary technique in quarantine laboratories, although it is not listed as a main selective technique in some countries.

Serological techniques such as latex particle agglutination tests were also carried out for diagnosis of the disease and the results are obtained in minutes. However, it

shows the detection values above 10^6 CFU/ml and often leads cross-reactions with other microorganisms, so the results must be verified by other tests [6].

1.5.5 Techniques Based on the Detection of Nucleic Acids

1.5.5.1 Conventional Polymerase Chain Reaction

In recent years, there has been an exponential growth in the design of protocols for the detection of nucleic acids. Among these, the PCR technique has been commonly used in detection methods of this bacterium worldwide due to its high level of specificity, sensitivity and rapidity [5, 88]. The detection limits of the PCR protocol depend on the type and nature of the sample used. It has been reported that, the detection limit of the technique between 10^1-10^2 CFU / ml from potato extracts [89]. However, these values can be affected by the presence of inhibitory substances in the soil particles in bacterial extracts and in the plant sap [90]. In this sense, quality protocol and correct performance of nucleic acid extraction play an important role [90, 91]. In addition to that, if the primers are failed to be specifically designed for the target organism, they can give false positives due to cross-reaction with other pathogenic bacteria or saprophytes [92].

The selective enrichment can be also used prior to the PCR step for detection of the pathogen in natural substrates such as plant material, soil and water currents. Lower detection limits can be obtained with the combination of the technique in comparison to the results obtained with the use of conventional PCR from these substrates [90]. Other authors used immuno-isolation combined with PCR for pathogen detection in plant samples and water reservoirs [83, 84].

Increase in the sensitivity and specificity of the technique was also achieved with the application of nested PCR protocol [93]. However, this method has the disadvantage of high risk of contamination since it is done in two steps of amplification in different tubes. A novel PCR procedure, cooperatively developed by Caruso et al. [82], were able to eliminate this problem. Increases in specificity and sensitivity have been obseved with the combination of three primers in a single reaction for the pathogen detection. In that study the pre-enrichment was also used and a detection limit of 1 CFU / ml of the bacteria was achieved from water samples without DNA extraction.

Other protocols were designed on the purpose of the characterization and identification of the pathogen, among which are the followings: Multiplex PCR [16] for the detection of *R. solanacearum* phylotypes and Rep-PCR procedures (repetitive regions of the genome) with the universal primers (ERIC and BOX) [94]

1.5.5.2 The Fluorescent in-situ Hybridization

The fluorescent *in-situ* hybridization (FISH) is another technique among the molecular tests for the detection of *R. solanacearum*. It is based on microscopic examination of fluorescence emission of a labeled probe, when hybridized to a specific sequence of the pathogen of interest. 23S rRNA gene, highly conserved across species, is used for the detection of *R. solanacearum* [95].

FISH shows a lower limit of detection than the IF does, because this test detects only living cells. However FISH has some disadvantages such the unintended effect of autofluorescence of plant tissues and the high cost of the technology. Despite these limitations, this method is relatively fast (1 day) and allows to discriminate potential false positives obtained by the IF. Moreover, it has a potential to detect the organism from natural substrates such as soil, water and plant samples and can be alternative to the PCR applications. Thus, it can be useful for study of disease in ecological niches where the detection of viable pathogens is important [95].

1.5.5.3 Isothermal DNA Amplification Methods

Isothermal DNA amplification methods have also been applied for the detection of *R. solanancearum*. Kubota et al. [96] designed a LAMP (Loop-Mediated Isothermal Amplification) protocol to detect this bacterium in which the results are obtained within just 3 hours without the need for a thermocycler. The reaction may be visualized by agarose gel electrophoresis or even visualized by an increase in turbidity. However, it has the limitation that the result is obtained as a pattern of bands in the molecular weight marker has a lower sensitivity to the conventional PCR (10^4 -10^6 CFU/ml). Despite these drawbacks, this method is an alternative for laboratories that do not possess the necessary equipment to carry out a conventional PCR [88].

1.5.5.4 Real-time PCR Approaches

Real-time PCR has become a standard technique in the detection of pathogenic bacteria. There are two validated protocols for the pathogen detection in potato tubers with detection values $30x10^2$ [97] and 30 CFU/ml [98], respectively. By using this technique, the subsequent steps of PCR staining with ethidium bromide and agarose gel electrophoresis are removed, which allows analysis of larger numbers of samples with the best automated process and reduces the risk of contamination due to handling. However, the costs of equipment and reagents for their performance are very high. [88, 97, 98]

The selection of the suitable chemistry detection system is very important throughout the design of real-time PCR experiments. These kinds of assays are labelling systems for the DNA amplicons allows to detect and quantify desired targets of DNA. Fluorescent dye labeled probes (TaqMan, Molecular beacons, hybridization, and Eclipse probes etc. [99]) designed for specific sequences on the target are commonly used which are very sensitive but also expensive detection systems. Furthermore, if the template DNA is prone to vary, false negatives results can occur because of the mismatches in base pairs between the probe and particular DNA sequences [100, 101].

Another labeling system is the DNA intercalating dyes which bind the minor groove of DNA double helix. It has several advantages over DNA probes. The problem encountered with probes does not happen when DNA fluorescent dyes are used. Since DNA dyes do not need the design like a probe does, it is affordable and practicable. But they may also give false positive results since the primers might bind to non-specific sites of DNA template [102]. So, the melting curve analysis of each product is required to overcome this problem.

Interpretation of melt peaks is important when the DNA sample present at low numbers like in latent infections because it will most likely give high threshold cycles (C_T) which might be deduced as non-specific binding of primers or primer dimers and therefore, false negatives.

SYBR Green I is one of the most used DNA intercalating dye for qPCR. Although SYBR Green I is defined as a highly specific dye, it has some disadvantages; high

concentrations of this DNA dye may inhibit PCR reactions. It may adversely affect on melting temperature (Tm) and tend to bind DNA sequences with high G+C content [103-105]. EvaGreen is an alternative DNA intercalating dye. In contrast to SYBR Green I, it has sharper melt peaks and higher reaction efficiency at various concentrations [106].

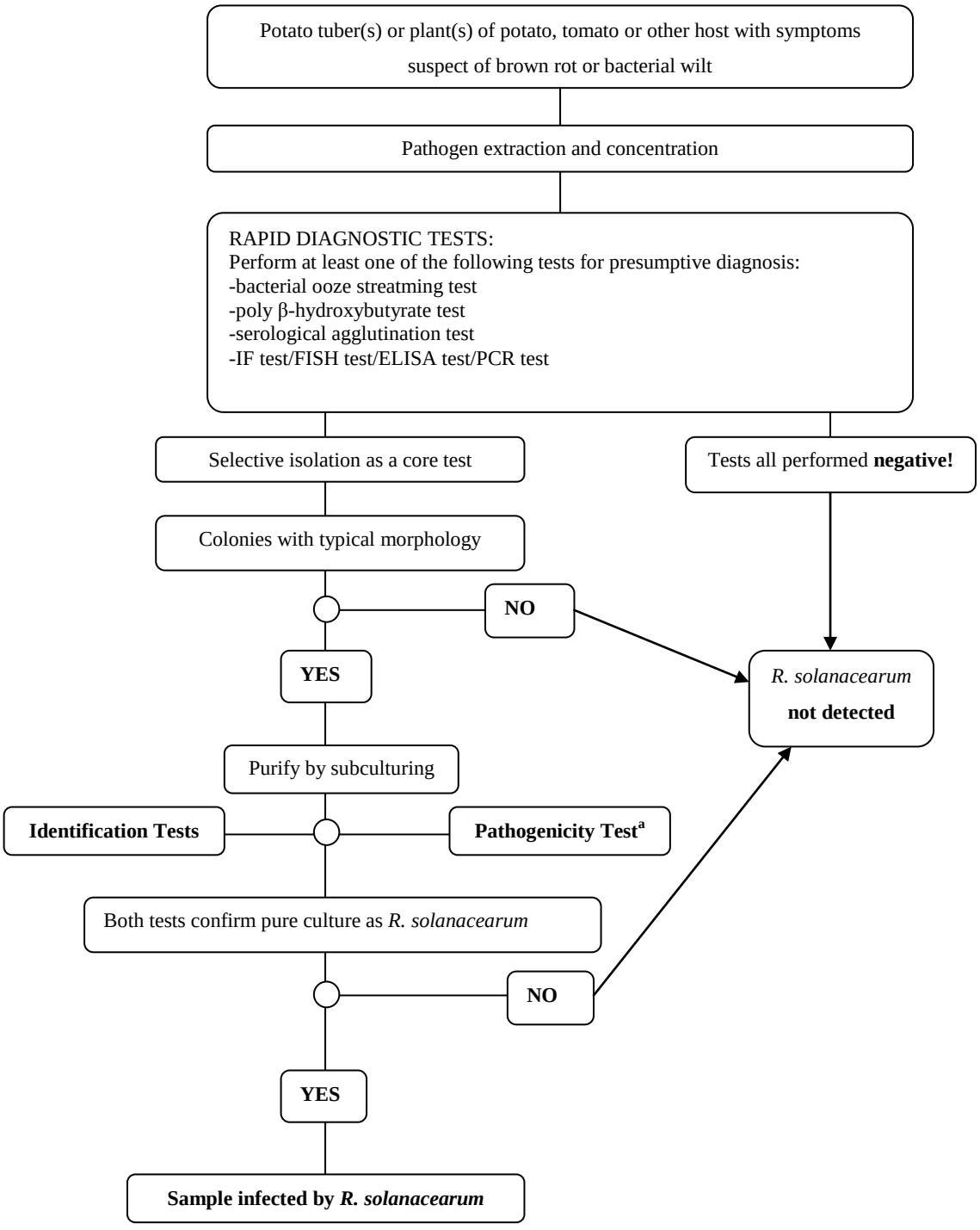

Figure 1.9 The detection scheme of *R. solanacearum* according to EU directives 2006/63/CE. ([a]) The Pathogenicity test must be reconfirmed by two of the core screening tests (see Figure 2.1).

1.6 AIM OF THE STUDY

The present official methods for individual detection of this bacterium in potato tubers are described in the EU directives 2006/63/CE (*R. solanacearum*) organized by EPPO. A great number of examinations on potato tubers are performed each year in each state members of EPPO. IF microscopy, conventional PCR, FISH or selective isolation are the main diagnostic assays and each of those is specific to be used for *R. solanacearum* detection. If the pathogen is detected by two of these methods, it is isolated and inoculated on a healthy tomato plant to confirm pathogenicity [6].

Ideally, the primary diagnostic tests should be fast, sensitive and reliable but the current official diagnostic procedures are time-consuming and need a lot of workers. Real-time PCR approach solves such problems and can be used as a main screening protocol: It is more sensitive, quick, and reproducible than conventional PCR. It does not require post-PCR processing step. Real-time PCR method is compatible with high throughput screening. Less number of workers and amount of time are required for a qPCR experiment. Moreover, carry-over contamination risk which may lead false-positive result is reduced by using this assay [100]. In previous studies, real-time PCR protocols using TaqMan fluorescent chemistry methodology were developed for the single detection of *R. solanacearum* [97, 98].

