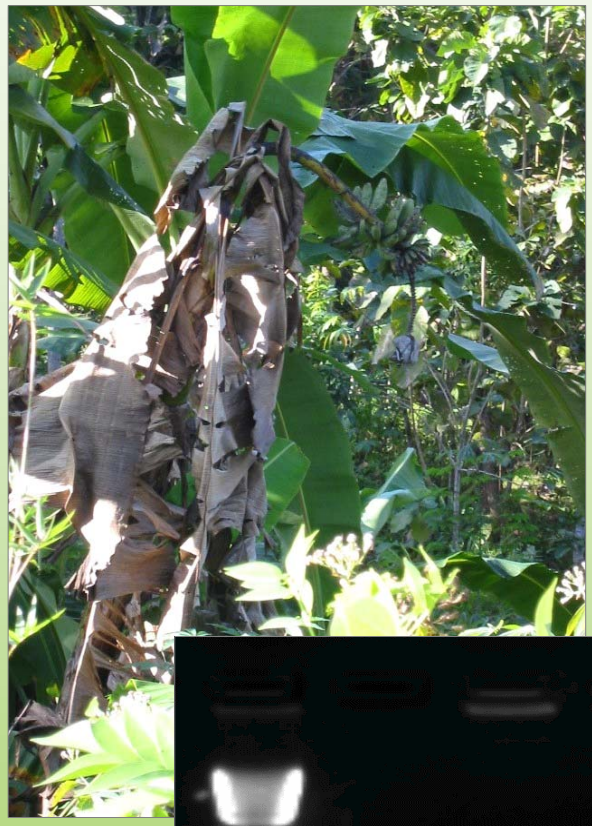
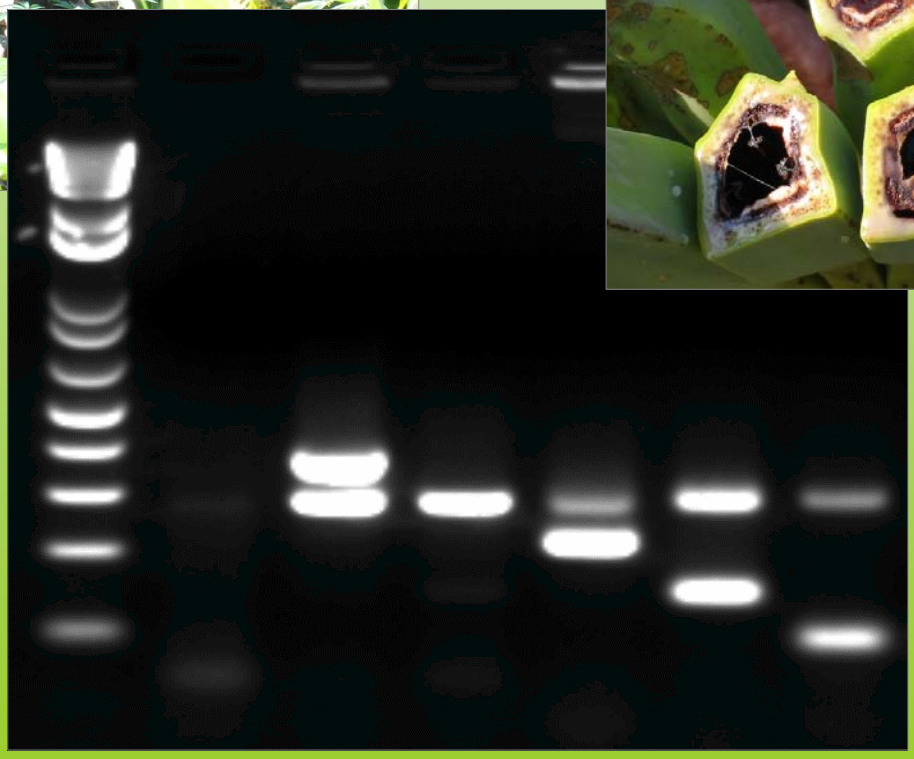
Bacterial Wilt of Banana

Diagnostics Manual





Bacterial Wilt Diagnostics Manual

2006

Table of Contents

1 Introduction

1.1. The Banana Industry in Australia

In 2003 the farm gate value of the Australian banana industry was $295 million dollars. This resulted from more than 1900 growers producing in excess of 300,000 tonnes of bananas from 15,500 Ha. More than 80% of this crop is produced in the wet tropics of Far North Queensland. The remaining 20% are grown predominantly in the subtropical regions of the eastern coast from Nambucca Heads, in New South Wales to Nambour in Queensland. There are also small producing regions in Darwin in the Northern Territory and also Kununurra and Carnarvon in Western Australia.

Approximately 95% of bananas grown in Australia are of the Cavendish subgroup with the remaining 5% comprising mainly the Ladyfinger cultivar but also some Goldfinger, Ducasse, Sucrier, FHIA 18, Red Dacca and Plantains. In Australia sales of banana are mostly that of the fresh fruit product. The market is mature and as such, notwithstanding changes to current trends, should follow population growth.

Currently the only other products from banana fruit and fibre are the small-scale production of banana puree, dried banana, banana wine and handbags. Although at this time there is little value added product emanating from the banana industry the development of a paper production facility at Mareeba, utilising the pseudostems of banana plants to provide the long fibres required for the paper production, has generated much interest by both the local council and also growers interested in supplying the pseudostems.

1.2. History of the Diseases

Although the three diseases Moko, Bugtok and Blood disease are closely related the distribution of each is quite distinct (Figure 1). All three however are limited to the tropics. Moko which has the widest distribution of the three has not been verified as being present in any regions outside of the area between latitudes 18º N and 18º S. Moko disease was first reported in Trinidad in the 1890s when it caused severe losses of Moko cooking bananas (ABB genotype, syn Bluggoe). The causal agent of the disease was described in 1911 by Rorer. Because this initial outbreak of the

disease was so severe the cultivation of Moko cooking bananas was completely abandoned. Due to this, outbreaks of Moko disease were no longer as devastating until the disease reached the large plantations of dessert bananas planted in the 1940s and 1950s when again severe losses were experienced. Much research carried out after this time has lead to good control measures being adopted within these commercial plantings resulting in low disease incidence.

Moko disease does still affect the smallholder farmers however and so has subsequently spread throughout many parts of Latin America and is also present in the Caribbean. It is officially recorded in Belize, Brazil, Colombia, Costa Rica, Ecuador, El Salvadore, Grenada, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Peru, Surinam, Trinidad and Venezuela (Stover 1972; Phelps 1987). In 2004 the disease reached the parish of St James in Jamaica. Restrictions have been placed on the movement of fruit and plants from St James in order to prevent further spread of the disease. An eradication program has also been instigated however to date no details regarding its progress are available. (http://www.radajamaica.com.jm/Technical/mokobanana.htm).

Outside of Central and South America and the Carribean the only other place that the presence of Moko disease has been verified is in the Philippines in the Mindanoa region. It has been postulated that it is likely that it was introduced to the region on banana material brought from Honduras for propagation in 1968 (Rillo 1979; Buddenhagen 1994). There are several reports of Moko from Africa and India although the details given are limited and so are not able to be substantiated (Thwaites, Eden-Green et al. 2000).

Bugtok is limited to the Philippines where it is present in Cardaba and Saba cooking bananas. It is thought that, although the disease is caused by the same pathogen as Moko, the disease, which differs in symptoms, is the result of the host reaction by these clones which are of the Balbisiana genotype.

The distribution of Blood disease is presently limited to Indonesia. The disease was first reported in 1906 in the Saleiren Islands off the coast of Sulawesi (Celebes). Further in investigations by Gäumann in 1921 established that the disease was

extensive throughout Southern Sulawesi. (Gäumann 1921). Quarantine restrictions preventing the exportation of fruit from that region appeared to have prevented spread of the disease until it appeared in West Java in 1987 (Eden-Green and Sastraatmadja 1990). The disease has since spread to Lombok Island, West Nusa Tenggara (Supriadi 2002). There are also reports that the disease has been identified in Sumatra, West Kalimantan the Moluccan Islands and Irian Jaya (Baharuddin 1994). Blood disease is estimated to be spreading at the rate of approximately 25 kilometres per year (Thwaites, Eden-Green et al. 2000; Supriadi 2002).

1.3. Global Distribution of the Diseases

Figure 1: Distribution map showing worldwide distribution of the bacterial wilt diseases of banana, Moko, Bugtok and Blood diseases.

1.4. The disease in Australia

Ralstonia solanacearum race 2 was first introduced into Australia in 1989 on infected rhizomes of *Heliconia* imported from Hawaii. Prior to December 1987, *Heliconia* plants required a 9-month post-entry quarantine period which included the screening of the imported material for *R. solanacearum* race 2 however after this date the post-entry quarantine period was reduced to only three months. The rhizomes, imported in April 1989, from Oahu, Hawaii had been screened for the required three-month quarantine period at a private post-entry quarantine facility in Cairns.

In response to advice from the Austalian Quarantine Inspection Service (AQIS) that *R. solanacearum* race 2 had been isolated from *Heliconia* Plants in Hawaii, a survey of properties growing *Heliconia* plants that had been recently imported from Hawaii was undertaken by the Queensland Department of Primary Industries in order to establish whether the pathogen was present or not. Wilted Heliconia plants were found during this survey and subsequent analysis showed they were infected by the SFR strain of *R. solanacearum* (Diatloff, Akiew et al. 1992). These plants were all part of the original consignment of plants imported from Hawaii. The property was placed under a quarantine order preventing movement of plant material onto or off the property. All of the 55 plants that were from the original infected consignment from Hawaii were removed and those within a 1 m radius of these were destroyed by Glyphosate injection. A further area within a 5 m radius of the infected plants was marked as a quarantine buffer zone. The plot was grassed and then left fallow for 26 months. No further disease was detected in this time and the Quarantine order was subsequently lifted (Hyde, McCulloch et al. 1992).

To date there have been no incursions of either Bugtok or Blood disease in Australia however the presence of Blood disease in Indonesia where it is spreading up to 25 km per year means that there is a high potential for this disease to enter Australia on propagation material brought in through our Northern borders during traditional activities by residents of these regions. The fact that the Blood disease bacterium is insect transmitted further increases the risk of accidental entry of this disease.

2 Disease Symptoms

2.1 Moko disease

Moko disease is characterised by wilt symptoms similar to those seen in a Fusarium infected banana with the yellowing of the leaf margins and collapse of the petiole in advanced infections (Figure 2(a)). Symptoms also vary depending on the strain causing the infection, mode of transmission of the pathogen, the age of the host plant and the reaction of the host plant itself. Disease symptoms in the fruit and in suckers distinguishes bacterial wilt from Fusarium wilt although these are not always present (Figure 2(b)). Infection via insect transmission produces symptoms of shrivelling and blackening in the flowers buds and the peduncle first with further discolouration of the vascular tissue occurring as the infection becomes systemic and moves through the entire plant. When infection is of the non-insect transmissible strains less bacterial ooze is produced and external wilt symptoms are visible much later in the disease cycle. As these strains generally transmitted to plants via wounds of the roots and rhizome the earliest external symptoms are the yellowing of the leaf margins of the oldest leaves.

In all infections of *R. solanacearum* race 2, once the infection is advanced the petioles collapse beginning with the oldest leaves first. If the pseudostem is cut open severe discolouration of the vascular tissue can be seen. Bacterial ooze is present (Figure 2(c)) and this is another distinguishing feature compared with Fusarium wilt. Moko also causes cessation of fruit development. The fruit pulp becomes discoloured and then rotten. This affliction of the fruit is also seen earlier in insect transmitted Moko than soil borne Moko.

As this pathogen also infects *Heliconia* species, it is important that these plants are also surveyed when surveys are being conducted for this pathogen. Symptoms are similar to those of banana with yellowing of the leaf margins and collapse of the petiole in advanced infections (Figure 3).

Figure 2: (a) Moko disease in a plantain in South America. Note the infected raceme and the male flower (initial inoculation point) and symptoms on the fruit. Note also the collapse of the petiole on the leaf visible behind the bunch. **(b)** Transverse sections of diseased fruit and peduncle showing characteristic discolouration. Note that not all fruit may be discoloured. Insect transmitted Moko produces symptoms in the male flower, fruit and peduncle before other symptoms are evident.

