ABSTRACT

*Ralstonia solanacearum* (*Rs*) is the causal organism of bacterial wilt of more than 200 species and 50 families of plants in tropical, subtropical, and warm regions in the world. *Rs* has been traditionally classified into five races based on differences and six biovars on the basis of biochemical properties for a long time. Several investigations employing molecular methods have confirmed this dichotomy within *Rs*. The current bacterial wilt situation and the relationship with the genetic diversity based on the recent genetic information of the Asian *Rs* strains are summarized. In relation to emerging diseases, a bacterial wilt disease of *Zingiberaceae* plants caused by *Rs* strains occurred in ginger, mioga and curcuma fields of Kochi Prefecture, Japan is described. The disease has been expanding ever since to outside of the Prefecture. Characterization of the pathogenic *Rs* strains revealed that the disease was caused by two types of the genetically different exotic *Rs* strains from different origins and has been spreading in epidemic proportions through separate routes. To control bacterial wilt disease, a wide range of disease management and control methods have been applied. As an important component of integrated disease management to be successfully achieved, case study of cultural, chemical, and biological (including resistant host) controls were outlined.

Keywords: *Ralstonia solanacearum*: diversity: emerging disease: disease management

INTRODUCTION

Bacterial wilt is a lethal vascular disease in the family Solanaceae, attacking economically important crops such as potato, tomato, pepper and eggplant (Hayward 1991, 1994). The importance of the disease has been widely recognized in tropical, subtropical and warm temperate regions of the world, including Asian countries.

*Ralstonia solanacearum* (*Rs*) is the causal organism of bacterial wilt of more than 200 species and 50 families of plants (Hayward 1994). Strains of *Rs* differ in host range, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties (Buddenhagen and Kelman 1964). For more than four decades, this pathogen mainly employed a binary system using two different approaches, race and biovar, one placing emphasis on host range characterization and the other making use of selected biochemical properties (Hayward 1991). Thus five races and six biovars have been described and designated so far according to the host or hosts primarily affected (Buddenhagen and Kelman 1964, He et al. 1983) and to metabolism of different carbon sources (Hayward 1964; Hayward 1994), respectively.

The development of molecular biology has shifted in the types of approaches used to
characterize, identify plant pathogens and enable to devise disease management strategies (Schaad et al. 2003). Consequently, more efficient approaches have been devised, the most popular and possibly the most accurate of which is the use of DNA analysis. Studies of DNA-DNA homology of Rs strains have revealed that the relatedness between isolates of this species is often less than the 70% threshold level commonly expected with in a species, which become defined Rs as a species complex (Gillings and Fahy 1994, Poussier et al. 2000a, Villa et al. 2005).

**Diversity of bacterial wilt diseases and *Ralstonia solanacearum* in Asia: Geographical distribution and current status in Asia**

Based on pathogenic specialization, five races have been described, designated according to the host range; race 1 affects tobacco, tomato, many solanaceous crops and other weeds and certain diploid bananas; race 2 causes bacterial wilt of triploid bananas (Moko disease) and *Heliconia*; race 3 affects potato and tomato; race 4 and race 5 affect ginger and mulberry, respectively (Buddenhagen and Kelman 1964, He et al. 1983). On the other hand, six biovars (1, 2, N2, 3, 4 and 5) were differentiated based on metabolism of different carbon sources (Hayward 1964; Hayward 1994). Strains of biovars 3 or 4 (Phylotype 1) are widespread and affect a very wide range of economically important crops across Asia, and have been reported from at least 20 Asian and Middle Eastern countries; strains with biovars 3, 4 and N2 is reported in Japan, race 1 is present in 11 states in India. The most important crop affected is tomato in the major production areas of all affected countries. Bacterial wilt is also widely ranked among the top five diseases of other solanaceous crops, including eggplant, sweet pepper, and chili. Bacterial wilt of ginger has become very destructive in many parts of Asia in the last decade, including India, the Philippines, northern Thailand, Indonesia and just recently, in Japan. Bacterial wilt of groundnut is currently significantly affected in Vietnam, the Philippines, Thailand, and India. Rs has been reported on banana in several Asian countries. In the Philippines, it is confirmed that Rs is still widespread throughout the country and is currently among the top five pathogens. Moko disease and bugtok disease is commonly found in commercial banana plantation and on cooking banana cultivars, respectively. Although the symptoms differ, both diseases are caused by Phylotype II (race 2 biovar 1) strains which are indistinguishable genetically. In Indonesia, the banana blood disease bacterium (Phylotype IV) has been distinctly reported and is widely spreading the country, in addition to being an important threat to banana production in South East Asia (Elphinstone 2005).