The aim of this study was to develop a fast, reliable and cost-effective EvaGreen based real-time PCR method for quantative assay of *R. solanacearum*. A part of the thesis also covers evaluation and comparison of the EvaGreen and TaqMan based real-time PCR methods.

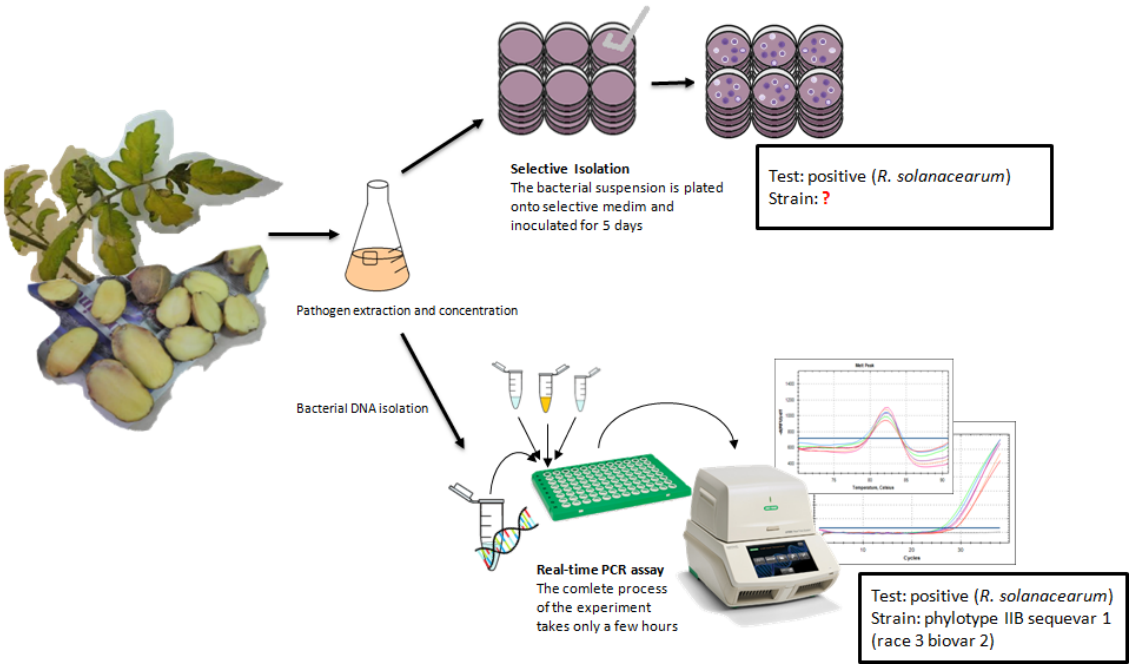

Figure 1.10 Real-time PCR method v.s. microbiological assay. The qPCR is a relatively fast and reliable technique in contrast to other microbiological (or serological) methods. It enables the sensitive and specific detection of the pathogen.

CHAPTER 2

MATERIALS AND METHODS

R. solanacearum (as a positive control) and the plant pathogens (*Xanthomonas spp., Pantoea stewartii subsp. stewartii, Erwinia amylovora, Clavibacter mischiganensis subsp. sepedonicus*, as negative controls) were cultured and the bacterial isolation from potato tubers with subsequent microbiological, serological and bioassay tests for the samples from Alpu (a district of Eskişehir province) were carried out by specialists in the bacteriology laboratories of Directorate of İstanbul Agricultural Quarantine. The culture of other non-related bacteria (*Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Citrobacter freundii, Escherichia coli*) and the real-time PCR tests of all bacteria used in this project were performed in the laboratories of Fatih University.

2.1 BIOLOGICAL MATERIAL AND GROWTH MEDIA

R. solanacearum PIIB-1 (NCPPB 4161) was purchased as a positive control from National Collection of Plant Pathogenic Bacteria (NCPPB). It was grown on King's B medium prepared as following; bacto proteose peptone (Difco) 20 g, anhydrous K2HPO4 1.5 g, glycerol 10.0 ml, MgSO4 1.5 g, agar 15 g Distilled water 1.0 L. The pH was adjusted to 7.2. Next, the solution was autoclaved. The bacteria were incubated at 28℃ for 3-5 days.

For selective isolation of bacteria from Alpu (a district of Eskişehir) SMSA medium was used which was prepared as following; Casamino acids (Difco) 1.0 g, Bacto-Peptone (Difco) 10.0 g, Glycerol 5.0 ml, Bacto-Agar (Difco) 15.0 g, Distilled water 1.0 L. Ingredients were dissolved and sterilized by autoclaving at 121 °C for 15

minutes. The solution cooled to 50 °C and filter-sterilized aqueous stock solutions were added which include following ingredients: Crystal Violet (Sigma) 5 mg per l, Polymixin-B-Sulphate (Sigma P-1004) 600 000 U (approximately 100 mg) per l, Bacitracin (Sigma B-0125) 1 250 U (approximately 25 mg) per l, Chloramphenicol, (Sigma C-3175) 5 mg per l, Penicillin-G (Sigma P-3032) 825 U (approximately 0,5 mg) per l 2,3,5-triphenyl tetrazolium chloride (Sigma) 50 mg per l. The bacteria were incubated at 28℃ for 2-5 days.

The non-related bacteria (*Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* [supplied from Veterinary Faculty of İstanbul University, İstanbul/Turkey], *Citrobacter freundii, Escherichia coli* [supplied from İnönü University, Malatya/Turkey]) were selected for specificity testing of EvaGreen real-time PCR assay. They were grown in LB broth (Acumedia, Prod. no.: 7279, USA) consisting of Enzymatic Digest of Casein 10.0 g, Yeast Extract 5.0 g, sodium chloride 10.0 g. H2O was added to first three ingredients to bring volume to 1 L and then autoclaved. The bacteria were incubated at 37℃ for overnight.

The other non-related plant pathogenic bacteria were purchased from NCPPB. *Xanthomonas spp.* were grown on YDC medium which was prepared as following Dextrose 20.0 g Yeast extract (Difco) 10.0 g CaCO3, light powder20.0 g Agar (Difco) 15.0 g Distilled water 1.0 L. Ingredients were dissolved and sterilized by autoclaving at 121 °C for 15 minutes. The bacteria were incubated at 28℃ for 3-5 days The others (*Pantoea stewartii subsp. stewartii, Erwinia amylovora, Clavibacter mischiganensis subsp. sepedonicus*) were grown on King's B media.

Table 2.1 The list of bacteria used in this study. ([a]) Ui abbreviations for Unknown isolate. ([b]) The isolates were determined as *R. solanacearum* by different methods such IF tests, microbiological culture and real-time PCR assay written below.

	Strain Number	**Microorganisms**
1	NCPPB 4161	*R. solanacearum*
2	Ui -3[a]	*R. solanacearum*[b]
3	Ui-21[a]	*R. solanacearum*[b]
4	Ui-22[a]	*R. solanacearum*[b]
5	Ui-26[a]	*R. solanacearum*[b]
6	Ui-40[a]	*R. solanacearum*[b]
7	NCPPB 3253	*Pantoea stewartii subsp. stewartii*
8	NCPPB 3361	*Xanthomonas oryzae pv. oryzicola*
9	NCPPB 3951	*Xanthomonas oryzae pv. oryzae*
10	NCPPB 4359	*Erwinia amylovora*
11	NCPPB 4229	*Clavibacter mischiganensis subsp. sepedonicus*
12	ATCC 4352	*Klebsiella pneumoniae*
13	CCM 1944	*Proteus mirabilis*
14	NRRL B-2643	*Citrobacter freundii*
15	JM103	*Escherichia coli*
16	ATCC 27853	*Pseudomonas aeruginosa*
17	ATCC 6633	*Bacillus subtilis*
18	ATCC 29213	*Staphylococcus aureus*

2.2 POTATO TISSUE EXTRACTS

Infected potato tubers were collected from different source and locations in Alpu (district of Eskişehir Province). A core of vascular tissue, heel-end, (~0.1 g) was aseptically removed from the stolon end of each potato tubers and then homogenized. A sample of 200 tubers stolon-end vascular tissue cores was macerated with approximately 40 ml of extraction buffer (50 mM phosphate buffer, pH 7,0) and agitated on a rotary shaker (Barnstead Lab-Line) (150 rpm) for 4 hours at room temperature. The supernatant was removed. The bacterial fraction was concentrated by centrifugation (Sigma 2-16 K) at 7 000 g for 15 minutes at a temperature between 4 to 10 °C was discardeded the supernatant without disturbing the pellet. The pellet was resuspend in 1.0 ml pellet buffer (10 mM phosphate buffer, pH 7,2).

2.3 SEROLOGICAL TEST

A total of 6 isolates from Alpu (a district of Eskişehir Province) were tested by using immunofluorescence (IF) against an antiserum produced from whole cells of *R. solanacearum*. Working dilutions of the antibody were prepared in IF buffer (10 mM phosphate buffered saline (PBS), pH 7.2)which is very important step since it affects the specificity of the test. Decimal dilutions (1/10, 1/100) of the resuspended pellet were also prepared in pellet buffer. 25µl of each suspension was placed on each 6 mm window diameter of a multi-wells slide and replicated two times. The slides were air-dried and fixed with 96% ethanol for 10 minutes. After drying , the slides were stained with 1/3200 and 1/6400 dilutions of *R.solanacearum* antiserum (ex goat, Prod. No. 07356/01, LOEWE® Biochemica GmbH, Germany) which was diluted in and then incubated in a moist chamber in the dark for 30 minutes. The slides were rinsed with IF buffer and then washed by submerging into IF buffer-tween (0,1 % Tween 20) and subsequently in IF buffer for 5 minutes. Then, the slides were stained with 1/200 dilution of second antiserum (rabbit anti-goat [H+L] conjugated FITC fluorescent dye, Prod. No 07200, LOEWE® Biochemica GmbH, Germany) and placed in a moist chamber in the dark for 30 minutes. Next, the slides were mounted in 0,1M phosphate-buffered glycerol and examined under the immunofluorescent microscope (Zeiss) at 100x magnification by using immersion oil.

2.4 SELECTIVE ISOLATION OF THE BACTERIA

100 µl from diluted bacterial suspension (1/100) were incubated on SMSA media in triplicates at 28 °C. Plates were observed after 48 hours and daily thereafter up to six days. After three days incubation *R. solanacearum* colonies on SMSA medium developed pink to blood-red coloration in the centre with whorling. Next, the bacterial colonies with typical morphology were subcultured onto King's B medium. IF test was performed from the pure culture.