(c) Cross section of a pseudostem showing vascular discolouration and bacterial ooze. The bacterial ooze and fruit symptoms distinguish Moko from Fusarium Wilt.

Photos coutesy of H.D. Thurston (http://www.tropag-fieldtrip.cornell.edu/docthurston/smokinhome.html)

Figure 3: (a) Heliconia in the field infected with *R. solanacearum* race 2/ biovar1 (Cairns, Qld, 1989). **(b)** Cross section of pseudostem showing vascular discolouration associated with the bacterial wilt disease. (Photos L. Diatloff, reproduced with permission DPI&F, Qld)

2.2 Bugtok

The symptoms of bugtok disease are similar to those of insect transmitted Moko in that the male bud and flowers are affected however the vascular discolouration is generally not found beyond the peduncle except in severe cases. Discolouration of the fruit pulp is also found along with areas of gelatinous or dry tissue (Figure 3). Bacterial ooze can be seen when the peduncle is cut. Unlike Moko however the infected plants do not wilt. Suckers are also generally not affected.

Figure 4: Symptoms of Bugtok disease. Note the discolouration of the fruit and also of the vascular tissue of the peduncle. Vascular discolouration is rarely seen beyond the peduncle.

2.3 Blood disease

Blood disease also causes yellowing of the leaf margins of the oldest leaves first and eventually the petioles collapse and become necrotic (Figure 4(a)). The presence of blackened and shrivelled males flower buds suggests that the disease is insect transmissible. Fruit may appear unaffected however when cut open the pulp displays reddish-brown discolouration and may be rotten or dry (Figure 4(b)). It is this discolouration which gives the disease its name. The discolouration extends from the fruit, through the peduncle down into the rest of the plant via the vascular tissue. Bacterial ooze is visible if the tissue is cut (Figure 4(c)). The discolouration often will be found throughout the mat and in the new suckers of an infected plant.

Figure 5: (a) Classic wilt symptoms produced by Blood Disease Bacterium infection of *Musa* sp. **(b)** Infected fruit showing characteristic red-brown discolouration which gives Blood disease its name. **(c)** Tranverse section of the pseudostem of an infected banana plant displaying the characterististic red-brown vascular discolouration and bacterial ooze produced during Blood disease bacterium infection.

The Pathogens

2.4 The Causal Agent

Moko disease is caused by the bacterium *Ralstonia solanacearum* (Yabuuchi, Kosako et al. 1995). Initially when it was identified by Rorer in 1911 he named the causal agent *Bacillus musae* however after the bacterium was shown to cause symptoms on solanaceous plants it was reclassified as *B. solanacearum* (Ashby 1926). This group has now been renamed *Ralstonia solanacearum*. The bacterium is described as an aerobic, gram-negative, non-fluorescent rod belonging to the rRNA homology group II (Palleroni 1984). There are various strains which have been identified and these are presented in Table 1. Bugtok disease is caused by the same bacterium however the symptoms and host range of this disease are distinctive compared with those of Moko (Thwaites, Eden-Green et al. 2000).

Although the Blood Disease Bacterium was originally named *Pseudomonas celebensis* by Gäumann in 1923 it was later considered to be an aberrant form of Moko (Thwaites, Eden-Green et al. 2000). After the appearance of the disease in Java further investigations showed that the bacterium was in fact different from that which causes Moko (Eden-Green and Sastraatmadja 1990).

2.5 Taxonomy

2.5.1 Diversity of the *R. solanacearum* species complex

A stable and meaningful taxonomy and nomenclature which accurately defines subspecific groups of *R. solanacearum* has been the aim of taxonomists working on the *R. solanacearum* species complex. Such a taxonomic system will aid plant breeders, plant pathologists and quarantine officials who require a system of classification where strains can be grouped into clusters of isolates that relate to epidemiology, pathogenicity, host range and/or geographic origin. The taxonomic framework and diagnostic methodology outlined below allows identification of subspecific groups within the *R. solanacearum* species complex and improves our ability to predict the properties of *R. solanacearum* species complex strains especially those strains which cause diseases of banana.

3.2.2 The *R. solanacearum* species complex

Traditionally *R. solanacearum* has been classified into five races on the basis of differences in host range (Buddenhagen, Sequeira et al. 1962; Pegg and Moffett 1971; He, Sequeira et al. 1983) and six biovars on the basis of biochemical properties (Hayward 1964; Hayward 1991; Hayward 1994). The work of Cook *et al*. (1989) and Cook and Sequeira (1994) was the first work assessing the genetic diversity of *R. solanacearum* and employed restriction fragment length polymorphism (RFLP) analysis. Their work showed that *R. solanacearum* can be divided into two divisions: division 1 comprising strains belonging to biovars 3, 4 and 5, primarily isolated in Asia and division 2 comprising strains belonging to biovars 1, 2 and N2, primarily isolated in the Americas. Several other investigations employing molecular methods have confirmed this dichotomy within *R. solanacearum* (Seal, Jackson et al. 1992; Gillings, Fahy et al. 1993; Taghavi, Hayward et al. 1996). Taghavi *et al*. (1996), using 16S rDNA sequence analysis, also revealed the existence of a subdivision within division 2 comprising isolates of *R. solanacearum* from Indonesia including the closely related organisms the blood disease bacterium (BDB) and *P. syzygii*. Further sequencing of the 16S-23S rRNA gene intergenic spacer region (ITS), the polygalacturonase gene and the endoglucanase gene (Fegan, Taghavi et al. 1998) has supported the existence of the two divisions and the existence of the group of strains originating in Indonesia.

PCR-RFLP analysis of the *hrp* gene region (Poussier, Vandewalle et al. 1999) has demonstrated that certain African biovar 1 strains did not cluster with other biovar 1 isolates as was expected. An extended PCR-RFLP analysis of the *hrp* gene region complemented by amplified fragment length polymorphism (AFLP) and sequencing of the 16S rRNA gene (Poussier, Trigalet-Demery et al. 2000) has provided further support for the existence of this group of strains. Phylogenetic analysis of the endoglucanase and *hrpB* genes has confirmed the presence of a group of strains originating in Africa (Poussier, Prior et al. 2000).
Hence the picture has emerged that the *R. solanacearum* species complex is comprised of four broad genetic groups corresponding with geographic origin of strains within these groups.

3.2.3 The Phylotyping scheme: a new scheme for classifying *R. solanacearum*

A hierarchical classification scheme has been proposed to reflect the known diversity within the *R. solanacearum* species complex (Fegan and Prior 2005). The scheme is outlined in Figure 5.

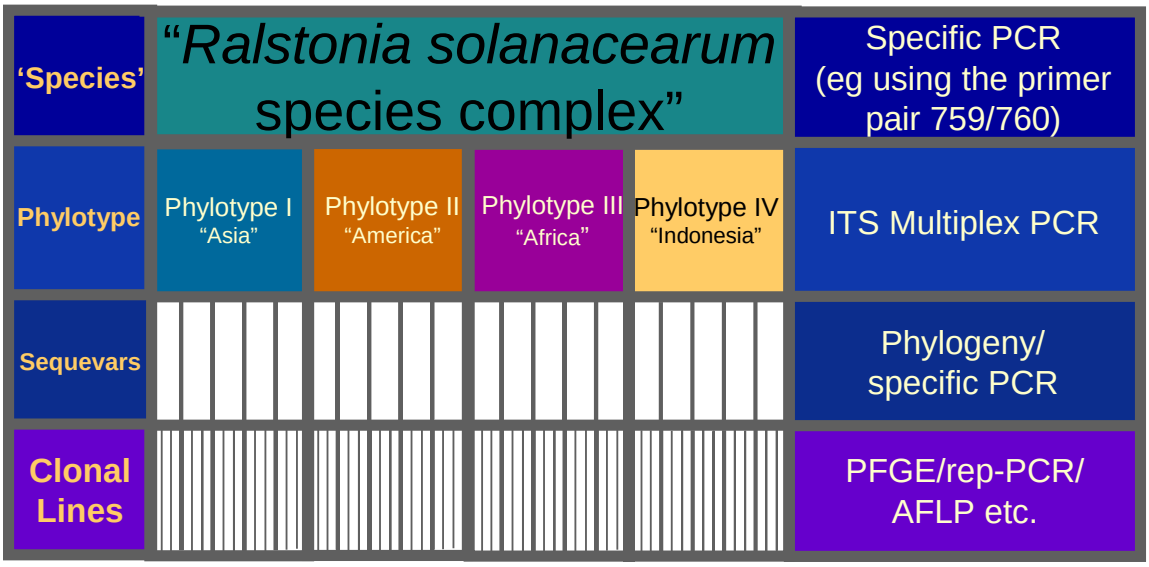


Figure 6: Diagrammatic representation of the hierarchical classification scheme proposed by Fegan and Prior (2005).

Under this classification system members of the *R. solanacearum* species complex can be subdivided into four phylotypes corresponding to the four genetic groups identified via sequence analysis (Figure 6). A phylotype is defined as a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data, in this case the ITS region, the *hrpB* gene and the endoglucanase gene. Phylotype I is equivalent to division 1 defined by Cook *et al*. (1989). This phylotype includes all strains belonging to biovars 3, 4, and 5; strains are isolated primarily from Asia (Figure 7). Phylotype II is equivalent to division 2 (1989), and includes strains belonging to biovars 1, 2 and 2T isolated primarily from America (Figure 7). Phylotype II contains the *R. solanacearum* race 3 potato pathogen, which has a world wide distribution, and the **race 2 banana pathogens** (Figure 7). Phylotype III contains strains primarily isolated from Africa and surrounding islands, strains belong to biovars 1 and 2T. Phylotype IV contains strains isolated primarily from Indonesia belonging to biovars 1, 2 and 2T (Figure 7). *R. solanacearum* strains in Phylotype IV have also been found in Australia and Japan. This phylotype also contains the two

close relatives of *R. solanacearum*, *P. syzygii* and **the Blood Disease Bacterium** (Figure 7).

Each phylotype is composed of a number of sequevars. A sequevar, or sequence variant, is defined as a group of strains with a highly conserved sequence within the area sequenced. Only if two or more strains sequenced have similar sequences has a sequevar been defined. Therefore single sequence clusters have not been given sequevar status (for example CIP10 in Figure 6). Sequevars, are primarily defined upon partial endoglucanase gene sequences as a large number of strains have been sequenced in this region. In the future it is hoped that sequence information from more strains from other areas of the genome, such as the *hrpB* gene, will be generated to confirm these sequevars. The endoglucanase gene of greater than 140 *R. solanacearum* isolates has been sequenced and over 20 sequevars have been identified (Figure 6).