**Characterization of *Ralstonia solanacearum* biovar N2 strains in Asia**

*R. solanacearum* Rs biovar N2 strains isolated in Asia were compared by biochemical tests with biovar N2 strains from South America and biovar 2 (race 3) strains from Africa, America, Asia and Europe. Distinct differences were found between Asian and South American strains of biovar N2, and between Asian biovar N2 and biovar 2 strains with respect to their ability to utilize several carbon sources. Using cluster analysis based on rep-PCR genomic fingerprints, the Asian biovar N2 strains were divided into two groups, group 1 containing Japanese strains and group 2 containing Indonesian and Philippine strains (Fig.1). The fingerprints showed the genetic diversity of biovar N2 strains in Asia (Horita et al., 2005).

So far molecular biological studies were conducted to characterize Rs strains from diverse origins. Accordingly, a classification scheme, based on analysis of restriction fragment length polymorphisms (RFLP) at the hypersensitive response and pathogenicity (hrp) locus and additional loci from the core genome (Cook et al. 1989), revealed the existence of two evolutionary divisions, corresponding to division I, comprised strains mainly isolated from Asia and Australia, and division II, with strains mainly originating from South and Central America. The genetic classification based on geographical origin, thus correlates nicely with the biovar classification because strains from division I match biovars 3, 4, and 5 and strains from division II match biovars 1, 2, and N2. The diversification of Rs into two major divisions later was confirmed using additional molecular criteria addressing various elements of the core genome including polymerase chain reaction based on PCR-RFLP analysis of polygalacturonase (*pgl*) and hrp genes (Gillings et al. 1994), Comparisons of rRNA sequences (Taghavi et al. 1996) by AFLP analysis on genomic DNA (Poussier et al. 2000b), and comparison of a partial nucleotide sequence of the *hrpB* and endoglucanase genes (*egl*) (Poussier et al. 2000a, Villa 2005) have confirmed the diversification
of the pathogen. Some of these studies allowed the identification of a third division for strains originating from Africa (Poussier et al. 1999, 2000a, 2000b).

Over the years, various genetic analyses have been carried out by many authors and confirmed the genetic variability of the pathogen both between and within the population of Rs (Horita and Tsuchiya 2000a, 2001, Tsuchiya 2004). Genomic fingerprints using repetitive DNA based PCR (Rep-PCR) have been employed to determine the clonal line of phytopathogenic bacteria including R. solanacearum (Fegan and Prior 2005, Louws et al. 1994).

Recently a new hierarchical classification scheme has been introduced by Fegan and Prior (2005), which redefines Rs as a species complex. They also subdivide the species complex into four phylotypes corresponding to the four genetic groups. A phylotype is further defined as a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data. The phylotype classification scheme has confirmed that phylotypes I and II are equivalent to divisions I and II defined by Cook et al. (1989). Phylotype III contains strains mainly from Africa and phylotype IV contains Indonesian strains (biovars 1, 2, and N2) (Villa et al. 2005). Furthermore, taxonomic classifications of R. solanacearum strains to sequevar and infrasubspecific groups have been outlined based on an endoglucanse gene sequencing (Fegan and Prior 2005).

According to Villa et al. (2003, 2005), the 16S rDNA, endoglucanase, and hrpB genes were partially sequenced for Asian strains of R. solanacearum spp. complex, including strains of Rs and each of the blood disease bacterium (BDB) and Pseudomonas syzygii. Various levels of polymorphisms were observed in each of these DNA regions. The highest polymorphism (approximately 25%) was found in the endoglucanase gene sequence. The hrpB sequence had about 22% polymorphism. The phylogenetic analysis consistently divided the strains into four clusters, as distinctly shown on the phylogenetic trees of 16S rDNA, hrpB gene and endoglucanase gene sequences. Cluster 1 contained all strains from Asia, which belong to biovars 3, 4, 5 and N2. Cluster 2 comprised the Asian strains of R. solanacearum (as biovar N2 and 1) isolated from potato and clove, as well as BDB and P. syzygii. Cluster 3 contained race 3 biovar 2 strains from potato, race 2 biovar 1 strains from banana, and race 1 biovar 1 strains isolated from America, Asia and other parts of the world. Cluster 4 was exclusively composed of African strains. The results of the study showed the distribution and diversity of the Asian strains, which are present in three out of the four clusters. The similarity of Asian strains to those in the other regions was also observed.