2.5 BIOASSAY TEST

The pathogenicity test was performed as final confirmation for the diagnosis of *R. solanacearum* and for assessment of virulence of cultures identified as *R. solanacearum*. The bacteria from the pure culture were suspended with susceptible tomato seedlings at the third true leaf stage overnight at room temperature. Then, they were planted into the pots and placed into growth chamber up to four weeks at 25 °C with high relative humidity (%75-80) and appropriately watered to prevent waterlogging or wilting through water deficiency. The bacterial isolate was regularly checked for degree of infectivity on tomato plants grown in earthen pots. Within a month. The plant stems of plant darkened and collapsed (see Figure 1.8/ sections a and b). Since the method takes a long time to see the results the other kind of bioassay was also performed.

The bacteria was inoculated with a sterile syringe needle into the stem of tomato plants, just above the cotyledon leaves. It was incubated for 5-6 days at 25°C and 80 % humidity with appropriate watering to avoid water logging or drought stress. Typical wilting was obtained within 5 days which confirmed as being a pathogenic form of *R. solanacearum* (see Figure 1.8/ sections c and d).

2.6 RECONFIRMATION OF THE BIOASSAY TEST

Reisolation of the bacteria from the infected plant tissue was performed to confirm that the pathogen was *R. solanacearum*, since the procedure is required for quarantine organisms. The plant parts of leaves showed hypersensitive response were cut and sterilized with 70% ethanol solution and dried with paper towel. Then, the tissue were pulverized in PBS buffer and soaked for 10 minutes. SMSA media were prepared in triplicates. 50 micro liters was pipetted from the solution into each of the media. The bacterial colonies with typical morphologies were subcultured onto King's B medium. IF test was carried out from the pure culture. After all, the pathogen was reconfirmed as *R.solanacearum* (see Figure 2.1).

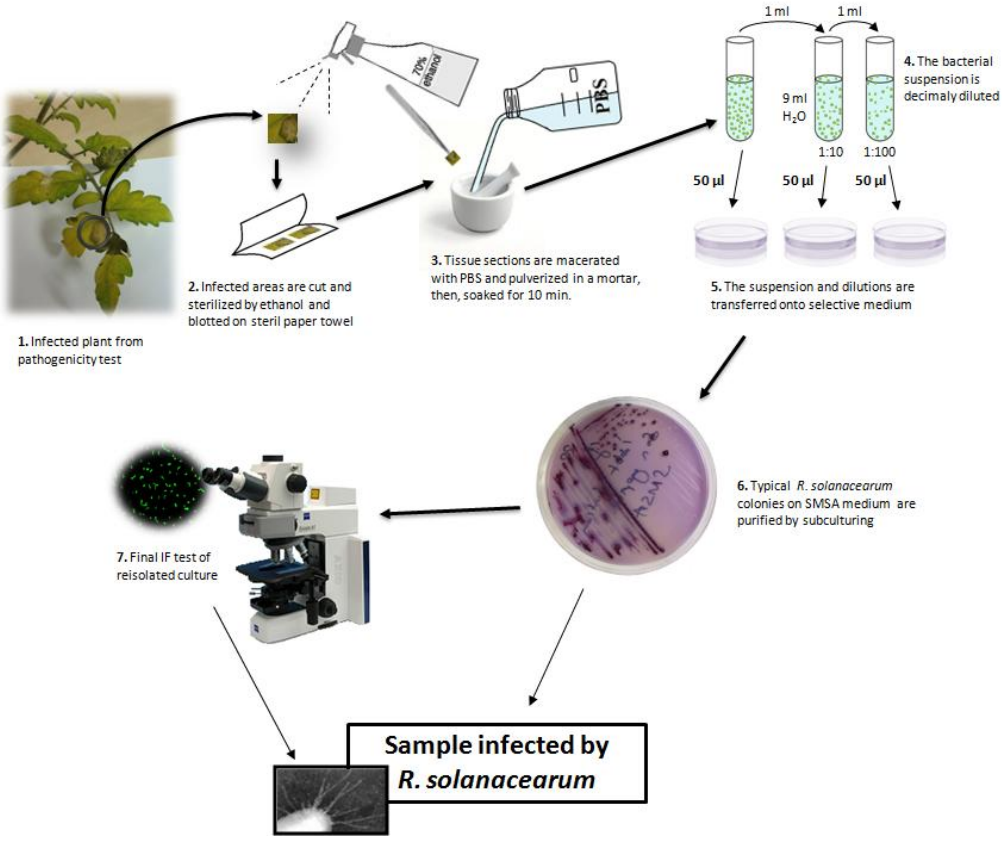

Figure 2.1 Reisolation of *R. solanacearum* from the bioassay test.

2.7 SAMPLE PREPARATION AND DNA EXTRACTION

Bacterial DNA was extracted either with High Pure PCR Template Preparation Kit (Roche) or with MagJET Genomic DNA Kit (Thermo Scientific) and quantified by using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and stored at -20˚C until use.

2.8 SELECTION OF PRIMERS AND PROBES

R. solanacearum phylotype IIB, sequevar 1 (race 3 biovar 2) specific primers and probes were selected from a previously published article [98] and supplied by Elips Company (Roche, Germany); Forward primer, RSC-F (5'-TTCACCGCAAACAGCG-3'); reverse primer, RSC-R (5'-TACGCCCCAGCAGATG-3') and probe RSC-P (5'-TTCGCCGATGCTTCCCA-3').

2.9 REAL-TIME PCR AMPLIFICATION

The real-time PCR reactions were carried out in a 20 µl volume with 3 different mastermix, which was composed by the following components:

1) 10 µl of 2x SsoFast™ EvaGreen® Supermix 2 (Bio-Rad, CA) (containing dNTPs, Sso7d fusion polymerase, MgCl2, EvaGreen® dye, and stabilizers), 2 µl of each primer (1 µM), 3,2 µl of H_2O and 2,8 µl of DNA template. For the amplification of *R. solanacearum* the following thermal conditions were used: incubation step at 98˚C for 2 min, 40 cycles of amplification at 98℃ for 5 s, 58℃ for 5 s. Melt curve; from 65˚ to 94℃ at 0.5℃/s.

2) 4 µl of 5x HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia)(containing ultrapure dNTPs, HOT FIREPol DNA polymerase, MgCl2, EvaGreen dye, and ROX dye), 0,5 µl of each primer (0,25 µM), 14,4 µl of H_2O and 0,6 µl of DNA template. For the amplification of *R. solanacearum* the following thermal conditions were used: incubation step at 95℃ for 15 min, 40

cycles of amplification at 95℃ for 15 s, 58℃ for 20 s, 72 ℃ for 20s. Melt curve; from 65℃ to 94 ℃ at 0.5℃/s.

3) 10 µl of 2x LightCycler® 480 Probes Master (Roche) (containing FastStart Taq DNA Polymerase, reaction buffer, dNTP mix [with dUTP instead of dTTP], and 6.4 mM MgCl2) 2 µl of each primer (1 µM), 2,2 µl of H_2O, 1 µl of 20x EvaGreen® dye (Biotium) and 2,8 µl of DNA template. For the amplification of *R. solanacearum* the following thermal conditions were used: incubation step at 95 ℃ for 10 min, 45 cycles of amplification at 95℃ for 10 s, 58℃ for 15 s, 72 ℃ for 2s. Melt curve; from 65℃ to 94 ℃ at 0.5℃/s.

CHAPTER 3

RESULTS

3.1 REAL TIME PCR USING SSOFAST™ EVAGREEN® SUPERMIX

In this assay, two products (51 bp at 80,50 ˚C and >200 bp at 88˚C) have been observed when annealing temperature was set at 58 ˚C (determined according to a previously published article [98]). Therefore the temperature gradient experiment was made to optimize and to obtain the desired DNA product for this mastermix. The annealing temperatures were set at 58,3°C;59 °C 60,3°C; 62,3°C with either 0,5 μM or 1 μM primer concentrations. Two peaks at 80,50 °C and 88,00 °C were derived from the annealing temperatures of 58,3°C;59 °C 60,3°C at both 1 μM and 0,5 μM primer concentrations. Although the single product was derived when the primer concentrations lowered to 0,5 μM at 62,3 degree Celsius, it was not of the desired DNA product which is 51 bp long. Amplification products were visualized under UV light.by agarose gel (2.5%) electrophoresis (see Figure 3.1).

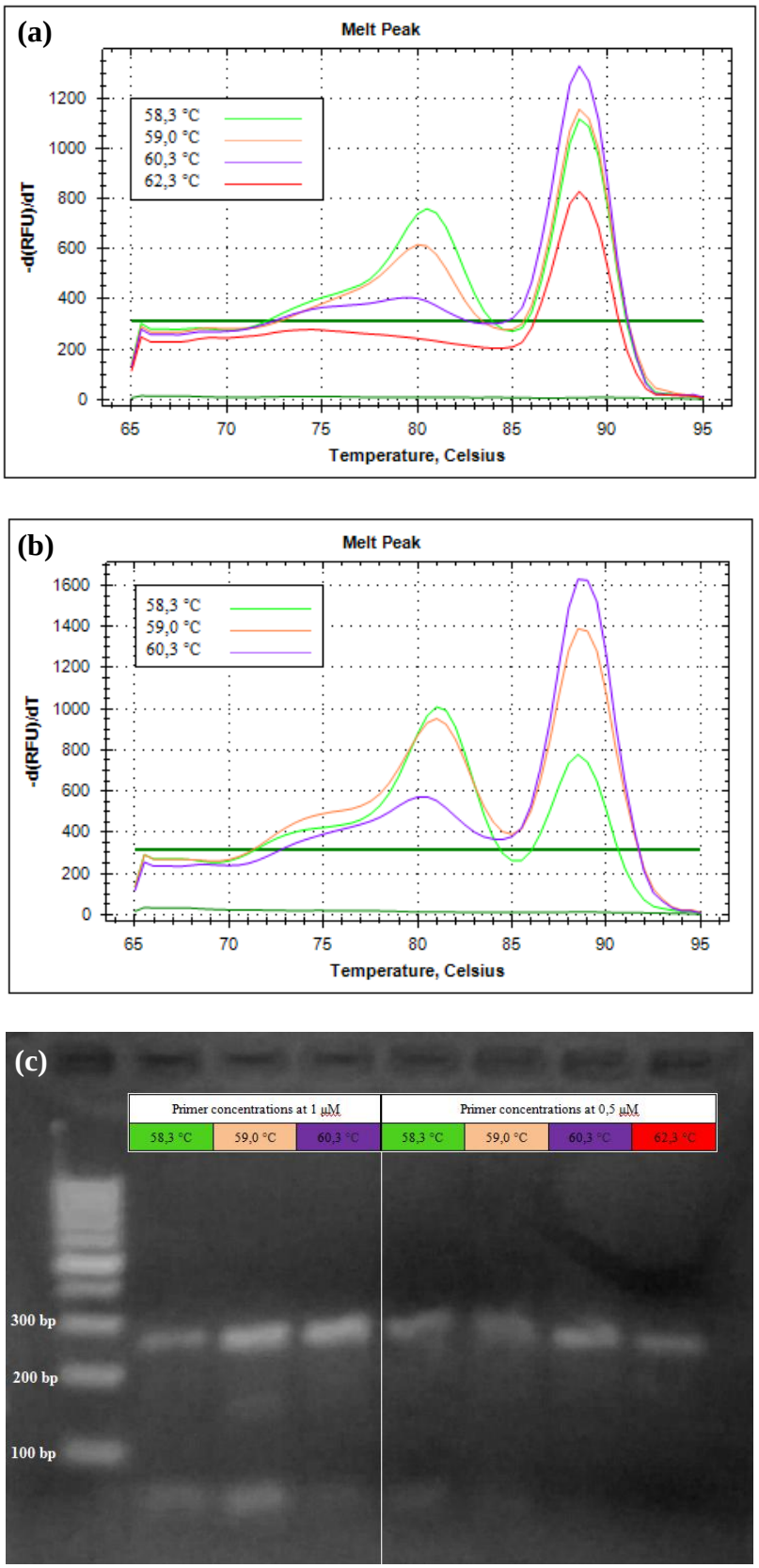

Figure 3.1 Primer optimization studies a) annealing temperature at 58,3°C;59 °C 60,3°C; 62,3°C with 0,5 µM primer concentrations. The same reaction conditions were performed in b) except the annealing temperature of 62,3°C. The primer concentrations were 1 µM c) Both sets of reactions examined under UV by gel (2.5%) electrophoresis.