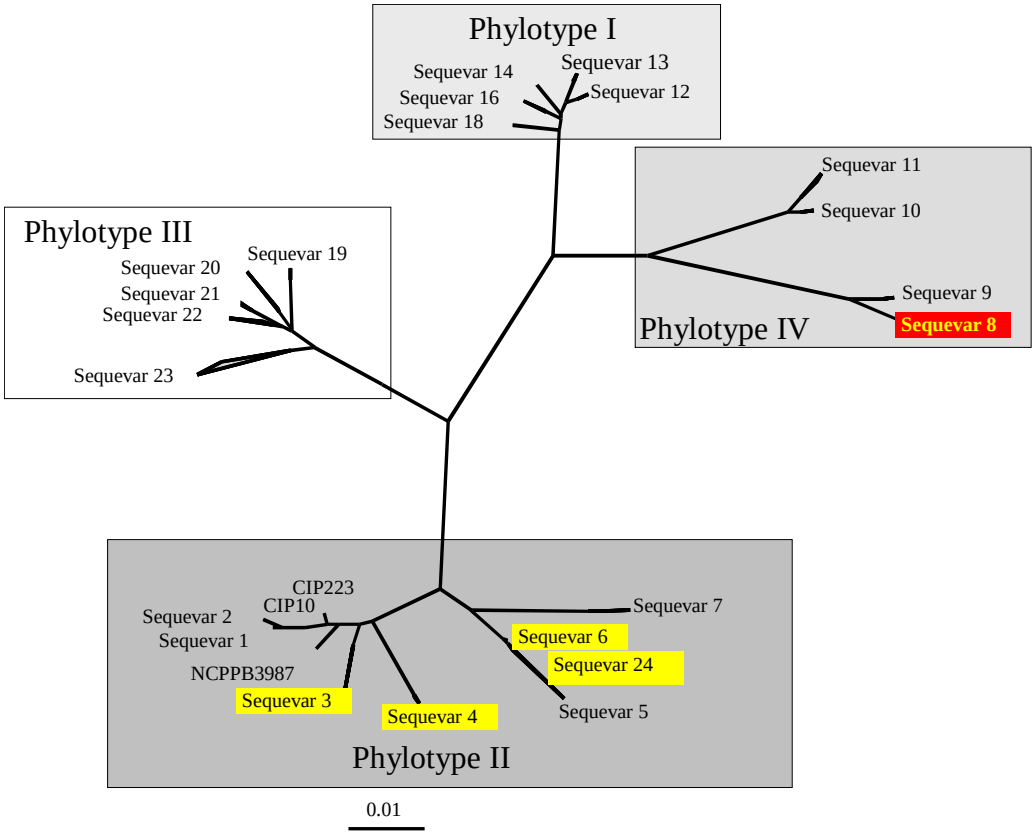


Figure 7: Phylogenetic tree generated from partial endoglucanase gene sequence data showing the phylogenetic relationships of sequevars and phylotypes. The bar indicates 1 nucleotide change per 100 nucleotide positions. Sequevars highlighted in yellow indicate those sequevars containing moko disease causing strains and the sequevar highlighted in red contains blood disease causing strains

Each sequevar may be composed of a number of clonal lines which may be identified using genomic fingerprinting methods such as PFGE, AFLP's or rep-PCR (Figure 5). In our experience rep-PCR is a fast and reproducible method for identification of clonal lineages within a sequevar. However, Pulsed Field Gel Electrophoresis (PFGE) is able to differentiate more closely related organisms and is therefore useful to resolve the diversity of strains in sequevars that a more closely related.

The phylotyping scheme is highly discriminatory, flexible and additive allowing identification of further sequevars or even phylotypes. This phylotyping scheme is based upon genetic variation that accumulates relatively slowly in the genome of organisms at the level of the phylotypes and sequevars thus giving a long term global epidemiological perspective. However, the scheme also incorporates the finer resolving power of the genomic fingerprinting techniques to identify clonal lines below the level of the sequevar.

2.5.2 Comparison of the phylotyping classification schemes

In comparison to the phenotypically based race and biovar classification schemes the phylotyping scheme more accurately reflects the diversity that we now know to be present in the *R. solanacearum* species complex.

Race 1, defined as strains "affecting tobacco, tomato, many solanaceous and other weeds, and certain diploid bananas" (Buddenhagen, Sequeira et al. 1962) is a very broad definition. Strains belonging to race 1 are found in phylotypes I and II and probably in phylotypes III and IV if host of origin can be used as a guide to which race a strain belongs. In contrast to Race 1, Races 2 (affect triploid banana and/or heliconia) and 3 (affect potato and tomato) have narrow host ranges and this is reflected in the relatively narrow genetic diversity included within races. Race 3 strains belong to phylotype II, sequevars 1 and 2 (Figure 6). **Strains belonging to race 2 belong to phylotype II, sequevars 3, 4, 6 and 24** (Figure 6).

Strains representing biovars 1 and 2T are present in three of the four phylotypes and it is clear that simply identifying a strain as biovar 1 or 2T does not tell you much about the strain (Figure 7). A large degree of phenotypic variation within strains of

biovar 1 had previously been recognised (Harris 1972) and this is mirrored in the genetic variation found in *R. solanacearum* strains belonging to biovar 1. Most strains belonging to biovar 2 are equvalent to race 3 and therefore belong to phylotype II sequevars 1 and 2. However, some biovar 2 strains do not belong to race 3 and are found in phylotype IV sequevars 8 and 9.

This new scheme largely confirms the RFLP typing scheme (Cook, Barlow et al. 1989). Phylotypes I and II are equivalent to the divisions 1 and 2 defined by Cook *et al*. (1989) (Figure 7). Phylotypes III and IV were not recognised by Cook *et al*. (1989) as they did not study strains belonging to these two phylotypes. It would be expected that if strains belonging to these two phylotypes had been analysed using RFLP's then these two groups would have been identified earlier. Below the level of the phylotype at the sequevar level the RFLP and phylotyping schemes are also congruent with strains which belong to different MLG's also belonging to distinct sequevars. For example **strains belonging to MLG's 24, 25 and 28, which contain moko disease causing strains of *R. solanacearum* are equivalent to sequevars 3, 4 and 6 respectively**.

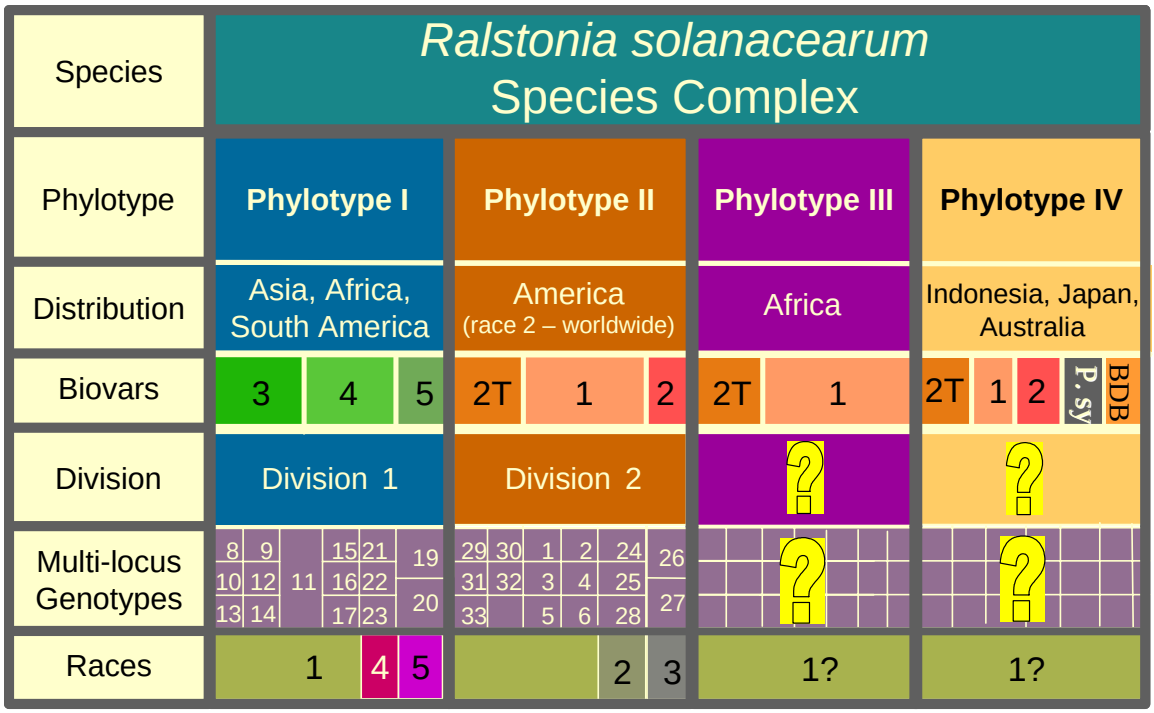


Figure 8: Relationship of the different schemes used to describe the infrasubspecific relationships of R. solanacearum including the phenotypic based schemes (biovars and races) and genotypic based schemes (RFLP analysis and phylotyping). (After Gillings and Fahy (1994))

3.3 Taxonomy and Diversity of R. solanacearum race 2 strains

R. solanacearum strains infecting *Musa* spp. pose a major threat to dessert and cooking banana (plantain) production worldwide (Sequeira 1998). Unravelling the phylogenetic relationships among these strains is essential for quarantine purposes and scientifically promising for investigation of the basis of host specificity. As discussed above *R. solanacearum* is a very heterogeneous species and even within race 2 strains, there is variation at both the phenotypic and genotypic level (French and Sequeira 1970; Cook, Barlow et al. 1989).

French and Sequeira (1970) defined five groups, or ecotypes, of strains of *R. solanacearum* race 2 causing bacterial wilt on banana, plantain and *Heliconia* sp. in Central and South America (See Table 1). These groups are: type A (Amazon basin), SFR (small, fluidal, round), B (banana), D (distortion), and H (heliconia). Strains within each group differ in virulence, some are pathogenic on both banana and plantain (A, SFR, B, and D types) and others are only pathogenic to plantain but not to banana (H). The groupings were also based on the major mode of transmission of strains either by insects (SFR) (Buddenhagen and Elsasser 1962) or by soil (B-type). Moreover, some groups consist of highly aggressive strains (SFR, A, B) and others of less aggressive strains (D-type). All the ecotypes are naturally pathogenic on *Musa* spp. and tested pathogenic to tomato and other solanaceous hosts if bacteria were directly injected into the stem (French and Sequeira 1970). However, they have never been isolated from a naturally wilted solanaceous plant in the field.

Table 1: Characteristics of strains of race 2 adapted from Thwaites et al. (2000), French and Sequeira (1970) and Prior and Fegan (2005)

Strain Type[a]	Distribution[a,b]	Characteristics[a]	Ecology[a]	MLG[b,c]
SFR (Small fluidal round)	Central America, Venezuela, Columbia, Caribbean	Small fluidal round, slight formazan pigment,	Highly pathogenic, Insect transmission high, soil transmission low	25, 28
B (banana rapid wilt)	Central and South America, The Philippines	Large elliptical colonies, slight formazan pigment	Highly pathogenic, Insect transmission high for bugtok disease, soil transmission high	24
D (Distortion)	Costa Rica, Surinam, Guyana	As for B strain above	Low pathogenicity for banana/ plantain / heliconia	24, 25
H	Costa Rica	As for B strain above	Pathogenic for plantain but not banana	24
A	Peru	Near round, slight formazan pigment	Highly pathogenic, Insect transmission high	25

a from Thwaites et al (2000)
b from Fegan and Prior (2005)
c from French and Sequeira (1970)

Using RFLP analysis Cook and Sequeira (1994) identified three genetic groups which they called multilocus genotypes (MLGs) 24, 25 and 28 which are equivalent to sequevars 3, 4 and 6 under the phylotyping scheme (Fegan and Prior 2005). Phylogenetic analysis of endoglucanase gene sequences of *R. solanacearum* race 2 strains has revealed a fourth genetic group of strains causing Moko disease from banana in Brazil which have been placed into a new seqeuvar (sequevar 24, Figure 6 and Figure 8); no banana pathogenic strains from Brazil have ever been fingerprinted using RFLP. This raises the possibility that there is greater,

undiscovered, genetic diversity present in *R. solanacearum* race 2 strains from South America.

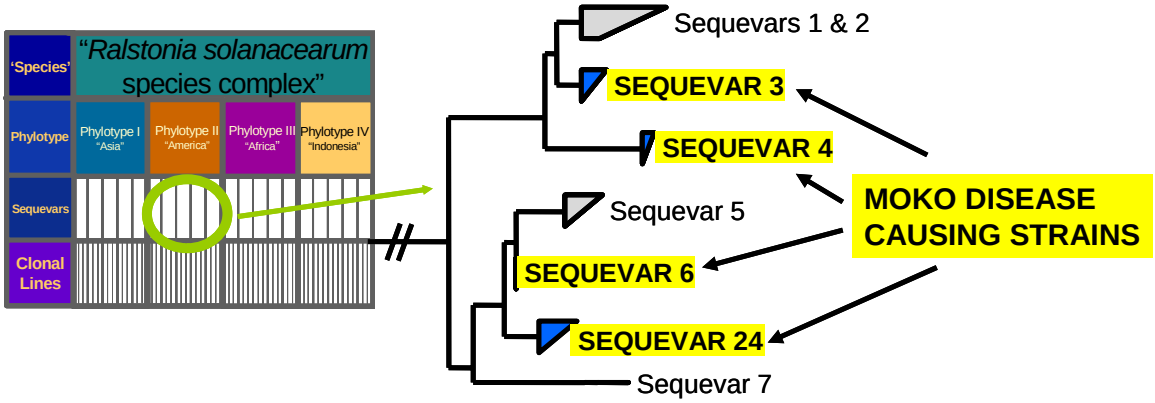


Figure 9: Phylogenetic tree based upon maximum likelihood analysis of partial endoglucanase gene sequences of phylotype II organisms within the R. solanacearum species complex. Sequevars highlighted in yellow contain R. solanacearum strains causing Moko disease.

3.3.2 Moko and Bugtok diseases in the Philippines

Phylogenetic analysis of endoglucanase gene sequence data from the Moko disease causing and Bugtok disease causing strains from the Philippines, shows that these strains cluster with sequevar 3 (MLG24) strains (Figure 9). A close relationship between Bugtok and Moko isolates in the Philippines has been identified previously (Eden-Green and Seal 1993; Eden-Green 1994) and this relationship has been confirmed by using genomic fingerprinting techniques (Raymundo, Aves-Ilagan et al. 1998; Raymundo and Ilagan 1999; Thwaites, Mansfield et al. 1999; Ilagan, Lavina et al. 2003). The similarity between Philippines Bugtok and Moko disease causing strains extends to the pathogenicity of strains which is indistinguishable when strains are inoculated onto Dwarf Cavendish and Saba plantlets (Ilagan, Lavina et al. 2003). Philippines Bugtok and Moko isolates have also been found to be closely related to a *R. solanacearum* race 2 strain isolated from Honduras (Raymundo, Aves-Ilagan et al. 1998), a country from where sequevar 3 (MLG24) strains have been isolated (Cook, Barlow et al. 1989). It is likely that Moko disease in the Philippines was the result of a single introduction of a strain of *R. solanacearum* sequevar 3 (MLG24) from South America. In contrast, it has been suggested that certain *R. solanacearum* race 2 strains may have originated in Southeast Asia (Eden-Green 1994), but this

seems unlikely. The greatest genetic diversity of strains causing Moko disease is found in strains from South America where it probably evolved associated with wild *Heliconia* species (Thwaites, Eden-Green et al. 2000). It is generally accepted that genetic diversity of a pathogen is usually greatest in the centre of origin. Also, sequevar 3 (MLG24) strains are closely related to sequevar 4 (MLG25) strains indicating an evolutionary link between these groups of strains (Figure 9), and sequevar 4 (MLG25) strains are only found in South America. It is therefore likely that a single genotype (sequevar 3) of the pathogen was moved to the Philippines on infected banana plants as has been previously suggested (Buddenhagen 1986).

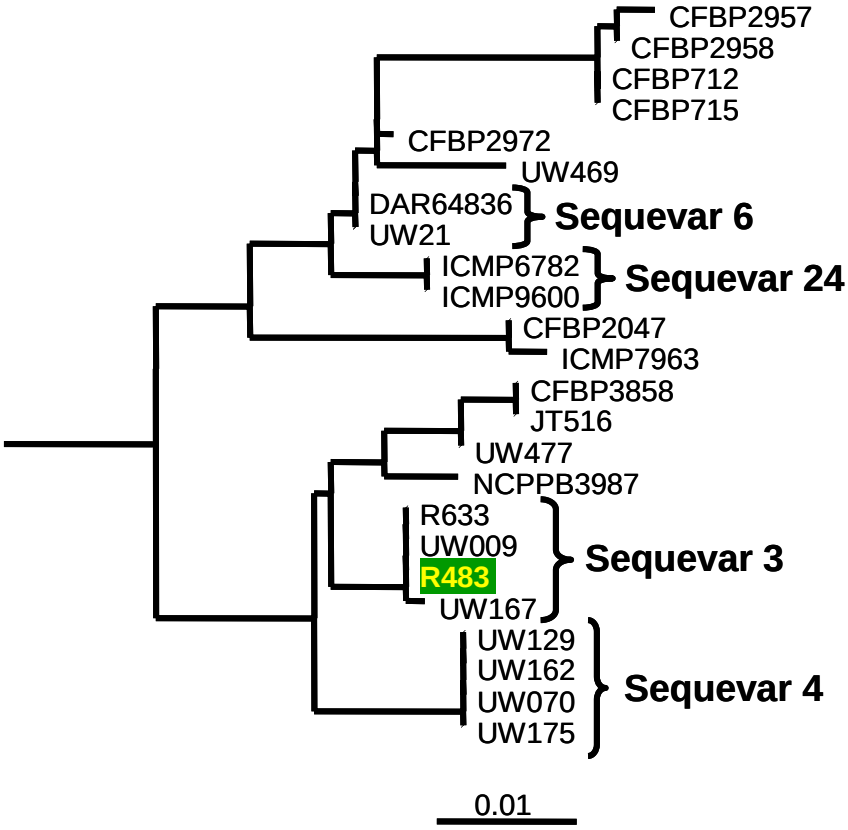


Figure 10: Phylogenetic tree based upon maximum likelihood analysis of partial endoglucanase gene sequences of phylotype II organisms within the R. solanacearum species complex. The strain highlighted in green is a R. solanacearum strains causing Bugtok disease. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

3.4 Taxonomy and Diversity of BDB strains

BDB strains, which cause blood disease of banana, belong to phylotype IV of the *R. solanacearum* species complex (Fegan and Prior 2005). Of the four phylotypes within the *R. solanacearum* species complex phylotype IV contains the most

phenotypically diverse range of strains, with *R. syzygii*, the BDB and strains of *R. solanacearum* all falling within this phylotype (Figures 6, 7 and 10) (Fegan and Prior 2005). Phylogenetic analysis of endoglucanase gene sequences shows that the closest relatives of BDB strains sequenced are strains of *R. solanacearum* isolated in Indonesia (Figure 10). In fact the endoglucanase gene sequence of certain *R. solanacearum* strains is identical to BDB strains (Figure 10). These *R. solanacearum* strains closely related to the BDB display colony morphology and growth rate differences to the BDB strains when cultured on TZC medium and although no information exists as to the pathogenicity of these *R. solanacearum* strains for banana no BDB strains are known to cause disease of solanaceous plants (Eden-Green 1994). The similarity of BDB strains to certain *R. solanacearum* isolated from Indonesia has also been found by other authors employing genetic fingerprinting

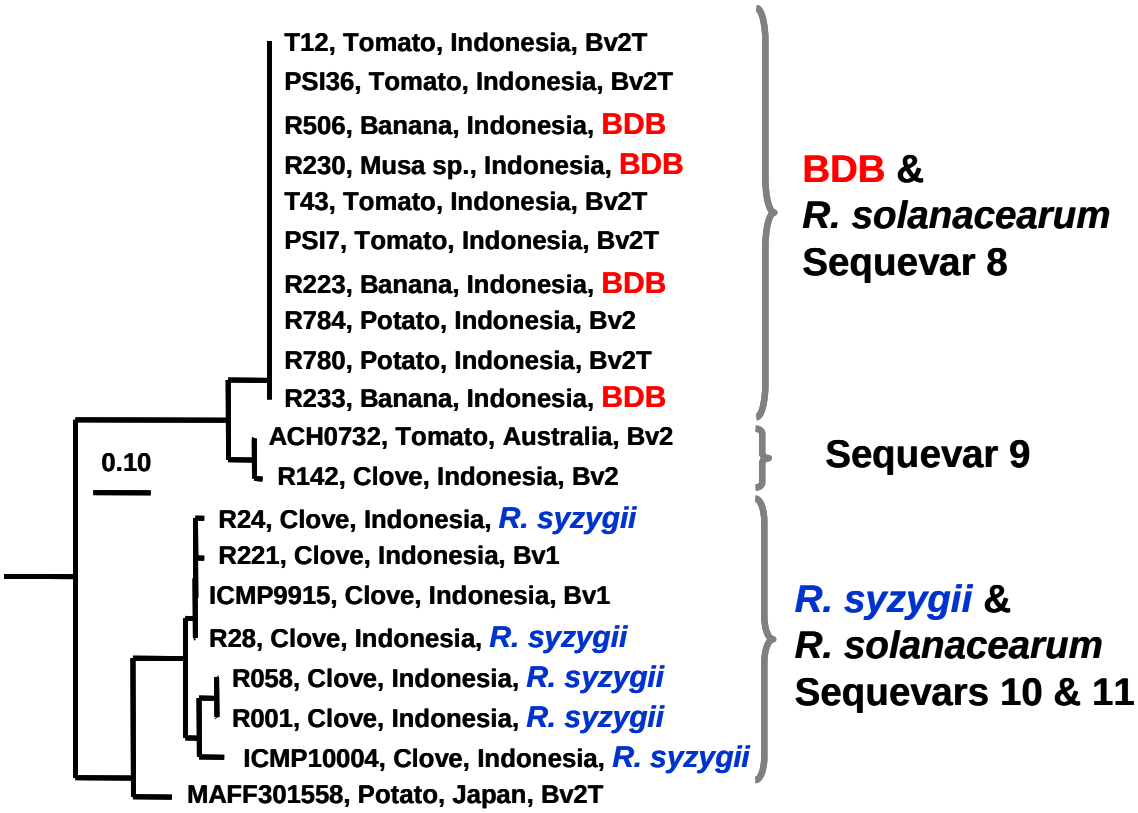


Figure 11: Phylogenetic tree based upon maximum likelihood analysis of partial endoglucanase gene sequences of phylotype IV organisms within the R. solanacearum species complex indicating the relationship of BDB strains to R. solanacearum and R. syzygii strains. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

techniques (Seal, Jackson et al. 1992; Eden-Green and Seal 1993; Thwaites, Mansfield et al. 1999). The close phylogenetic relationship of strains of *R. solanacearum* from Indonesia belonging phylotype IV to the BDB strains contrasts with the phenotypic variation between these organisms.

There are few studies assessing the genetic diversity between BDB strains. Thwaites *et al*. (1999) used rep-PCR and random amplified polymorphic DNA analysis of vascular pathogens of *Musa* sp. which included a set of six BDB strains. These authors found that the genetic fingerprinting patterns of these six BDB strains were nearly identical. Phylogenetic analysis of partial endoglucanase gene sequences (Figure 10) also shows a low level of diversity between BDB strains. The only method found to reveal genetic variation between BDB strains is Pulsed-Field Gel Electrophoresis (PFGE), the significance of this genetic variation has yet to be assessed.

3.5 Hosts

Musa spp., both bananas and plantains, and *Heliconia* spp. are susceptible to natural infections of the banana strains of *R. solanacearum* Race 2 (Sequeira and Averre 1961; Buddenhagen 1994) although there have reports that the cooking banana Pelipita (ABB) has resistance to the insect transmission of the bacterium. This clone has indehiscent flower bracts which prevents insect transmission. Pelipita was recommended as an alternative to Moko for planting in Central America (Stover and Richardson 1968). Natural infection of abaca (*Musa textiles*) by *R. solanacearum* Race 2 is unlikely (Rillo 1981) although artificial inoculation is possible (Rillo 1979).

Bugtok disease has been observed in the two Philippine cooking bananas Saba and Cardaba both of which are of the balbisiana genotype (INIBAP-MGIS database http://mgis.grinfo.net/) however other cooking banana (ABB genotype) have also been observed with Bugtok symptoms.

There are no known banana cultivars resistant to Blood disease. Gaumann tested over 100 cultivars for resistance in his original study of this disease however no resistance was found.