3.2 qPCR USING LIGHTCYCLER® 480 PROBES MASTER (LPM)

3.2.1 LPM with TaqMan Probes

The real time assay using standard recommended manufacturer's protocol with TaqMan probes was evaluated at DNA concentration 10 ng/µl. The assay indicated that the desired product was generated since the probe is specific to the target DNA. Later on, an agarose gel electrophoresis was also performed to verify the data monitored on the real-time experiment as shown in Figure 3.2.

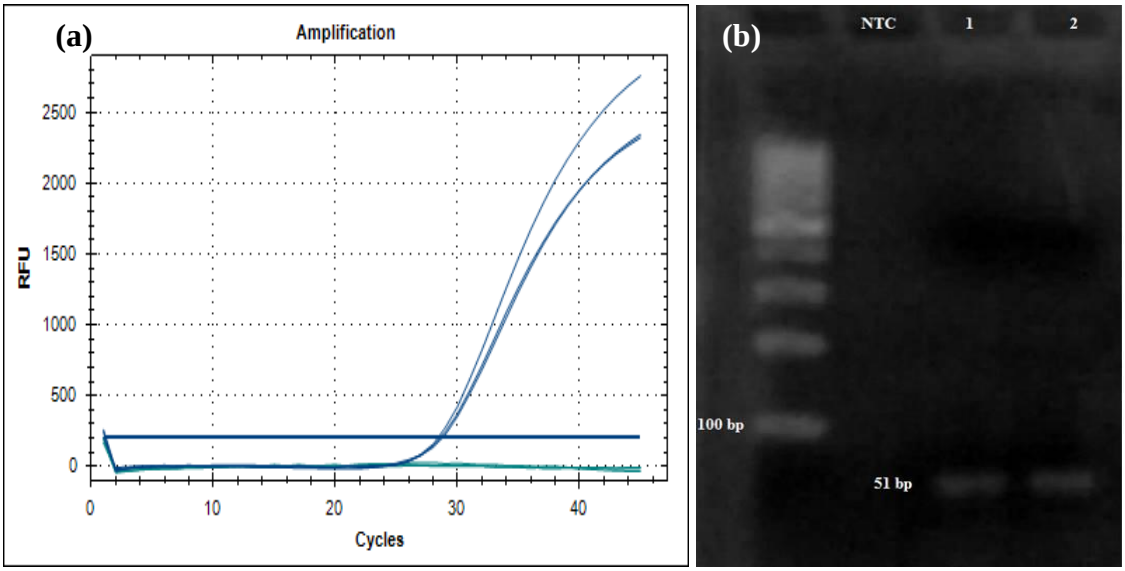

Figure 3.2 Use of LPM with probe in Real-time PCR a) The amplification curve of LPM with probe. The reaction was carried out in triplicates. b) Visualisation of the reaction under UV by agarose gel (2.5%) electrophoresis.

3.2.2 LPM with EvaGreen Dye

EvaGreen dye was used instead of DNA probes. The results have shown that the C_T values of both assays are nearly the same, so it does not inhibit the reaction when added. The target DNA has melted at 82,50˚C (see Figure 3.3).

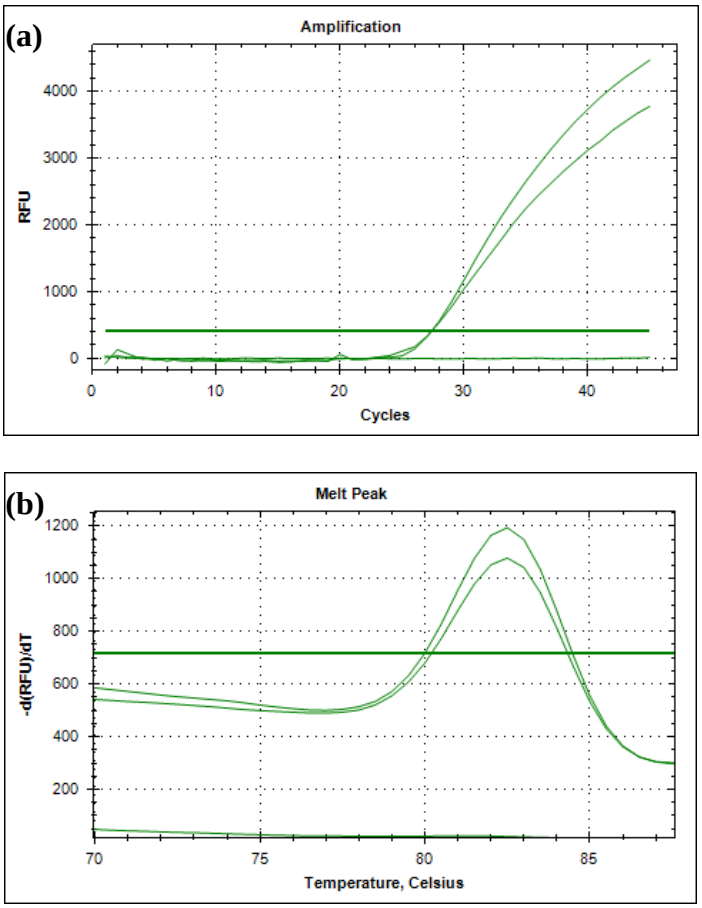

Figure 3.3 Real time PCR experiment by using LPM with EvaGreen dye a) The amplification curves of the product. b) The melt curve of the reaction in a) which gave peak at 82,50 ℃.

For EvaGreen real-time PCR assay; the optimal primer concentrations (200 nM 300 nM 400 nM 500 nM and 1000 nM) were determined. So, the best result was obtained when each primers was at 1µM which provided the lowest C_T value (data not shown).

3.2.2.1 Construction of the Standard Curve

10-fold serial dilutions from *R. solanacearum* genomic DNA was carried out. The assay accuracy was assessed by standard dilution and running of the sample in three replicates. A linear Standard curve of the logarithm of the template quantity against the C_T was constructed. Its slope was -3.222 while its correlation coefficient (r^2) was 0.982 demonstrated an efficiency of 104 %. The real-time PCR system were linear over a range of six logarithms (see Figure 3.4).

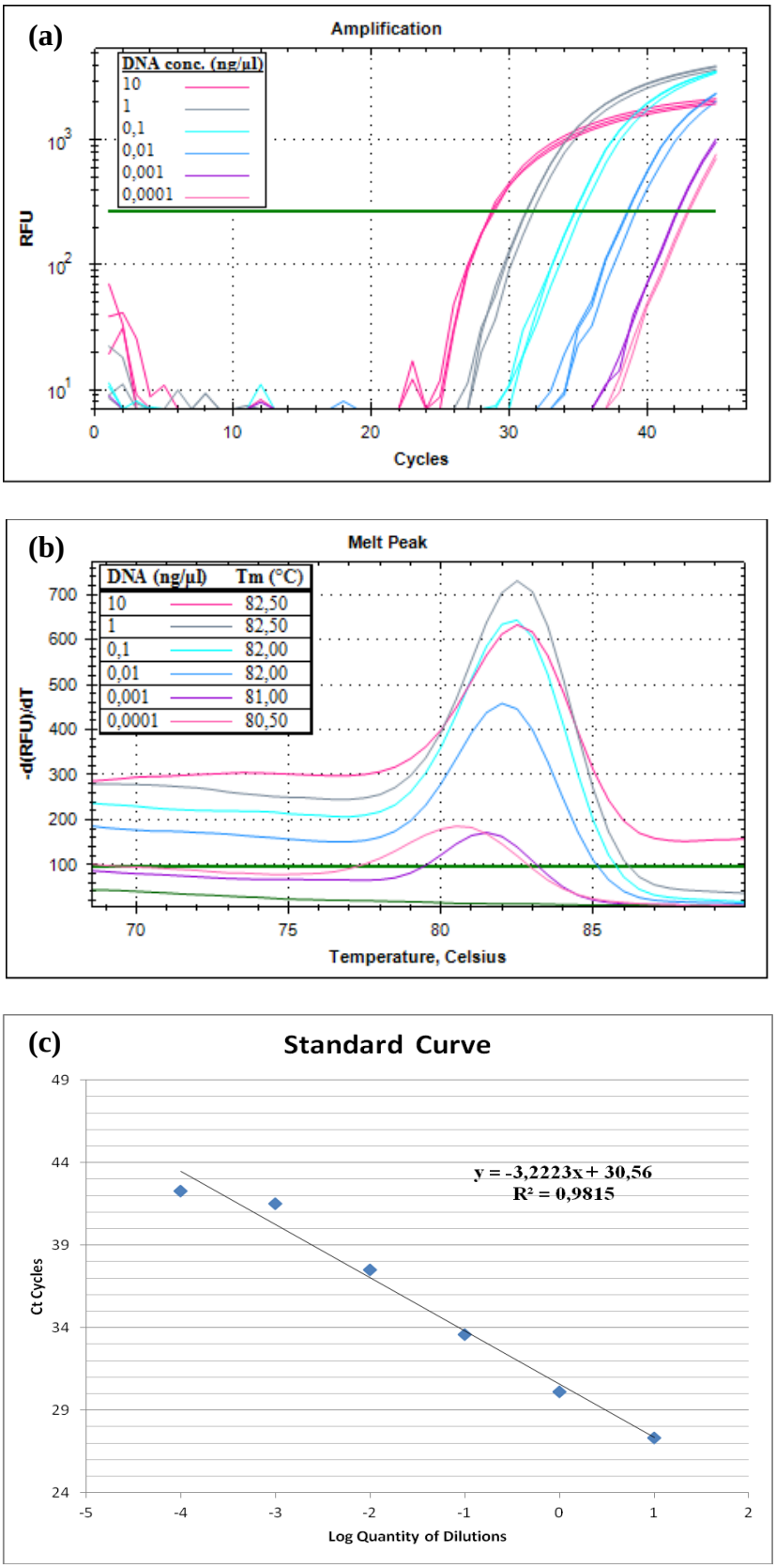

Figure 3.4 Logarithmic dilutions of genomic DNA of *R. solanacearum* and the construction of standard curve a) the amplification curve of the diluted sample (10^{1}-10^{-4}). b) The melt curve of the reaction in b). c) the standard curve of the diluted samples.

Table 3.1 Titration data for LPM-EvaGreen system using *R. solanacearum* race 3 biovar 2 specific primers. Purified *R. solanacearum* genomic DNA was serially diluted and then three replicates of each sample were run using the same polymerase chain cycling conditions. C_T values = cycle threshold values. StDev = Standard deviation.

Sample no.	DNA(pg)	C_T Value	StDev
1	10,000	27.34	0.05
2	1,000	30.12	0.25
3	100	33.60	0.36
4	10	37.52	0.38
5	1	41.49	0.27
6	0,1	42.29	1.60

3.2.2.2 Specificity Test of EvaGreen Real-time PCR Assay

EvaGreen real-time PCR assay of 12 samples was carried out to check the specificity of the primer pair Rsc-F/Rsc-R. No amplification curves were observed in the reactions with *Bacillus subtilis* and *Pseudomonas aeruginosa*. On the contrary, the rest of non-related bacteria was also amplified and gave different melt peaks.

A single peak at 82,50 ˚C for *R. solanacearum* revealed from the analysis of the melting (dissociation) curve, while no peaks were observed for the other bacteria used in this experiment at that temperature (data not shown).

3.3 qPCR USING 5X HOT FIREPOL® EVAGREEN®qPCR MIX PLUS (ROX)

The experiment was performed by using HOT FIREPol® EvaGreen®qPCR Mix Plus (ROX). The melt peak has shown at 82,50 ˚C which was the same as the one carried out by LPM with EvaGreen dye (see Figure 3.5). The data shown below indicates that the product was amplified in the reaction. An agarose gel electrophoresis was carried out to confirm the results obtained from this experiment and compared with the other reactions with two different master mixes mentioned above (see Figure 3.6).

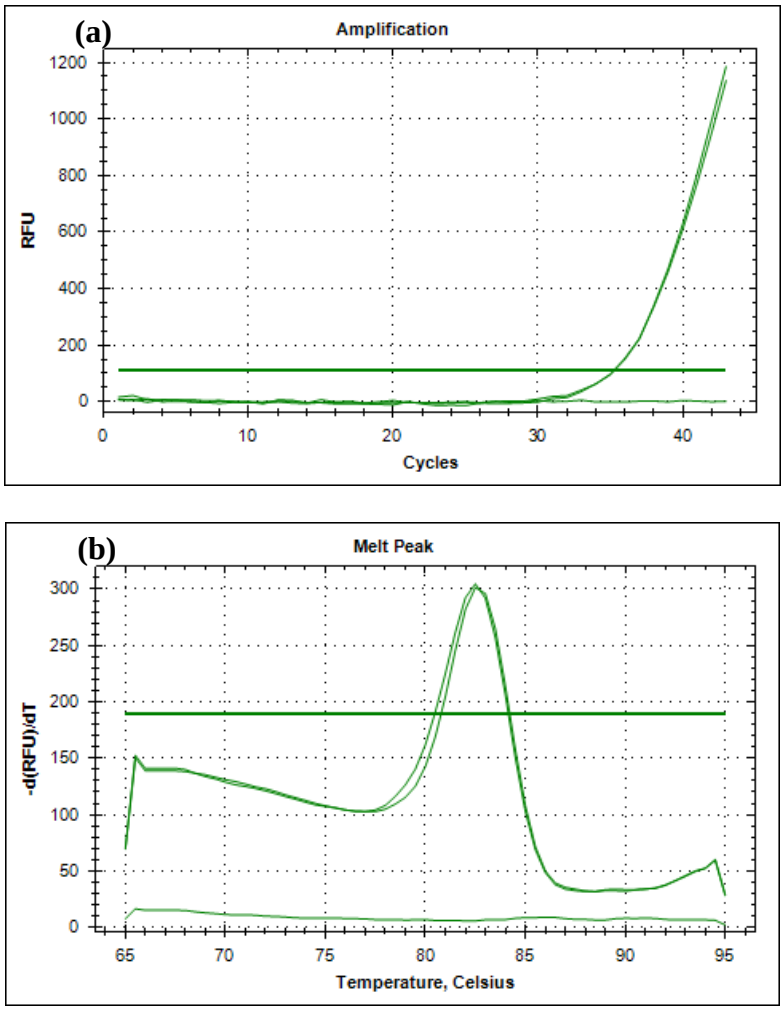

Figure 3.5 Real time PCR experiment by using HOT FIREPol® EvaGreen®qPCR Mix Plus (ROX) a) The amplification curve of the product. b) The melt curve of the reaction in a).

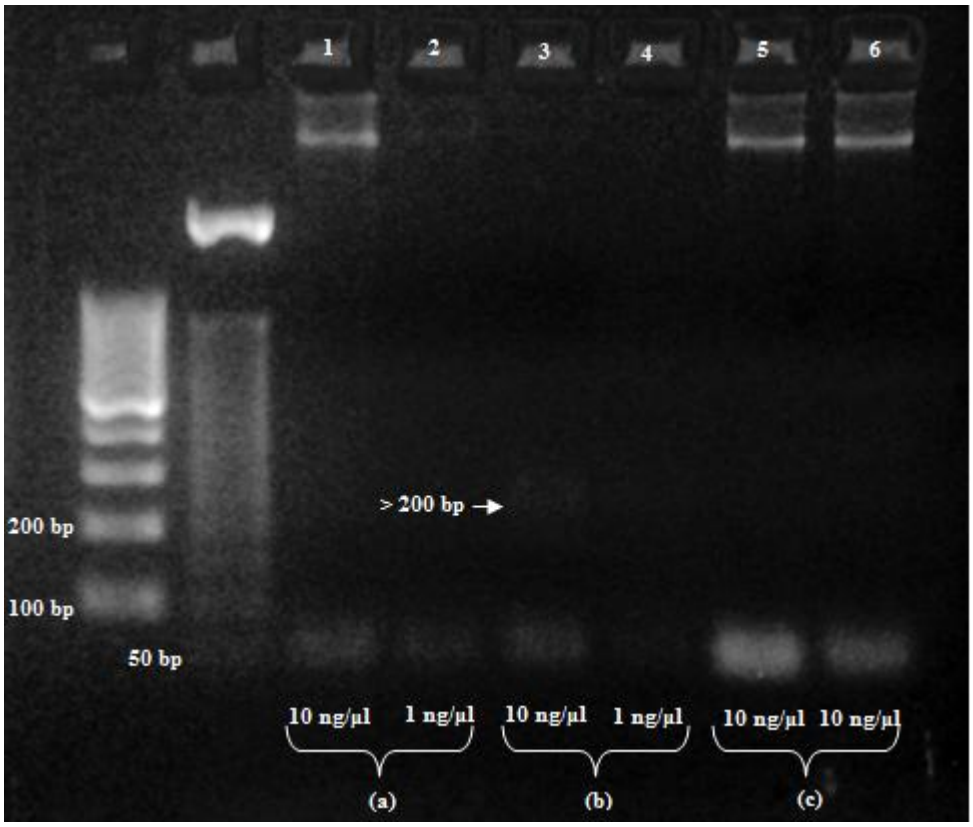

Figure 3.6 Agarose gel electrophoresis of the reactions carried out with three different mastermixesa) in lanes 1 and 2, the real-time PCR reactions with 10 ng/µl and 1 ng/µl template DNA concetrations by using HOT FIREPol® EvaGreen®qPCR Mix Plus (ROX) b) Lanes 3 and 4 show the amplification products from 10 ng/µl and 1 ng/µl template DNA concetrations by using SsoFast™ EvaGreen® Supermix. c) Amplified target DNA by LPM with EvaGreen dye (Lanes 5 and 6).

3.4 EVAGREEN REAL-TIME PCR ASSAY OF ISOLATES FROM ALPU

We have carried out the EvaGreen real time PCR assay on the 5 isolates from Alpu (a district of Eskişehir Province) given by Directorate of İstanbul Agricultural Quarantine Laboratories. All the isolates were amplified and gave melt peak at 82,50˚C (see Figure 3.7). That means they all belong to phylotype IIB sequevar 1 (race3 biovar 2).

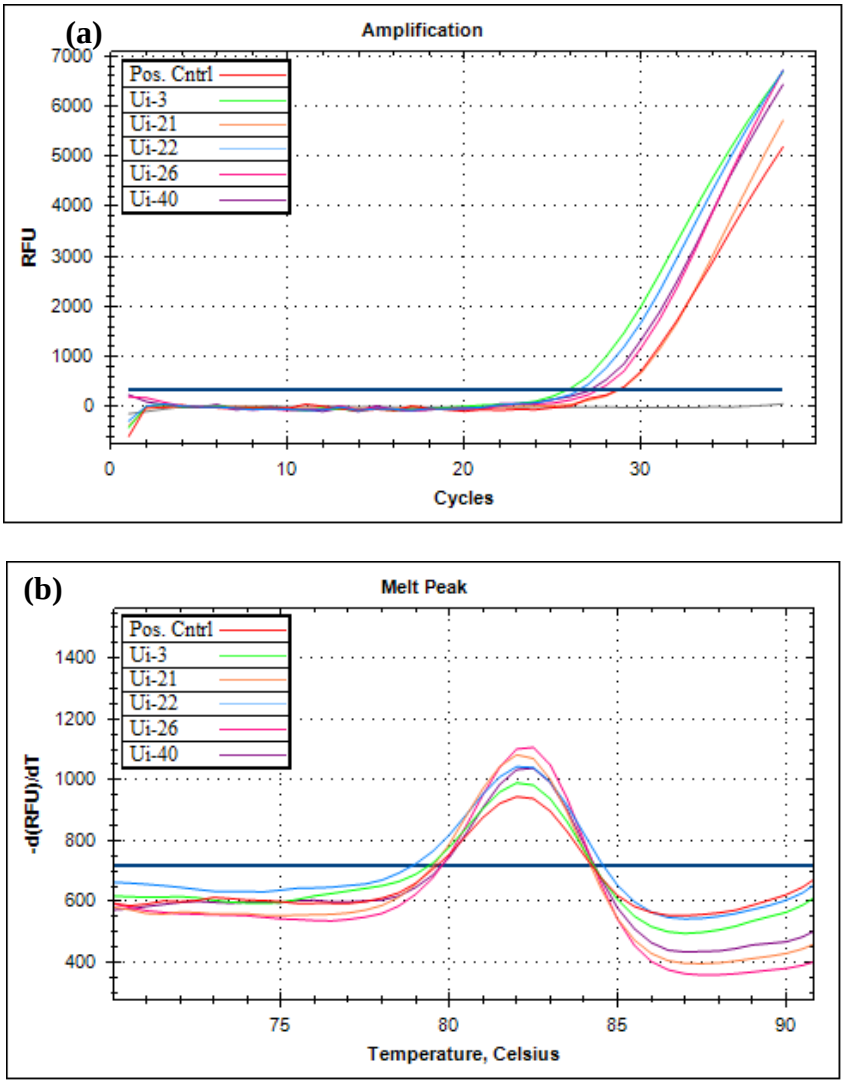

Figure 3.7 Real time PCR assay of samples from Eskişehir (Alpu) by using LPM with EvaGreen dye a) The amplification curve of the products. b) The melt curve of the reaction in a).