4 Disease Development and Epidemiology

4.2 Disease Cycle

The bacterium causing Moko, Bugtok and Blood disease have similar life cycles although less is known about the latter. The cycle of Bugtok disease is very similar to that of insect transmitted Moko. The disease is spread from plant to plant by both humans and insects. Figure 11 shows mains methods of transmission of *R. solnacearum.*

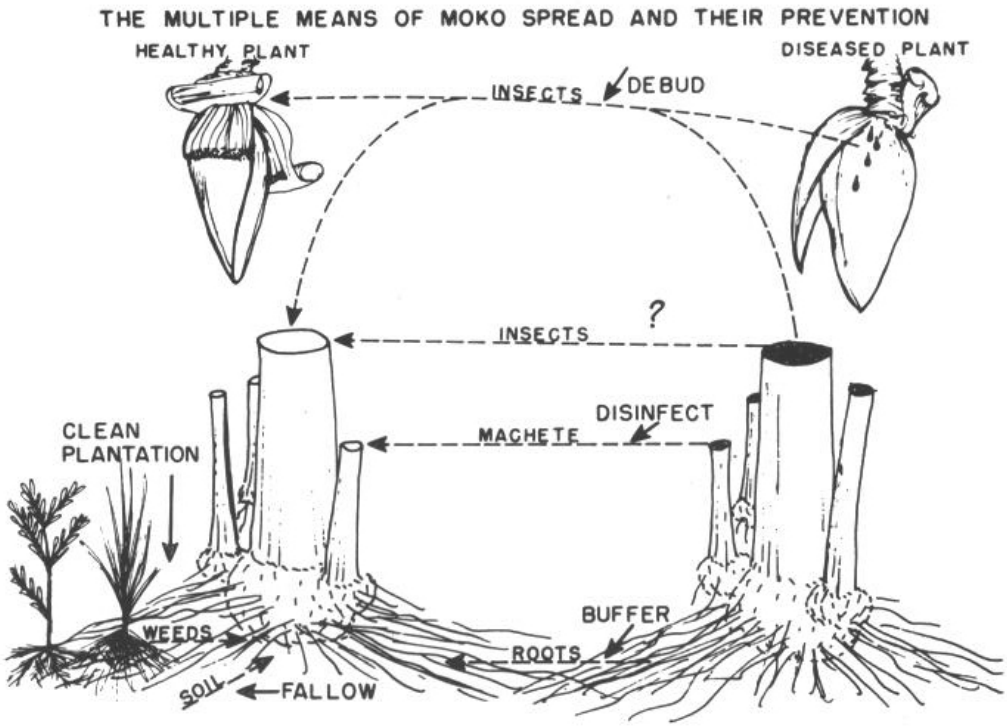


Figure 12: Diagram of the main transmission pathways for *R. solanacearum* between banana plants as proposed by Stover (1972). Reproduced with permission from CABI Publishing, CAB International.

4.3 Survival of the Inoculum

R. solnacearum is present not only within the plant but also within the soil in which the plant is growing. In order to control the disease the soil must be allowed to fallow. Studies have shown that the B strain can survive in soil for up to 18 months (Stover, 1972) although the SFR strain generally only survives for about 6 months (Sequeira, 1962). As R. solanacearum is unable to resist desiccation the lower the water

content of the soil the shorter the life of the bacterium. Good drainage or shallow tilling assists in lowering the inoculum levels in infected soils. (Lozano,et al, 1969; Sequeira, 1958; Wardlaw, 1972).

5 Disease Control

5.1 Cultural Control

The bacterial wilt diseases of banana are systemic and so once infected a plant must be destroyed in order to prevent further spread of the disease. Although some strains of these bacteria are insect transmissible much can still be done to holt the spread of the disease by good cultural practices. Disinfection of all work tools to being used in the field is of paramount importance. Various methods for the in field disinfection of tools have been devised (Buddenhagen & Sequeira, 1958; Sequeira, 1958; Stover 1972; Wardlaw, 1972). Workers must ensure that tools are disinfected between tending each plant. Work boots must also be disinfected on departing an infected area.

A practice of breaking off the male bud from Bluggoe plants was also devised in order to prevent the insect transmission of Moko in Honduras (Stover & Richardson, 1968).

Where infected plants are found the general eradication method used to date involves the injection of the diseased plant with the herbicide Glyphosate as well as all those plants within a 5 m radius of these infected ones. This creates a buffer zone (Stover, 1972). The soil within this buffer zone must then be allowed to fallow. It has been suggested that this period be up to 2 years (Hunt, 1987).

5.2 Chemical Control

There are no useful chemical control measures available for the management of any of these bacterial wilt diseases. Sequiera (1958) investigated the use of bactericidal chemicals for the treatment of infected soil however none of those trialled effectively reduced the incidence of Moko disease in previously infected areas. The only chemical that is effective is methyl bromide used as a soil fumigant. Fallow periods required for disease management were significantly reduced (Stover and Simmonds 1987) however this treatment is impractical for smallholder farmers. The use of this chemical not only has serious health issues associated with it but also has serious environmental repercussions. Methyl Bromide used as an agricultural chemical is to be phased out in developed countries by 2005 and in developing countries by 2015 under the Montreal Protocol (http://www.undp.org/seed/eap/montreal/).

6 Molecular Diagnosis of Diseases

6.1 Introduction

The PCR tests described below are designed to detect/identify *R. solanacearum* race 2/biovar 1 strains that cause moko disease of banana. The moko disease pathogens are found in at least four genetic groups within the *R. solanacearum* species complex. The four genetic groups identified to contain *R. solanacearum* race 2/biovar 1 moko disease causing strains are designated as sequevar 3 (MLG 24), sequevar 4 (MLG 25), sequevar 6 (MLG 28) and sequevar 24. As there are four genetic groups of *R. solanacearum* race 2/biovar 1 moko disease causing strains four separate pairs of oligonucleotide primers have been designed to identify each of these four genetic groups. A multiplex PCR protocol to identify all of these groups in a single PCR has also been developed. This allows the detection of all *R. solanacearum* race 2/biovar 1 strains causing moko disease in a single amplification reaction.

The following methods describe preparation of template for PCR and PCR protocols to be employed to detect/identify the presence of *R. solanacearum* race 2/biovar 1 moko disease causing strains in infected plant tissue. A Preferred protocol is also provided and it is suggested that this protocol be used wherever possible.

6.2 Preparation of Samples to be tested by PCR

6.2.1 Materials

A small beaker containing 5-10% sodium hypochlorite
3 large beakers containing at least 100 mL distilled water
A small beaker containing 95-100% ethanol
2 or more scalpels
Sterile gloves
Forceps
Bunsen burner
microcentrifuge
micropipettors and sterile tips
2 mL sterile microcentrifuge tubes (screw capped)
sterile distilled water
selective media– modified –SMSA (Appendix 1)

6.2.2 Sampling Protocols

6.2.2.1 Sterilisation of scalpels and forceps

To remove all DNA before sampling and between samples

1. Place scalpels in hypochlorite solution for at least 5 min prior to use to degrade any DNA present on the scalpel.
2. Rinse the scalpel free of hypochlorite by placing successively into the three beakers containing the distilled water
3. Place the scalpel into the 95-100% ethanol and flame using the Bunsen burner.

6.2.2.2 Sampling of vascular tissue of plant material

1. Cut a portion of the vascular tissue of an infected plant small enough to fit into a 2 mL microcentrifuge tube.
2. Place the tissue into a 2 mL microcentrifuge tube and add sterile distilled water into the minimum amount of water necessary to cover the cut end of the tissue.
3. Leave for 10 to 20 min until bacteria can be seen oozing out of the tissue.
4. Sterilise a pair of forceps by flaming with alcohol and use these to remove the plant tissue.
5. Centrifuge the sample and remove the supernatant.
6. Resuspend the pelleted bacteria in 1 mL of sterile distilled water, centrifuge and decant the supernatant leaving approximately 50-100 µL and resuspend the pellet in this remaining volume of water.
7. Use the bacterial suspension as template directly added to PCR. Alternatively the suspension can be boiled in a boiling water bath for 10 min followed by centrifugation to remove cell debris and the supernatant can be used as template for PCR.
8. Prior to boiling (if performed) take a loopful of the suspension and plate (16 streak dilution) onto SPA and modified-SMSA (see appendix 1 for recipes). Place plates in a 28^{o}C for 48-72 h.

6.2.2.3 Alternative method for sampling vascular tissue

1. Remove a small piece of vascular tissue from a potentially infected plant.
2. Place in a sterile Petri dish.
3. Add the minimum amount of sterile distilled water to the sample so that the tissue is covered by the water and gently macerate the tissue using a sterile scalpel

4. Leave for 10 min.
5. Remove the water using a micropipette and place into a microcentrifuge tube.
6. Make volume to 1 mL with sterile distilled water and centrifuge.
7. Resuspend the pelleted bacteria in 1 mL of sterile distilled water and centrifuge.
8. Decant the supernatant leaving approximately 50-100 µL and resuspend the pellet
9. Use the bacterial suspension as template directly added to PCR. Alternatively the suspension can be boiled in a boiling water bath for 10 min followed by centrifugation to remove cell debris and the supernatant can be used as template for PCR.
10. Prior to boiling (if performed) take a loopful of the suspension and plate onto SPA and modified-SMSA (see appendix 1 for recipes). Place plates in a 28^{o}C for 48-72 h.

6.2.2.4 Testing of cultures

1. From the modified-SMSA or SPA plate make a dense suspension of bacteria in 1 mL of sterile distilled water.
2. Centrifuge the bacterial suspension and resuspend in a further 1mL of sterile distilled water. Centrifuge again, remove the supernatant and add enough sterile distilled water to make a dense suspension of the bacteria.
3. Use this suspension directly as template for PCR.

6.2.2.5 Soil samples

This PCR test is not directly applicable to soil samples because of the presence of inhibitory substances.

The method of Ito *et al*. (1998) should be used to assess the presence of *R. solanacearum* in soil samples. However, modified-SMSA (Appendix 1) should be substituted as the selective medium.

6.3 R. solanacearum *race 2/biovar 1 PCR protocols*

6.3.1 Solutions required

Solution	Stock Concentration
dNTP's	1.25 mM each dNTP
$MgCl_2$	25mM (supplied with polymerase
PCR Buffer	10X (Supplied with the polymerase)
Tris-Acetate-EDTA buffer	50X (appendix 2)
Loading Dye	6X (appendix 2)
Ethidium Bromide (EtBr)	10 mg mL^{-1}

6.3.2 Setting up individual PCR reactions

Individual R. solanacearum race 2/biovar 1 sequevar specific PCR

To minimise the chance of contaminating the PCR always wear gloves (other important procedures to minimise contamination are give below). All PCR reactions are carried out in a total volume of 25 µL. Each reaction consists of:

Solution	Stock Concentration	Final Concentration	Volume added
Sterile Distilled Water	-	-	12.8 µL
dNTP's	1.25 mM	200 µM	4 µL
PCR Buffer *	10X	1X	2.5 µL
$MgCl_2$ *	25 mM	1.5µM	1.5 µL
Forward Primer(s) (see Table 1)	6 mM	24 µM	1 µL
Reverse Primer (see Table 1)	6 mM	24 µM	1 µL
Polymerase*	5U/µL	1U/reaction	0.2 µL
Template (cell suspension as prepared above)	-	-	2 µL

*** Amplitaq Gold DNA Polymerase is recommended for use in these PCR protocols. The concentration and volumes used in the PCR indicated in the Table are for Amplitaq Gold DNA Polymerase. PCR buffer and $MgCl_2$ are supplied with the polymerase.**

Table 2: Primer pairs and specificities

Primer	Primer Sequence	Specificity
SI28F	CGTTCTCCTTGTCAGCGATGG	Sequevar 6 (MLG 28)
SI28R	CCCGTGTGACCCCGATAGC	
MUS20F	CGGGTGGCTGAGACGAATATC	Sequevar 4 (MLG 25)
MUS20R	GCCTTGTCCAGAATCCGAATG	
IS24F	TCGGGCGTAAGAGGCAGAC	Sequevar 3 (MLG 24)
IS24R	GGAGGTGTGCGCCATCAACTG	
VC46F	CTCCTGGGAGTCGGTTGGGTC	Sequevar 24
VC46R	AGGGAACCTAGGCGTGACTG	
759	GTCGCCGTCAACTCACTTTCC	*R. solanacearum*
760	GTCGCCGTCAGCAATGCGGAATCG	

Table 2. Preparation of 10x primer mix (containing 2 µM each primer)

Concentration of primer stock	50 µM (50 pmol/µl)	100 µM (100 pmol/µl)
Each primer (SI28F, SI28R, MUS20F, MUS20R, IS24F, IS24R, VC46F, VC46R)	20 µl	10 µl
Primers 759, 760	10 µl	5 µl
TE buffer (See Chapter 11)	Variable	Variable
Total volume	500 µl	500 µl

6.3.2.1 Making a MasterMix

It is essential to always include positive and negative controls for each PCR. Hence a minimum of 3 PCR reactions should always be set up. It is more convenient and much more accurate to make up a MasterMix which includes all components for the

total number of samples required in one tube and then subsequently aliquot the mixture into the PCR tubes for thermocycling.