CHAPTER 4

DISCUSSIONS & CONCLUSION

4.1 DISCUSSION

Two different commercial DNA extraction kits were used; High Pure Template DNA purification kit, enzymatic lysis with column based isolation, and Thermo Magjet kit, enzymatic lysis with magnetic bead purification. DNA quality (purity and intactness) was measured using UV spectroscopy, in order to assess the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios which are markers of different contaminants. A_{260}/A_{280} ratios (above and below 1.8 and 2.0) are indicator of RNA and protein contamination whereas A_{260}/A_{230} below accepted range (1.8 to 2.2) is an indicator of phenol, salt, protein, and polysaccharide contamination. In both isolation kits, A_{260}/A_{280} ratios were nearly 1,8 and A_{260}/A_{230} ratio were 1,9 or above showed that DNA quality within the accepted range in the samples [107]. The reason of having the good DNA quality may be that the bacterial DNA was isolated from pure culture. So, less clear results would be derived if the bacterial DNA was directly isolated from the environmental samples (soil, water, plant material etc.) [90]. In that case the DNA isolation method used in the experiment would be much more important since in practice inhibition problems are reducing the theoretical sensitivity of molecular detection [107]. If the contaminants are not removed in the DNA isolation step, the melt characteristics of DNA template may also be affected in samples and cause to misinterpretation of results.

The situation specifically occurs when home-brew DNA purification methods are used. In such methods various salts or ethanols that are used for precipitation and washing of pellets. The typical contaminants that home-brew DNA purification methods consist are NaCl, KOAc, EDTA, ETOH, isopropanol, sodium citrate, phenol. The presence of contaminants may give clear shifts in melt peaks . For example; salts

increase the Tm whereas ethanol decrases Tm [91]. So, the effect may be easily observed when the samples are diluted. Since the substances in the crude extract are also reduced by dilution and therefore Tm changes can occur. In this experiment, the commercial DNA extraction kits were used in order to obtain high quality and quantity of DNA template. Even though the A_{260}/A_{280} and A_{260}/A_{230} ratios were around the optimal values and amplification curves were in the expected C_T numbers in the dilution experiment, Tm began to decrease when the DNA template was diluted below 1 ng/µl (see Figure 3.4/section b). The reason may be that Tm is very sensitive to subtle differences occur in the buffer when samples are diluted. DNA concentration might also directly or indirectly influence on melt peaks. So, in normal PCR reactions it is recommended that all DNA samples should be quantified and then adjusted to the same concentration using the same dilution buffer [91].Consequently, DNA quality and purity has a key role when determining Limit of Detection (LOD) process and suboptimal assay performance [107].

Three different master mixes including or with EvaGreen dye were used. Each PCR reactions were carried out according to the manufacturer's recommended protocols. 40 cycles of PCR amplification and then melting-curve analysis (using 0,5 ° C temperature increments with 5 s hold in each step) were carried out. The melt-curve analysis is a method to distinguish double-stranded DNA (dsDNA) based on their melting (disassociation) behavior which is a transition from dsDNA to single-stranded DNA (ssDNA) as Tm increases. Amplification of the product is processed in PCR reactions before melting-curve analysis. A specific single melt peak of PCR products is generated according to the nucleotide sequence, length, G+C content, and strand complementarity. Thus, one specific PCR product shows single melt-peak whereas multiple peaks of melting temperature may be a sign of the presence of non-specific products in addition to the specific one [108].

Interestingly, the DNA amplification reaction with SsoFast^{TM} EvaGreen® Supermix gave two melt peaks instead of one as expected. In order to optimize the reaction, primer concentrations were reduced to 0,5 µM and primer annealing temperature was increased from 58℃ to 62,5℃. Surprisingly the data showed that as the temperature increased, a single peak was obtained from the unexpected non-specific product which was then resolved on 2.5 % gel electrophoresis. When the reaction was

set up with the LPM using either probe or EvaGreen dye a single desired product was amplified showed that the primers were actually specific to the target DNA. We evaluated the result with HOT FIREPol® EvaGreen®qPCR Mix Plus (ROX) to check if the condition is the same as with the LPM reaction or not. The target DNA was successfully amplified without generating any non-specific products. So, this result can be shown as a supportive data to the LPM.

It has been concluded that the DNA polymerase (Sso7d fusion polymerase) in ssofast mix might be the causative factor for generating two products. It is conjugated with polypeptides called Sso7 DNA binding domain which has a non-specific double-stranded binding activity therefore providing increased processivity. Eventhough non-specific amplification activity of the Sso7 polymerase conjugate protein has been reduced by amino acid sequence substitutions in order to obtain improved specificity to the target sequences [108]. We suspect that the modified enzyme may be tolerable to a few primer base mismatches thus it generates the non-specific product in qPCR reaction. But further studies are required to be done to verify this condition.

TaqMan probes previously designed by Özakman et al.[98] were also used to ensure that the mastermix amplify the product since they highly specific to bind target sequence. LightCycler® 480 Probes Master was used with probe according to manufacturer's instructions. The target sequence was successfully amplified and then resolved on 2.5 % gel electrophoresis. The slightly modified protocol by using the same mastermix was performed but this time EvaGreen dye was used instead of TaqMan probe. The melting-curve analysis was used to evaluate PCR specificity and a single product was generated and then melted at 82,50 ˚ C which was later visualized and confirmed under UV by 2.5 % gel electrophoresis. When the reactions with EvaGreen dye and with probe were compared, the C_T values were very similar to each other and cycle differences between replicates were below 0.5 indicating that the reactions were efficiently amplified. Thus, it has been deduced that the EvaGreen dye does not interfere the reaction efficiency when added to LPM. The sensitivity of EvaGreen real-time PCR assay was evaluated. The standard curve using LPM with EvaGreen dye was constructed. The DNA template was serially diluted from 10 ng/µl to 100 fg /µl. Reactions were run in triplicates. A slope of -3.222 and R^2>0.981 demonstrated an efficiency of 104 % which showed very satisfactory linear fit curve.

PCR specificity was evaluated by the melting curve analysis. Although a single peak was derived at 82.5℃ for *R. solanacearum* and was not exhibited in other non-related bacteria, some products were generated and disassociated at different melt temperatures in some of other bacteria. Recently, in silico analysis [109] and Comparative Genomic Hybridization (CGH) studies [92] found specific sequences from brown rot strains of *R. solanacearum* and different primer pairs targeting these sequences were proposed. Thus, the primers used in this study can be replaced by one those primer pairs from the published articles [92, 109]. Then, the primers can be evaluated for degree of specifity and optimized for the EvaGreen real-time PCR assay.

Moreover, qPCR was mostly performed on Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. It has quite robust, with fast ramp times and a user-friendly software program with a flexible interface. Plate setups done in Excel can be imported into the program which provides customization of report printouts, and figures and tables can be easily exported to other programs such Microsoft word, Microsoft powerpoint. In addition, it features thermal gradient functionality which is not found in most of real-time PCR machines from other brands. A few experiments were also carried out by using Rotor-Gene™ 6000 instrument which based on centrifugal air-heated plastic tube format [110]. Since the plastic PCR tubes are used in this machine, it is more practical than the plate-based platforms. It has faster Melting curve (dissociation) analysis especially for High-resolution melt experiments but has poorer data visualization than Bio-Rad CFX96 Touch™ Real-Time PCR Detection System.

4.2 CONCLUSION

EvaGreen dye has been increasingly used since it was released to the market. It can be used in various applications such double-stranded DNA tracing, DNA conformation detection, qPCR, real-time isothermal DNA amplifications, capillary electrophoresis quantitation and disassociation analysis on a Lab-on-Chip. Even though probe based chemistry are more specific to target sequence than this DNA intercalating dye, it is cost-effective and practicable. It reduces assay setup and costs since it only needs two PCR primers to be designed and be synthesized. In addition, EvaGreen technology has many advantages over SYBR Green I. In 2007, its physicochemical

features were compared to SYBR Green I [111].The study showed EvaGreen dye has a higher reproducibility, the low tendency to produce non specific amplification through short dsDNA fragments and a less PCR inhibitory effect. Also, Eischeid et al. [106] has reported its melting peaks are more narrow and higher than SYBR Green I's. So, findings from this work confirmed the efficiency of EvaGreen dye and its application in real-time PCR amplification and melting-curve analysis, which is necessary when DNA intercalating dye is used in a qPCR assay.

In this work, a real-time PCR assay, based on the use of EvaGreen intercalating dye with a primer pair, previously designed [98], has developed for the specific detection and quantification of *R. solanacearum* PIIB-1. We have also confirmed that isolates from Eskişehir (Alpu) are the strains of phylotpe IIB, sequevar 1. In contrast to other identification methods, Evagreen-based quantitative PCR for the detection of *R. solanacearum* is a rapid, reliable and cost effective assay. The application of this technique will be useful for assisting in the elimination of pathways of introduction of this quarantine pathogen and in the management of *R. solanacearum* eradication programs.

REFERENCES

1. FAOSTAT., *Rankings*, 2014, http://faostat3.fao.org/browse/rankings/commodities_by_regions/E.

2. Fiers, M., et al.,"Potato soil-borne diseases. A review", *Agronomy for sustainable development*, Vol. 32, pp. 93-132, 2012.

3. Allen, C., Prior, P., and Hayward, A.C.,*Bacterial Wilt Disease and the Ralstonia Solanacearum Species Complex*, American Phytopathological Society, 2005.

4. Floyd, J., *New Pest Response Guidelines: Ralstonia solanacearum race 3 biovar 2*, 2008, http://www.aphis.usda.gov/import_export/plants/manuals/emergency/downloads/nprg-ralstonia.pdf.

5. López, M.M., et al.,"Are molecular tools solving the challenges posed by detection of plant pathogenic bacteria and viruses?", *Current issues in molecular biology*, Vol. 11, pp. 13, 2009.

6. Anonymous,"Commission Directive 2006/63/EC of 14 July 2006: amending Annexes II to VII to Council Directive 98/57/EC on the control of R. solanacearum (Smith) Yabuuchi et al.", *Off J Eur Communities*, Vol. L206, pp. 36-106, 2006.

7. Kelman, A.,"The bacterial wilt caused by Pseudomonas solanacearum", *Technical Bulletin of North Carolina Agricultural Experiment Station*, 1953.

8. Pötter, M., et al.,"The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in Ralstonia eutropha", *Microbiology*, Vol. 150, pp. 2301-2311, 2004.

9. EPPO,"Diagnostic protocol for regulated pests. Ralstonia solanacearum", *EPPO Bull.*, Vol. 34, pp. 173-78, 2004.

10. Hayward, A.,"Biology and epidemiology of bacterial wilt caused by Pseudomonas solanacearum", *Annual review of phytopathology*, Vol. 29, pp. 65-87, 1991.

11. Denny, T., et al.,"Phenotype conversion of Pseudomonas solanacearum: its molecular basis and potential function", *Bacterial wilt: the disease and its causative agent, Pseudomonas solanacearum.*, pp. 137-155, 1994.

12. Poussier, S., et al.,"Host plant-dependent phenotypic reversion of Ralstonia solanacearum from non-pathogenic to pathogenic forms via alterations in the phcA gene", *Molecular microbiology*, Vol. 49, pp. 991-1003, 2003.

13. Brumbley, S.M. and Denny, T.P.,"Cloning of wild-type Pseudomonas solanacearum phcA, a gene that when mutated alters expression of multiple traits that contribute to virulence", *Journal of Bacteriology*, Vol. 172, pp. 5677-5685, 1990.

14. Kelman, A.,"The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in a tetrazolium medium", *Phytopathology*, Vol. 44, pp. 693-695., 1954.

15. Yabuuchi, E., et al.,"Transfer of Two Burkholderia and An Alcaligenes Species to Ralstonia Gen. Nov", *Microbiology and immunology*, Vol. 39, pp. 897-904, 1995.

16. Fegan, M. and Prior, P.,"How complex is the Ralstonia solanacearum species complex", 2005.

17. Gillings, M. and Fahy, P.,"Genomic fingerprinting: towards a unified view of the Pseudomonas solanacearum species complex", 1994.

18. Taghavi, M., et al.,"Analysis of the phylogenetic relationships of strains of Burkholderia solanacearum, Pseudomonas syzygii, and the blood disease bacterium of banana based on 16S rRNA gene sequences", *International Journal of Systematic Bacteriology*, Vol. 46, pp. 10-15, 1996.

19. Allen, C., Prior, P., and Hayward, A.,*Bacterial wilt disease and the Ralstonia solanacearum species complex*, American Phytopathological Society (APS Press), 2005.

20. Buddenhagen, I., Sequeira, L., and Kelman, A.,"Designation of Races in *Pseudomonas-solanacearum*", *Phytopathology*, Vol. 52, pp. 726, 1962.

21. Hayward, A.,"Characteristics of Pseudomonas solanacearum", *Journal of Applied Bacteriology*, Vol. 27, pp. 265-277, 1964.

22. Hayward, A.,"Systematics and phylogeny of Pseudomonas solanacearum and related bacteria", 1994.

23. Prior, P. and Fegan, M.,"Recent Developments in the Phylogeny and Classification of Ralstonia solanacearum", *Acta horticulturae*, 2005.

24. Poussier, S., et al.,"Partial sequencing of the hrpB and endoglucanase genes confirms and expands the known diversity within the Ralstonia solanacearum species complex", *Systematic and applied microbiology*, Vol. 23, pp. 479-486, 2000.

25. Wicker, E., et al.,"Contrasting recombination patterns and demographic histories of the plant pathogen Ralstonia solanacearum inferred from MLSA", *The ISME journal*, Vol. 6, pp. 961-974, 2012.

26. N'Guessan, C.A., et al.,"Development of variable number of tandem repeats typing schemes for Ralstonia solanacearum, the agent of bacterial wilt, banana Moko disease and potato brown rot", *Journal of microbiological methods*, Vol. 92, pp. 366-374, 2013.

27. Salanoubat, M., et al.,"Genome sequence of the plant pathogen Ralstonia solanacearum", *Nature*, Vol. 415, pp. 497-502, 2002.

28. Boucher, C.A., Barberis, P.A., and Demery, D.A.,"Transposon mutagenesis of Pseudomonas solanacearum: isolation of Tn5-induced avirulent mutants", *Journal of general Microbiology*, Vol. 131, pp. 2449-2457, 1985.

29. Genin, S. and Boucher, C.,"Ralstonia solanacearum: secrets of a major pathogen unveiled by analysis of its genome", *Molecular plant pathology*, Vol. 3, pp. 111-118, 2002.

30. Genin, S. and Boucher, C.,"Lessons learned from the genome analysis of Ralstonia solanacearum", *Annu. Rev. Phytopathol.*, Vol. 42, pp. 107-134, 2004.

31. Elsas, J.D.v., et al.,"Effects of ecological factors on the survival and physiology of Ralstonia solanacearum bv. 2 in irrigation water", *Canadian Journal of Microbiology*, Vol. 47, pp. 842-854, 2001.

32. Gabriel, D.W., et al.,"Identification of open reading frames unique to a select agent: Ralstonia solanacearum race 3 biovar 2", *Molecular Plant-Microbe Interactions*, Vol. 19, pp. 69-79, 2006.

33. Remenant, B., et al.,"Genomes of three tomato pathogens within the Ralstonia solanacearum species complex reveal significant evolutionary divergence", *BMC genomics*, Vol. 11, pp. 379, 2010.

34. González, A., et al.,"Detection and functional characterization of a large genomic deletion resulting in decreased pathogenicity in Ralstonia solanacearum race 3 biovar 2 strains", *Environmental microbiology*, Vol. 13, pp. 3172-3185, 2011.

35. Elphinstone, J., Stanford, H., and Stead, D., *Detection of Ralstonia solanacearum in potato tubers, Solanum dulcamara and associated irrigation water, Bacterial Wilt Disease*, Springer, pp. 133-139, 1998.

36. Anonymous,"Council Directive 98/57/EC of 20 July 1998 on the control of R. solanacearum (Smith) Yabuuchi et al.", *Off J Eur Communities*, Vol. L235, pp. 1-39, 1998.

37. Hayward, A. and Hartman, G.L.,*Bacterial wilt: the disease and its causative agent, Pseudomonas solanacearum*, CAB International, 1994.

38. EPPO, *PQR-EPPO database on quarantine pest*, 2014, http://www.eppo.int

39. Swanson, J.K., et al.,"Behavior of Ralstonia solanacearum race 3 biovar 2 during latent and active infection of geranium", *Phytopathology*, Vol. 95, pp. 136-143, 2005.

40. Williamson, L., et al.,"Ralstonia solanacearum race 3, biovar 2 strains isolated from geranium are pathogenic on potato", *Plant Disease*, Vol. 86, pp. 987-991, 2002.

41. Ciampi, L., Sequeira, L., and French, E.,"Latent infection of potato tubers byPseudomonas solanacearum", *American Potato Journal*, Vol. 57, pp. 377-386, 1980.

42. Araud-Razou, I., et al.,"Detection and visualization of the major acidic exopolysaccharide of Ralstonia solanacearum and its role in tomato root infection and vascular colonization", *European journal of plant pathology*, Vol. 104, pp. 795-809, 1998.

43. Vasse, J., Frey, P., and Trigalet, A.,"Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by Pseudomonas solanacearum", *Molecular plant-microbe interactions: MPMI (USA)*, 1995.

44. Genin, S.,"Molecular traits controlling host range and adaptation to plants in Ralstonia solanacearum", *New Phytologist*, Vol. 187, pp. 920-928, 2010.

45. Janse, J.,"Potato brown rot in Western Europe–history, present occurrence and some remarks on possible origin, epidemiology and control strategies", *EPPO Bulletin*, Vol. 26, pp. 679-695, 1996.

46. van Overbeek, L.S., et al.,"The low-temperature-induced viable-but-nonculturable state affects the virulence of Ralstonia solanacearum biovar 2", *Phytopathology*, Vol. 94, pp. 463-469, 2004.

47. Oliver, J.D., *Formation of viable but nonculturable cells*, *Starvation in bacteria*, Springer, pp. 239-272, 1993.

48. Grey, B.E. and Steck, T.R.,"The viable but nonculturable state ofRalstonia solanacearum may be involved in long-term survival and plant infection", *Applied and environmental microbiology*, Vol. 67, pp. 3866-3872, 2001.

49. van Elsas, J.D., et al.,"Survival of Ralstonia solanacearum biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates", *Phytopathology*, Vol. 90, pp. 1358-1366, 2000.

50. Álvarez, B., Biosca, E.G., and López, M.M.,"On the life of Ralstonia solanacearum, a destructive bacterial plant pathogen", *Current research, technology and education topics in applied microbiology and microbial biotechnology. Volume*, Vol. 1, 2010.

51. Burgess, G.,"Bacteria in oligotrophic environments: starvation survival lifestyle", *World Journal of Microbiology and Biotechnology*, Vol. 14, pp. 305-305, 1998.

52. Álvarez, B., López, M.M., and Biosca, E.G.,"Survival strategies and pathogenicity of Ralstonia solanacearum phylotype II subjected to prolonged starvation in environmental water microcosms", *Microbiology*, Vol. 154, pp. 3590-3598, 2008.

53. Kelman, A. and HRUSCHKA, J.,"The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of Pseudomonas solanacearum", *Journal of general Microbiology*, Vol. 76, pp. 177-188, 1973.

54. Yao, J. and Allen, C.,"The plant pathogen Ralstonia solanacearum needs aerotaxis for normal biofilm formation and interactions with its tomato host", *Journal of Bacteriology*, Vol. 189, pp. 6415-6424, 2007.

55. Agrios, G.N.,"Plant pathology", *New York: Academic*, pp. 146-149, 2005.

56. Saile, E., et al.,"Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by Ralstonia solanacearum", *Phytopathology*, Vol. 87, pp. 1264-1271, 1997.

57. Denny, T.P. and Baek, S.-R.,"Genetic evidence that extracellular polysaccharide is a virulence factor of Pseudomonas solanacearum", *Mol. Plant-Microbe Interact*, Vol. 4, pp. 198-206, 1991.

58. Gijsegem, F., et al.,"The hrp gene locus of Pseudomonas solanacearum, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex", *Molecular microbiology*, Vol. 15, pp. 1095-1114, 1995.

59. Liu, H., et al.,"Pyramiding unmarked deletions in Ralstonia solanacearum shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence", *Molecular Plant-Microbe Interactions*, Vol. 18, pp. 1296-1305, 2005.

60. Tans-Kersten, J., Brown, D., and Allen, C.,"Swimming motility, a virulence trait of Ralstonia solanacearum, is regulated by FlhDC and the plant host environment", *Molecular Plant-Microbe Interactions*, Vol. 17, pp. 686-695, 2004.