For example if you have 2 plant samples, make a mix for 2 + 2 controls. In this case you should make up a mixture for 5 samples to ensure enough MasterMix for the required number of samples (the greater the number of samples to be tested the greater the number of extra mixture you will have to make eg. if 30 samples + 2 controls are to be tested make a mixture for 35 or 36).

For example a MasterMix for 6 samples + 2 controls – make a MasterMix for 10 PCR

Solution	**Volume for 1 reaction**	**Volume for 10 reactions**
Sterile Distilled Water	12.8 µL	128 µL
dNTP's	4 µL	40 µL
PCR Buffer*	2.5 µL	25 µL
$MgCl_2$*	1.5 µL	15 µL
Forward Primer (see Table 1)	1 µL	10µL
Reverse Primer (see Table 1)	1 µL	10 µL
Polymerase*	0.2 µL	2 µL

*** Amplitaq Gold DNA Polymerase is recommended for use in these PCR protocols. The concentration and volumes used in the PCR indicated in the Table are for Amplitaq Gold DNA Polymerase. PCR buffer and $MgCl_2$ are supplied with the polymerase.**

All solutions are stored frozen at -20^{o}C so make sure that all solutions are completely thawed and gently mixed prior to use. Mix all the ingredients together using a set of micropipettes which are only used for preparation of PCR (this minimises the chance of contaminating the MasterMix with template). Make sure that after addition of the polymerase the MasterMix is well mixed by rapid shaking of the tube (Note: some PCR buffers include detergents, if too much frothing occurs quickly spot spin the MasterMix prior to aliquoting).

Aliquot 23 µL of MasterMix into each of 8 PCR tubes. Add 2 µL of template and place in a programmable thermocylcer programmed with the following PCR protocol.

6.3.2.2 PCR Protocol

Initial Denaturation	96°C	10 min*	1 cycle
Denaturation	92ºC	15 s	30 cycles
Annealing	59ºC	15 s	
Extension	72ºC	30 s	
Final extension	72ºC	10 min	1 cycle
Hold	11ºC	-	-

** reduce to 5 min if not using AmpliTaq Gold*

6.3.3 Multiplex PCR reaction

To identify of R. solanacearum race 2/biovar 1 sequevars in a single reaction

All procedures are as above apart from the making of the MasterMix which instead of the one primer pair (2 primers) for the PCR's outlined above contains 5 primer pairs (10 primers) in a multiplex reaction. The five primer pairs include all three sequevar specific PCR primer pairs and an *R. solanacearum*-species specific PCR primer pair, 759/760 (Opina, Tavner et al. 1997).

The reaction Mixture for a single PCR is:

Solution	**Stock Concentration**	**Final Concentration**	**Volume for 1 reaction**
Sterile Distilled Water	-	-	10.55 µL
dNTP's	1.25 mM	200 µM	4.0 µL
PCR Buffer*	10X	1X	2.5 µL
Primer Mix (Table 2)	10X	1X	2.5 µl
$MgCl_2$*	25 mM	2µM	2.0 µL
DMSO	100%	5%	1.25 µl
Polymerase*	5U/µL	1U/reaction	0.2 µL

*** Amplitaq Gold DNA Polymerase is recommended for use in these PCR protocols. The concentration and volumes used in the PCR indicated in the Table are for Amplitaq Gold DNA Polymerase. PCR buffer and $MgCl_2$ are supplied with the polymerase.**

6.3.4 Gel electrophoresis

1. After the PCR is completed remove tubes and add 5 µL of loading dye (Appendix 2) to each tube and mix well using the pipette.
2. Load 5 µL of the mixture into the wells of a prepared 2% agarose gel in TAE (Appendix 2). Always use a molecular weight marker in a least one lane on the gel. The marker should be chosen to allow identification of product sizes of between 600 and 100 bp.
3. Run the gel at 10V/cm (divide the number of volts by the distance between the electrodes eg. for a 10 cm distance between the electrodes of the electrophoresis tank apply a voltage of 100V) for 20-30 min.
4. Stain the gel with 0.5 µg mL^{-1} EtBr solution for 10-15 min. Alternatively add 1 µL of 10mg mL^{-1} EtBr per 100 mL of 2% TAE agarose gel directly to the gel prior to electrophoresis.
5. The expected amplicon sizes for each *R. solanacearum* race 2/biovar 1 sequevar are presented in Table 2.

Table 3: Primer pairs, specificities and expected product sizes.

Primer	**Specificity**	**Product size**
SI28F SI28R	Sequevar 6 (MLG 28)	220bp
MUS20F MUS20R	Sequevar 4 (MLG 25)	351bp
IS24F IS24R	Sequevar 3 (MLG 24)	490bp
VC46F VC46R	Sequevar 24	100bp
759 760	*R. solanacearum*	282bp

6.4 Precautions During PCR Assays

Due to the exquisite sensitivity of PCR small amounts of contaminating template DNA either from a cell culture or, more importantly, PCR amplified DNA fragments samples can be contaminated and this can lead to false PCR positives. Most commonly cross-contamination is caused by the presence of contaminating DNA on gloves or micropipette's. Always ensure that clean gloves are used and always avoid contact with cell cultures and PCR amplified DNA. Micropipette's are commonly contaminated by aerosols generated during the pipetting operation. These aerosols commonly end up in the barrel of the micropipettes where they are transferred to other PCR tubes. To prevent this from occurring filter tips should be used and a dedicated area for the set-up and aliquoting of the PCR MasterMix should be employed. This set-up area should never be used for addition of template, gel-electrophoresis or any other stage of the PCR process.

6.5 Preferred Molecular Protocol
For the Identification of *R. solanacearum* in Infected Plant Tissue

Due to the relatively low sensitivity of the PCR tests developed it is suggested that the PCR test be applied to a culture of *R. solanacearum* rather than directly to a plant extract. Therefore it is suggested that a two stage process be used. Firstly the plant extract should be tested directly by PCR. Secondly this result should be confirmed by culturing of the plant extract on semi-selective media and testing of the cultures cells by PCR. It is also suggested that the multiplex PCR protocol be used to minimise the time and number of PCR tests required to test a sample.

6.5.1 Preparation of template for PCR

1. Cut a portion of the vascular tissue of an infected plant small enough to fit into a 2 mL microcentrifuge tube.
2. Place the tissue into a 2 mL microcentrifuge tube and add sterile distilled water into the minimum amount of water necessary to cover the cut end of the tissue.
3. Leave for 10 to 20 min until bacteria can be seen oozing out of the tissue.
4. Sterilise a pair of forceps by flaming with alcohol and use these to remove the plant tissue.
5. Centrifuge the sample and remove the supernatant.
6. Resuspend the pelleted bacteria in 1 mL of sterile distilled water, centrifuge and decant the supernatant leaving approximately 50-100 µL and resuspend the pellet in this remaining volume of water.
7. Use the bacterial suspension as template directly added to PCR.
8. Take a loopful of the suspension and plate (16 streak dilution) onto SPA and SMSA (see appendix 1 for recipes). Place plates in a 28^{o}C for 48-72 h.
9. From the SMSA or SPA plate make a dense suspension of bacteria in 1 mL of sterile distilled water.
10. Centrifuge the bacterial suspension and resuspend in a further 1mL of sterile distilled water. Centrifuge again, remove the supernatant and add I mL sterile distilled water to make a dense suspension of the bacteria.
11. Use this suspension directly as template for PCR.

6.5.2 Setting up the multiplex PCR reaction

The reaction Mixture for a single PCR is

Solution	Stock Concentration	Final Concentration	Volume for 1 reaction
Sterile Distilled Water	-	-	8.2 µL
dNTP's	1.25 mM	200 µM	4 µL
PCR Buffer*	10X	1X	2.5 µL
$MgCl_2$*	25 mM	2.0µM	2.0 µL
DMSO	100%	5%	1.25 µl
10X Primer Mix	10X	1X	2.5
AmpliTaq Gold DNA Polymerase*	5U/µL	1U/reaction	0.2 µL

*** Amplitaq Gold DNA Polymerase is recommended for use in these PCR protocols. The concentration and volumes used in the PCR indicated in the Table are for Amplitaq Gold DNA Polymerase. PCR buffer and $MgCl_2$ are supplied with the polymerase.**

Set up a MasterMix for the number of samples required. Aliquot 23 µL of PCR mixture into PCR tubes and add 2 µL of template (Plant extract or bacterial culture). Place tubes in the thermocycler and apply the PCR protocol listed below.

6.5.3 PCR Protocol

Initial Denaturation	96°C	10 min*	1 cycle
Denaturation	94ºC	30 s	30 cycles
Annealing	59ºC	90 s	
Extension	72ºC	90 s	
Final extension	72ºC	20 min	1 cycle
Hold	11ºC	-	-

**reduce to 5 min if not using AmpliTaq Gold*

6.5.4 Gel electrophoresis

1. After the PCR is completed remove tubes and add 5 µL of loading dye to each tube and mix well using the pipette.
2. Load 5 µL of the mixture into the wells of a prepared 2% TAE agarose gel. Always use a molecular weight marker in a least one lane on the gel. The marker should be chosen to allow identification of product sizes of between 600 and 100 bp.
3. Run the gel at 10V/cm (divide the number of volts by the distance between the electrodes eg. for a 10 cm distance between the electrodes of the electrophoresis tank apply a voltage of 100V) for 20-30 min.
4. Stain the gel with 0.5 µg mL^{-1} EtBr solution for 10-15 min. Alternatively add 1 µL of 10mg mL^{-1} EtBr per 100 mL of 2% TAE agarose gel directly to the gel prior to electrophoresis.
5. The expected amplicon sizes for each *R. solanacearum* race 2/biovar 1 sequevar are presented in Table 2 and an example gel is given in Figure 12.

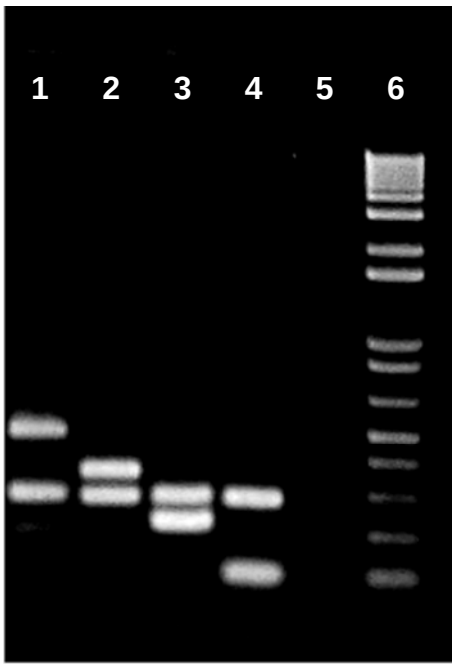


Figure 13: Amplification of *R. solanacearum* race 2/biovar 1 strains with the Moko multiplex PCR. Lane 1 – *R. solanacearum* race2/biovar 1 sequevar 3, Lane 2 - *R. solanacearum* race2/biovar 1 sequevar 4, Lane 3 - *R. solanacearum* race2/biovar 1 sequevar 6, Lane 4 - *R. solanacearum* race2/biovar 1 sequevar 24, Lane 5 negative control and Lane 6 molecular weight marker.

7 BDB PCR

Primer Sequences

121F – 5’ CGT ATT GGA TGC CGT AAT GGA 3’

121R – 5’ AAG TTC ATT GGT GCC GAA TCA 3’

Amplifies a 317bp amplicon from BDB strains

Master Mix

Solution	**Stock Concentration**	**Final Concentration**	**Volume added per reaction**
Sterile Distilled Water	-	-	12.8 µL
dNTP’s	1.25 mM	200 µM	4 µL
PCR Buffer*	10X	1X	2.5 µL
$MgCl_2$*	25 mM	1.5µM	1.5 µL
121F	6 mM	24 µM	1 µL
121R	6 mM	24 µM	1 µL
Polymerase*	5U/µL	1U/reaction	0.2 µL
Template (cell suspension as prepared above)	-	-	2 µL

*** Amplitaq Gold DNA Polymerase is recommended for use in these PCR protocols. The concentration and volumes used in the PCR indicated in the Table are for Amplitaq Gold DNA Polymerase. PCR buffer and $MgCl_2$ are supplied with the polymerase.**

Cycling conditions:

Initial Denaturation	96^{o}C	10 min*	1 cycle
Denaturation	94ºC	15 s	30 cycles
Annealing	59ºC	30 s	
Extension	72ºC	30 s	
Final extension	72ºC	10 min	1 cycle
Hold	11ºC	-	-

** reduce to 5 min if not using AmpliTaq Gold*

When visualised on an agarose gel according to the protocol (6.5.4, p39) a 317bp amplicon from BDB strains is produced as in Figure 13.

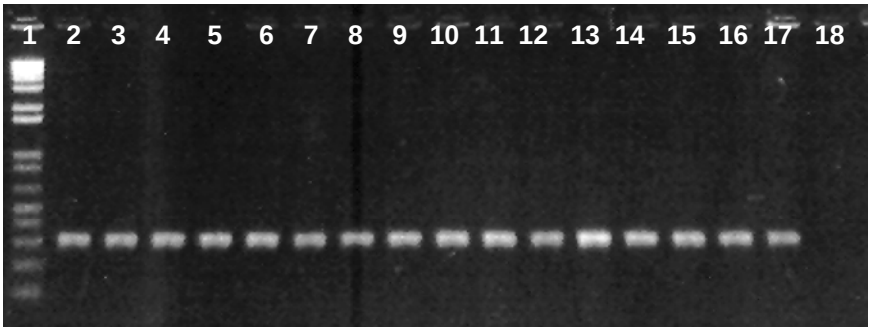


Figure 14: Amplification of 317 bp amplicon from banana samples infected with BDB. Lane 1 – molecular weight marker, Lanes 2-16 BDB positive samples, Lane 17 positive control and Lane 18 negative control.

8 Phylotype multiplex PCR

Primer Sequences

Primer Name	Primer Sequence
Nmult:21:1F	CGTTGATGAGGCGCGCAATTT
Nmult:21:2F	AAGTTATGGACGGTGGAAGTC
Nmult:22:InF	ATTGCCAAGACGAGAGAAGTA
Nmult:23:AF	ATTACSAGAGCAATCGAA
Nmult:22:RR	TCGCTTGACCCTATAACGAGTA
759	GTCGCCGTCAACTCACTTTCC
760	GTCGCCGTCAGCAATGCGGAATCG

Table 4: Expected amplicon sizes from the various diagnostic PCRs.

Primer	Specificity	Amplicon size when paired with Nmult:22:RR
Nmult:21:1F	Phylotype I	144bp
Nmult:21:2F	Phylotype II	372bp
Nmult:23:AF	Phylotype III	91bp
Nmult:22:InF	Phylotype IV	213bp
Nmult:22:RR	All phylotypes	NA[a]
759 760	*R. solanacearum*	182bp

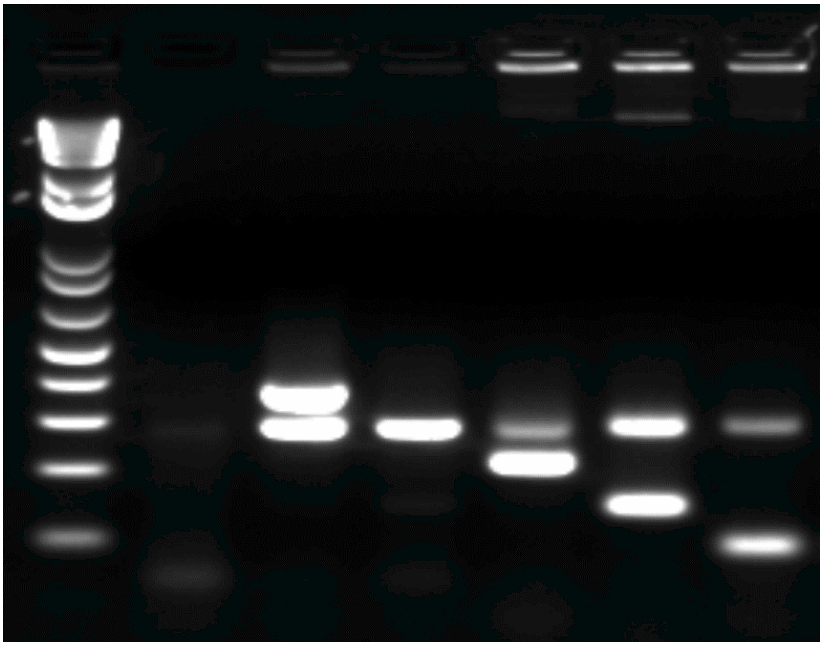


Figure 11: Example of a multiplex PCR gel. Lane 1 molecular weight marker (1kb plus DNA ladder; Life Technologies); Lane 2, negative control; Lane 3, a representative phylotype II strain; Lane 4 ACH0732; Lane 5 a representative phylotype IV strain; Lane 6, a representative phylotype I strain; Lane 7, a representative phylotype III strain

Preparation of 10X primer mix

Concentration of primer stock	**50 µM (50 pmol/µl)**	**100 µM (100 pmol/µl)**
Each primer (Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, Nmult:23:AF, Nmult:22:RR)	20 µl	10 µl
Primers 759, 760	10 µl	5 µl
TE buffer	Variable	Variable
Total volume	500 µl	500 µl

Master mix

Solution	**Stock Concentration**	**Final Concentration**	**Volume for 1 reaction**
Sterile Distilled Water	-	-	10.55 µL
dNTP's	1.25 mM	200 µM	4.0 µL
PCR Buffer*	10X	1X	2.5 µL
Primer Mix	10X	1X	2.5 µl
$MgCl_2$*	25 mM	2µM	2.0 µL
DMSO	100%	5%	1.25 µl
Polymerase*	5U/µL	1U/reaction	0.2 µL

*** Amplitaq Gold DNA Polymerase is recommended for use in these PCR protocols. The concentration and volumes used in the PCR indicated in the Table are for Amplitaq Gold DNA Polymerase. PCR buffer and $MgCl_2$ are supplied with the polymerase.**

Cycling conditions:

Initial Denaturation	96°C	10 min*	1 cycle
Denaturation	94ºC	30 s	30 cycles
Annealing	59ºC	90 s	
Extension	72ºC	90 s	
Final extension	72ºC	20 min	1 cycle
Hold	11ºC	-	-

** reduce to 5 min if not using AmpliTaq Gold*

9 Troubleshooting

9.1 Troubleshooting Guide 1 - Gel Electrophoresis

Problem	Possible Causes	Suggestions
Sample escapes from bottom of the wells	• Gel comb height set too low on adjustable comb holders • Bottom of the well pierced with a tip during loading • Gel not set when comb removed	• Check comb height is 2-3mm above tray before pouring gel • Do not place tip low into well when loading • Wait until gel is set before removing comb (15-20 min)
Samples have run backwards	• Electrodes or gel tank lid has been fitted backwards (possible on older gel rigs)	• DNA samples are –‘ve charged and so must always run from the black (-‘ve) cathode to the red (+’ve) anode
Samples have not run out of well	• Power pack failure • Incorrect fitting of electrodes or tank lid	• Always check for voltage by looking for bubbles rising from cathode • Check current (should be 40-50 mA at 100V) • Ensure correct dilution of TBE is used in the agarose gel and tank

Gel bands wavy, fuzzy or absent	• Incorrect concentration of TBE buffer in gel or tank • Water used instead of TBE (common error) • Air bubble in agarose gel • Incomplete dissolving of agarose before pouring gel	• Take care to remove air bubbles before gel begins to set • Ensure all agarose is dissolved before cooling and pouring gel • Ensure correct dilution of TBE is used in the agarose gel and tank
Sample flows up and out of well	• Insufficient glycerol or sucrose in gel loading buffer (GLB) • Too little GLB added to sample • Air bubble in gel loading tip • Sample loaded too fast • Sample well too shallow for volume added	• Check concentration of sucrose/glycerol in loading buffer or use commercially prepared loading buffer • Add GLB: sample in 1:6 ratio • Check pipetting technique • Check height that gel comb is set at
No bands visible on agarose gel when viewed under UV light (including molecular weight markers)	• Incorrect concentration of EtBr added to gel • Post-staining EtBr solution old	• Check concentration of EtBr added to gel • Post-stain in newly prepared EtBr solution
Uneven EtBr staining of gel (seen as patchiness or regions of intense fluorescence)	• EtBr added to agarose was not mixed properly • Post-staining with multiple gels in the tank does not allow good contact with the solution	• Swirl EtBr through cooled agarose (60 C) before pouring gel • Do not overload post-stain tank with many gels • Destain (water) and restain

9.2 Troubleshooting Guide 2 – PCR Diagnostic Results

Problem	Possible Causes	Suggestions
Failed positive control reaction	• Degraded template • Inhibitors in template • Too high or too low template concentration • PCR Primers degraded • dNTPs too old • PCR primer or dNTP preparation errors • *Taq* DNA polymerase inactive • Enzyme buffer or $MgCl_2$ not completely mixed • PCR cycler error or power outage	• Repeat PCR with new template (preferably extracted from single-spored culture to avoid PCR inhibition) • Use 1-10ng DNA as control • Repeat PCR with new aliquots of primers, dNTPs, buffers, $MgCl_2$ making sure to mix components thoroughly before addition
Failed negative control reaction	• Contamination of one or more PCR reagents	• Do not spend time trying to determine the exact source of the contamination • PCR reagents should be aliquotted. When PCR contamination occurs, a fresh aliquot canthen be used. • Repeat experiment with new aliquots of reagents

Unexpected positive result: • Symptoms not typical for disease • Unexpected incursion of the disease • First record of disease in a region	• Cross-contamination of templates • The PCR diagnostic assay is both highly specific and sensitive: the result is most likely real	• Ensure +'ve control is loaded on the gel after test samples • Check extraction buffer and negative PCR controls • To further confirm the result, the diagnostic PCR product may be cloned and sequenced

9.3 Limitations of the Technology

Where there is any doubt as to the validity of the results then the sequence of the diagnostic region should be obtained in order to ascertain whether or not the result is due to a false positive or a feint positive sample. If the result is still positive and the integrity of the primers is intact then refer to the trouble shooting guide above in order to ascertain the possible causes of this. Should the result be negative then the integrity of the primers need to be checked. Unfortunately although every effort has been made to ensure that these primers are specific for these pathogens, without obtaining the sequence of every bacterium that may ever infect a banana leaf the specificity cannot be guaranteed.

Due to the nature of PCR-based protocols, specific issues arise because of the high level of sensitivity and amplification of millions of copies of the target sequence. A few molecules of PCR-generated fragments can contaminate samples of subsequent PCR runs and result in false positives. On the other hand, a low copy number of initial target DNA sequences makes the first amplification cycles critical e.g. PCR inhibition can result in false negatives.

False positives can result from cross-amplification of non-target DNA, exogenous DNA from cells/cultures or aerosols, or from contaminating DNA originating from carry-over of previous experiments. A negative control (that contains no template DNA) should be included in all PCR diagnostic tests to identify false positive results.

False negatives can arise for many reasons, including the presence of compounds derived from extracted substrates that inhibit *Taq* polymerase, degradation of the DNA target sequence, or reagent problems. Including a positive control for DNA (a known positive DNA sample) and the presence of an amplified internal standard for each sample can protect against false negatives.

Sample size

The sampling strategy is another critical aspect of a PCR based diagnostic test, particularly because a very large initial sample size needs to be consolidated to a very small (50 µl) sample size endpoint. A high level of sensitivity is required because the persistence of latent infections and the multicyclic nature of disease spread can result in the high risk of disease, even with low inoculum levels. However, as mentioned above, sometimes zero tolerance is not necessary and proper detection thresholds need to be determined by correlating pathogen populations with disease risk.