61. Peeters, N., et al.,"Ralstonia solanacearum, a widespread bacterial plant pathogen in the post-genomic era", *Molecular plant pathology*, Vol. 14, pp. 651-662, 2013.

62. Hikichi, Y., et al.,"Global regulation of pathogenicity mechanism of Ralstonia solanacearum", *Plant Biotechnology*, Vol. 24, pp. 149-154, 2007.

63. Elphinstone, J., et al.,"The current bacterial wilt situation: a global overview", *Bacterial wilt disease and the Ralstonia solanacearum species complex*, pp. 9-28, 2005.

64. Persley, G. "Ecology of Pseudomonas solanacearum, the causal agent of bacterial wilt". *Bacterial wilt disease in Asie and the South Pacific. Proceedings of an International Workshop held at PCARRD, Los Banos, Philippines, 8-10 October 1985*. 1986 pp. 71-76.

65. Nakaho, K., et al.,"Distribution and multiplication of Ralstonia solanacearum in tomato plants with resistance derived from different origins", *Journal of General Plant Pathology*, Vol. 70, pp. 115-119, 2004.

66. Bohlmann, H. and Broekaert, W.,"The role of thionins in plant protection", *Critical reviews in plant sciences*, Vol. 13, pp. 1-16, 1994.

67. Jaynes, J.M., et al.,"Increasing bacterial disease resistance in plants utilizing antibacterial genes from insects", *BioEssays*, Vol. 6, pp. 263-270, 1987.

68. Broekaert, W.F., et al.,"Antimicrobial peptides from plants", *Critical reviews in plant sciences*, Vol. 16, pp. 297-323, 1997.

69. VUNNAM, S., JUVVADI, P., and Merrifield, R.,"Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine", *The Journal of peptide research*, Vol. 49, pp. 59-66, 1997.

70. Nakajima, H., et al.,"Fungal and bacterial disease resistance in transgenic plants expressing human lysozyme", *Plant Cell Reports*, Vol. 16, pp. 674-679, 1997.

71. Hartman, G., Hong, W., and Hayward, A. "Potential of biological and chemical control of bacterial wilt". *ACIAR Proceedings*. 1993 pp. 322-322, Australian Centre for International Agricultural Research 1993.

72. Autrique, A. and Potts, M.,"The influence of mixed cropping on the control of potato bacterial wilt (Pseudomonas solanacearum)", *Annals of Applied Biology*, Vol. 111, pp. 125-133, 1987.

73. Grimault, V., Schmit, J., and Prior, P. "Some characteristics involved in bacterial wilt (Pseudomonas solanacearum) resistance in tomato". *ACIAR Proceedings*.Australia 1993 pp. 112-119, 1993.

74. Pradhanang, P., et al.,"Effects of plant essential oils on Ralstonia solanacearum population density and bacterial wilt incidence in tomato", *Plant Disease*, Vol. 87, pp. 423-427, 2003.

75. Azcón-Aguilar, C. and Barea, J.,"Arbuscular mycorrhizas and biological control of soil-borne plant pathogens–an overview of the mechanisms involved", *Mycorrhiza*, Vol. 6, pp. 457-464, 1997.

76. Fujiwara, A., et al.,"Biocontrol of Ralstonia solanacearum by treatment with lytic bacteriophages", *Applied and environmental microbiology*, Vol. 77, pp. 4155-4162, 2011.

77. Guo, J.-H., et al.,"Biocontrol of tomato wilt by plant growth-promoting rhizobacteria", *Biological Control*, Vol. 29, pp. 66-72, 2004.

78. French, E.B., et al.,"Culture media for Ralstonia solanacearum isolation, identification and maintenance", *Fitopatologia*, Vol. 30, pp. 126-130, 1995.

79. Ito, S., et al.,"Detection of viable cells of Ralstonia solanacearum in soil using a semiselective medium and a PCR technique", *Journal of Phytopathology*, Vol. 146, pp. 379-384, 1998.

80. Pradhanang, P., Elphinstone, J., and Fox, R.,"Sensitive detection of Ralstonia solanacearum in soil: a comparison of different detection techniques", *Plant Pathology*, Vol. 49, pp. 414-422, 2000.

81. Caruso, P., et al.,"Enrichment double-antibody sandwich indirect enzyme-linked immunosorbent assay that uses a specific monoclonal antibody for sensitive detection of Ralstonia solanacearum in asymptomatic potato tubers", *Applied and environmental microbiology*, Vol. 68, pp. 3634-3638, 2002.

82. Caruso, P., et al.,"A new and sensitive co-operational polymerase chain reaction for rapid detection of Ralstonia solanacearum in water", *Journal of microbiological methods*, Vol. 55, pp. 257-272, 2003.

83. Dittapongpitch, V. and Surat, S.,"Detection of Ralstonia solanacearum in soil and weeds from commercial tomato fields using immunocapture and the polymerase chain reaction", *Journal of Phytopathology*, Vol. 151, pp. 239-246, 2003.

84. Peckham, G.D., et al.,"Immuno-capture of Ralstonia solanacearum by an EPS-specific monoclonal antibody enhances sensitivity of PCR", *Phytopathology*, Vol. 99, pp. S101-S101, 2009.

85. Machmud, M. and Suryadi, Y.,"Detection and identification of Ralstonia solanacearum strains using the indirect ELISA technique", *Indonesian J Agric*, Vol. 1, pp. 13-21, 2008.

86. Vaneechoutte, M., et al.,"Wautersia gen. nov., a novel genus accommodating the phylogenetic lineage including Ralstonia eutropha and related species, and proposal of Ralstonia [Pseudomonas] syzygii (Roberts et al. 1990) comb. nov", *International journal of systematic and evolutionary microbiology*, Vol. 54, pp. 317-327, 2004.

87. Priou, S., Gutarra, L., and Aley, P.,"An improved enrichment broth for the sensitive detection of Ralstonia solanacearum (biovars 1 and 2A) in soil using DAS–ELISA", *Plant Pathology*, Vol. 55, pp. 36-45, 2006.

88. Palacio-Bielsa, A., Cambra, M., and López, M.,"PCR detection and identification of plant-pathogenic bacteria: updated review of protocols (1989-2007)", *Journal of Plant Pathology*, pp. 249-297, 2009.

89. Pastrik, K.H. and Maiss, E.,"Detection of Ralstonia solanacearum in potato tubers by polymerase chain reaction", *Journal of Phytopathology*, Vol. 148, pp. 619-626, 2000.

90. Poussier, S., et al.,"Evaluation of procedures for reliable PCR detection of Ralstonia solanacearum in common natural substrates", *Journal of microbiological methods*, Vol. 51, pp. 349-359, 2002.

91. Anonymous, *Critical Success Factors for HRM Performance*, 2014, http://www.qiagen.com/tr/resources/technologies/hrm/critical%20success%20factors%20for%20hrm%20performance/.

92. Guidot, A., et al.,"Specific genes from the potato brown rot strains of Ralstonia solanacearum and their potential use for strain detection", *Phytopathology*, Vol. 99, pp. 1105-1112, 2009.

93. Poussier, S. and Luisetti, J.,"Specific detection of biovars of Ralstonia solanacearum in plant tissues by nested-PCR-RFLP", *European journal of plant pathology*, Vol. 106, pp. 255-265, 2000.

94. Norman, D.J., et al.,"Genetic diversity and host range variation of Ralstonia solanacearum strains entering North America", *Phytopathology*, Vol. 99, pp. 1070-1077, 2009.

95. Wullings, B., et al.,"Detection of Ralstonia solanacearum, which causes brown rot of potato, by fluorescent in situ hybridization with 23S rRNA-targeted probes", *Applied and environmental microbiology*, Vol. 64, pp. 4546-4554, 1998.

96. Kubota, R., et al.,"Detection of Ralstonia solanacearum by loop-mediated isothermal amplification", *Phytopathology*, Vol. 98, pp. 1045-1051, 2008.

97. Weller, S., et al.,"Detection of Ralstonia solanacearumStrains with a Quantitative, Multiplex, Real-Time, Fluorogenic PCR (TaqMan) Assay", *Applied and environmental microbiology*, Vol. 66, pp. 2853-2858, 2000.

98. Ozakman, M. and Schaad, N.,"A real-time BIO-PCR assay for detection of Ralstonia solanacearum race 3, biovar 2, in asymptomatic potato tubers", *Canadian Journal of Plant Pathology*, Vol. 25, pp. 232-239, 2003.

99. Anonymous, *PCR Primer and Probe Chemistries*, 2014, http://www.bio-rad.com/en-tr/applications-technologies/pcr-primer-probe-chemistries.

100. Mackay, I.M.,"Real-time PCR in the microbiology laboratory", *Clinical Microbiology and Infection*, Vol. 10, pp. 190-212, 2004.

101. Anderson, T.P., et al.,"Failure to genotype herpes simplex virus by real-time PCR assay and melting curve analysis due to sequence variation within probe binding sites", *Journal of clinical microbiology*, Vol. 41, pp. 2135-2137, 2003.

102. Papin, J.F., Vahrson, W., and Dittmer, D.P.,"SYBR green-based real-time quantitative PCR assay for detection of West Nile Virus circumvents false-negative results due to strain variability", *Journal of clinical microbiology*, Vol. 42, pp. 1511-1518, 2004.

103. Ririe, K.M., Rasmussen, R.P., and Wittwer, C.T.,"Product differentiation by analysis of DNA melting curves during the polymerase chain reaction", *Analytical biochemistry*, Vol. 245, pp. 154-160, 1997.

104. Nath, K., et al.,"Effects of ethidium bromide and SYBR® Green I on different polymerase chain reaction systems", *Journal of biochemical and biophysical methods*, Vol. 42, pp. 15-29, 2000.

105. Giglio, S., Monis, P.T., and Saint, C.P.,"Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR", *Nucleic Acids Research*, Vol. 31, pp. e136-e136, 2003.

106. Eischeid, A.C.,"SYTO dyes and EvaGreen outperform SYBR Green in real-time PCR", *BMC research notes*, Vol. 4, pp. 263, 2011.

107. Olson, N.D. and Morrow, J.B.,"DNA extract characterization process for microbial detection methods development and validation", *BMC research notes*, Vol. 5, pp. 668, 2012.

108. Cheng, M., *SSO7-polymerase conjugates with decreased non-specific activity*, Google Patents. 2014.

109. Kubota, R., et al.,"In silico genomic subtraction guides development of highly accurate, DNA-based diagnostics for Ralstonia solanacearum race 3 biovar 2 and blood disease bacterium", *Journal of General Plant Pathology*, Vol. 77, pp. 182-193, 2011.

110. Josefsen, M.H., et al., *Instrumentation and Fluorescent Chemistries Used in qPCR* in M. Filion(Ed.) *Quantitative real-time PCR in Applied Microbiology*, Horizon Scientific Press2012.

111. Mao, F., Leung, W.-Y., and Xin, X.,"Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications", *BMC biotechnology*, Vol. 7, pp. 76, 2007.