Detecting non-viable cells

PCR is a proxy measurement, “an indirect indicator of cell presence”, which raises the criticism that DNA from dead cells (i.e. no disease risk) may be amplified and result in false positives

10 Sources of Reference Material

10.1 DNA/Bacterial Cultures

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11 Media and Stock Solutions

11.1 Media

11.1.1 Sucrose Peptone Agar (SPA)

Composition g L^{-1}

Sucrose	20.0
Peptone	5.0
Potassium hydrogen orthophosphate ($K_2HPO_{4)}$	0.5
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	0.25
Agar	15.0

Adjust pH to 7.2 – 7.4 with 40% sodium hydroxide (NaOH)

11.1.2 Modified – SMSA

Composition g L^{-1}

Casamino acid	1.0
Peptone	10.0
Glycerol	5.0
Agar	15

Sterilise the medium by autoclaving and cool to 55ºC and add 5 mL of a 1% stock solution of 2,3,5-triphenyl tetrazolium chloride. The stock is prepared in distilled water and filter sterilised through a 0.2 µM membrane. While the medium is aat 55 ºC also add the following antibiotics dissolved in 70% alcohol.

Crystal violet 1%	0.5 mL	(final conc. 5 mg/L)
Polymyxin B sulfate 1%	10 mL	(final conc. 100 mg/L)
Bacitracin 1%	2.5 mL	(final conc. 25 mg/L)
Chloromycetin 1%	0.5 mL	(final conc. 5 mg/L)
Penicillin 0.1%	0.5 mL	(final conc. 0.5 mg/L)

When using for isolation of *R. solanacearum* from soil also add 2.5 mL of Cyclohexamide 1% (in 70% ethanol) (final conc. 100 mg/L).

This semi-selective medium is particularly useful to improve detection of *R. solanacearum* when the bacterium is in low numbers in plant material or soil by first culturing the organism and then applying a diagnostic PCR.

11.2 Stock Solutions

11.2.1 6X Loading Buffer

Xylene Cyanol FF 0.25%

Ficol (Type 400; Pharmacia) 15%

Store at room temperature

11.2.2 Tris-Acetate-EDTA buffer – 50X stock

Tris base 242 g/L

Glacial Acetic Acid 57.1 mL/L

0.5M EDTA (ph8.0) 100 mL/L

12 Further Reading

Allen C., Prior, P. and Hayward A.C. (Eds) Bacterial Wilt: The Disease and the *Ralstonia solanacearum* species complex. (2005) APS Press, St. Paul, Mn, USA.

Jones, D.R. (Ed) Diseases of Banana, Abacá and Enset. (2000) CABI Publishing, Wallingford, Oxon, UK.

13 References

Ashby, S. F. (1926). "A wilt disease of bananas." Tropical Agriculture **3**: 127-129.

Baharuddin, B. (1994). Pathological, biological and serological characterization of the blood disease bacterium affecting banana and plantain (*Musa* spp.) in Indonesia. Institute of Plant Pathology and Plant Protection, Faculty of Agricultural Sciences. Göttingen, Georg-August-University, Göttingen**:** 129.

Buddenhagen, I., L. Sequeira, et al. (1962). "Designation of races in *Pseudomonas solanacearum*." Phytopathology **52**: 726.

Buddenhagen, I. W. (1986). Bacterial wilt revisited. Bacterial wilt disease in Asia and the South Pacific: proceedings of an international workshop held at PCARRD, Los Banos, Philippines, 8-10 October 1985. G. Persley. Canberra, Australia, ACIAR**:** 126-143.

Buddenhagen, I. W. (1994). Banana diseases caused by bacteria. Compendium of Tropical Fruit Diseases. R. C. Ploetz, G. A. Zentmyer, W. T. Nishijima, K. G. Rohrbach and H. D. Ohr. St. Paul, Minnesota, APS Press**:** 15-17.

Buddenhagen, I. W. and T. A. Elsasser (1962). "An insect-spread bacterial wilt epiphytotic of bluggoe banana." Nature **194**: 164-165.

Cook, D., E. Barlow, et al. (1989). "Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment polymorphisms with DNA probes that specify virulence and hypersensitive response." Molecular Plant-Microbe Interactions **2**: 113-121.

Cook, D. and L. Sequeira (1994). Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A. C. Hayward and G. L. Hartman. Wallingford, United Kingdom, CAB International**:** 77-93.

Diatloff, A., E. Akiew, et al. (1992). "Characteristics of isolates of Pseudomonas solanacearum from Heliconia." Australasian Plant Pathology **21**(4): 163-168.

Eden-Green, S. and S. Seal (1993). Bacterial diseases of banana and plantain in Southeast Asia. Breeding banana and plantain for resistance to diseases and pests. J. Ganry. Monpellier, France, CIRAD, INIBAP**:** 115-121.

Eden-Green, S. J., Ed. (1994). Banana Blood Disease. *Musa* Disease Fact Sheet No. 3. Montpellier, France, INIBAP.

Eden-Green, S. J. (1994). Diversity of *Pseudomonas solanacearum* and related bacteria in South East Asia: new direction for moko disease. Bacterial wilt: the disease and its causative organism, *Pseudomonas solanacearum*. A. C. Hayward and G. L. Hartman. Wallingford, United Kingdom, CAB International**:** 25-34.

Eden-Green, S. J. and H. Sastraatmadja (1990). "Blood disease present in Java." FAO Plant Protection Bulletin **38**: 49-50.

Fegan, M. and P. Prior (2005). How complex is the "*Ralstonia solanacearum* species complex". Bacterial Wilt: The Disease and the *Ralstonia solanacearum* species complex. C. Allen, P. Prior and A. C. Hayward. St. Paul, Mn, USA, APS Press**:** In Press.

Fegan, M., M. Taghavi, et al. (1998). Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum*. Bacterial Wilt Disease: Molecular and Ecological Aspects. P. Prior, C. Allen and J. Elphinstone. Paris, France, INRA Editions**:** 19-33.

French, E. R. and L. Sequeira (1970). "Strains of *Pseudomonas solanacearum* from Central and South America: a comparative study." Phytopathology **70**: 506-512.

Gäumann, E. (1921). "Onderzoekingen over de bloedziekte der bananen op Celebes I." Mededeelingen van het Instituut voor Plantenziekten No. 48.

Gillings, M., P. Fahy, et al. (1993). "Restriction analysis of an amplified polygalacturonase gene fragment differentiates strains of the phytopathogenic bacterium *Pseudomonas solanacearum*." Letters in Applied Microbiology **17**: 44-48.

Gillings, M. R. and P. Fahy (1994). Genomic Fingerprinting: towards a unified view of the *Pseudomonas solanacarum* species complex. Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A. C. Hayward and G. L. Hartman. Wallingford, United Kingdom, CAB International**:** 95-112.

Harris, D. C. (1972). Intra-specific variation in *Pseudomonas solanacearum*. Proceedings of the Third International Conference on Plant Pathogenic Bacteria Wageningen 14 - 21 April 1971. H. P. Mass Geesteranus. Wageningen, Centre for Agricultural Publishing and Documentation**:** 289-292.

Hayward, A. C. (1964). "Characteristics of *Pseudomonas solanacearum*." Journal of Applied Bacteriology **27**: 265-277.

Hayward, A. C. (1991). "Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*." Annual Review of Phytopathology **29**: 67-87.

Hayward, A. C. (1994). Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A. C. Hayward and G. L. Hartman. Wallingford, CAB International**:** 123-135.

He, L. Y., L. Sequeira, et al. (1983). "Characteristics of strains of *Pseudomonas solanacearum*." Plant Disease **67**: 1357-1361.

Hyde, K. D., B. McCulloch, et al. (1992). "Strategies used to eradicate bacterial wilt of Heliconia (race 2) in Cairns, Australia, following introduction of the disease from Hawaii." Australasian Plant Pathology **21**(1): 29-31.

Ilagan, Y. A., W. A. Lavina, et al. (2003). "Genetic homogeneity of the banana-infecting strains of *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. in the Philippines." Philippine Agricultural Scientist **86**(4): 394-402.

Ito, S., Y. Ushijima, et al. (1998). "Detection of viable cells of *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique." Journal of Phytopathology-Phytopathologische Zeitschrift **146**(8-9): 379-384.

Opina, N., F. Tavner, et al. (1997). "A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*)." Asia Pacific Journal of Molecular Biology and Biotechnology **5**: 19-30.

Palleroni, N. J. (1984). Pseudomonadaceae. Bergey's Manual of Systematic Bacteriology. N. R. Krieg and J. G. Holt. Baltimore, Williams and Wilkins**:** 141-219.

Pegg, K. G. and M. Moffett (1971). "Host range of the ginger strain of *Pseudomonas solanacearum* in Queensland." Australian Journal of Experimental Agriculture and Animal Husbandry **11**: 696-698.

Phelps, R. H. (1987). The status of moko disease of banana in the caribbean. Improving citrus and banana production in the Caribbean through phytosanitation: seminar proceedings, St. Lucia West Indies 2-5 December 1986. Wageningen, The Netherlands, CARDI.

Poussier, S., P. Prior, et al. (2000). "Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the known diversity within the *Ralstonia solanacearum* species complex." Systematic and Applied Microbiology **23**(4): 479-486.

Poussier, S., D. Trigalet-Demery, et al. (2000). "Genetic diversity of *Ralstonia solanacearum* as assessed by PCR- RFLP of the hrp gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision." Microbiology **146**: 1679-1692.

Poussier, S., P. Vandewalle, et al. (1999). "Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the hrp gene region." Applied and Environmental Microbiology **65**(5): 2184-2194.

Prior, P. and M. Fegan (2005). Diversity and molecular detection of Ralstonia solanacearum race 2 strains by multiplex PCR. Bacterial Wilt: The Disease and the Ralstonia solanacearum species complex. C. Allen, P. Prior and A. C. Hayward. St. Paul, Mn, USA, APS Press**:** In Press.

Raymundo, A. K., Y. Aves-Ilagan, et al. (1998). Analysis of genetic variation of a population of banana infecting strains of *Ralstonia solanacearum.* Bacterial Wilt Disease: Molecular and Ecological Aspects. P. Prior, C. Allen and J. Elphinstone. Paris, France, INRA Editions**:** 56-60.

Raymundo, A. K. and Y. A. Ilagan (1999). "Cloning of a repetitive element which distinguishes banana strains of *Ralstonia solanacearum* in the Philippines." The Philippine Agricultural Scientist **82**(3): 338-350.

Rillo, A. R. (1979). "Bacterial wilt of banana in the Philippines." FAO Plant Protection Buletin **27**: 105-108.

Rillo, A. R. (1981). "Differences of Pseudomonas solanacearum EFS isolates in Abacá and Enset." The Philippine Agriculturalist **64**: 329-334.

Seal, S. E., L. A. Jackson, et al. (1992). "Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification." Applied and Environmental Microbiology **58**: 3759-3761.

Sequeira, L. (1998). Bacterial wilt: the missing element in international banana improvement programs. Bacterial Wilt Disease; Molecular and Ecological Aspects. P. Prior, C. Allen and J. Elphinstone. Paris, France, INRA Editions**:** 6-14.

Sequeira, L. and C. W. Averre (1961). "Distribution and pathogenicity of strains of *Pseudomonas solanacearum* from virgin soils in Costa Rica." Plant Disease Reporter **45**: 435-440.

Stover, R. H. (1972). Banana, Plantain and Abaca diseases. Surrey, England, Commonwealth Mycological Institute.

Stover, R. H. and D. L. Richardson (1968). "Pelipita, an ABB Bluggoe-type plantain resistant to bacterial and fusarial wilts." Plant Disease Reporter **52**: 901-903.

Stover, R. H. and N. W. Simmonds (1987). Bananas. New York, Longman Scientific and Technical.

Supriadi (2002). Present status of blood disease in Indonesia. 3rd International Bacterial Wilt Symposium, White River, South Africa.

Taghavi, M., C. Hayward, et al. (1996). "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 **46**: 10-15.

Thwaites, R., S. J. Eden-Green, et al. (2000). Diseases caused by bacteria. Diseases of Banana, Abaca and Enset. D. R. Jones. Wallingford, United Kingdom, CABI Publishing**:** 213-239.

Thwaites, R., J. Mansfield, et al. (1999). "RAPD and rep PCR-based fingerprinting of vascular bacterial pathogens of *Musa* spp." Plant Pathology **48**(1): 121-128.

Yabuuchi, E., Y. Kosako, et al. (1995). "Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia picketti* (Ralston, Palleroni and Douderoff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov." Microbiology and Immunology **39**: 897-904.