Fig. 1. Genetic diversity of strains of Ralstonia solanacearum biovar N2 on the basis of rep-PCR.
(Horita et al., 2005)
Genetic and biological characters of Japanese potato strains of *Ralstonia solanacearum*

Geographical distribution, biovar, phylotype, DNA fingerprints (rep-PCR), and/or endoglucanase sequence of potato bacterial wilt pathogen (Fig. 2), *R. solanacearum* (*Rs*), in Japan were assessed (Horita *et al.* 2010). *Rs* have been isolated from potato cultivated fields in southwestern, warm, temperate regions. Of the 188 isolates, 74 belonged to biovars N2 (39%), 44 to biovar 3 (24%), and 70 to biovar 4 (37%). Biovars N2 and 4 strains were widely distributed, from Northern (Hokkaido) to Southern (Okinawa) Japan. Based on the results of multiplex-PCR analysis, every potato strains belonged to either phylotypes I or IV. Phylotype I comprised both biovars 3 and 4 strains. On the other hand, phylotype IV included biovar N2 strains (Fig. 3). None of the strains belonged to phylotypes II or III or biovars 1 or 2. Phylogenetic analysis based on DNA fingerprints and endoglucanase gene sequences clarified the genetic diversity of the Japanese potato strains and the close genetic relationship between the Japanese strains and the Asian strains in phylotypes I and IV.

Emergent occurrence and dissemination of ginger bacterial wilt in Japan

*Rs* strains that affect Zingiberaceae crops have been widespread and exceedingly destructive in many parts of the world, including Hawaii, Africa, several Asian countries and Australia (no severe recurrence since 1970) in the last decades (Hayward 1994, Elphinstone 2005, Tsuchiya *et al.* 2005, Zehr 1969, 1970). In Japan, bacterial wilt of Zingiberaceae plants was first reported in *Curcuma alismatifolia* cultivation fields of Kochi prefecture in 1995 (Morita *et al.* 1996). Subsequently, outbreaks of the disease have occurred in ginger and mioga cultivation fields in 1997 and 1999, respectively, throughout neighboring areas of the same prefecture (Tsuchiya *et al.* 2005, Yano *et al.* 2005, Fig. 4). The causal pathogens were identified as race 4 based on their specific pathogenicity to...
Zingiberaceae crops (Morita et al. 1996, Tsuchiya et al. 1999 &2005, Yano et al. 2005 & 2011). All of the strains from infected plants of the three zingiberaceous plants were identical and have been identified as biovar 4 by comparing with those strains from Thailand and Indonesia (biovar 3 and 4) and of Australia and China (biovar 4). Genetic diversity analyzed by rep-PCR revealed that the Japanese race 4 strains are sub-classified into two DNA fingerprint types; type I showed quite similar to that of ginger strains from Thailand, and type II is also homogeneous to ginger strains from China and Australia (Fig. 5) (Horita et al. 2004, Tsuchiya et al. 2005). Determination of the phylotype tested by multiplex PCR according to Fegan and Prior (2005) revealed that it was Phylotype I. Strains were further examined using two PCR primer sets (Horita et al. 2004), each specially amplify a 165-bp and a 125-bp band from Japanese race 4 strains representing type I or type II DNA fingerprints, respectively (Fig. 5).

So it is concluded that the disease caused by these two exotic R. solanacearum strains started from different origins and has been spreading in
epidemic proportions through separate routes.

Regarding pathogenicity of the strains, all type I isolates were highly virulent on ginger, mioga and curcuma; weakly virulent on tomato, eggplant and sweet pepper; and avirulent to *Musa velutina*. On the other hand, type II isolates were highly virulent on ginger, mioga, tomato, eggplant, sweet pepper and *Musa velutina* and weakly virulent on curcuma. The Phylogenetic types of the isolates from Zingiberaceae plants in 1995-2009 were determined by the PCR method using above primer sets. All isolates from mioga and curcuma belonged to type I and the ginger isolates belonged to type I or type II. These results demonstrate a relationship between the Phylogenetic types of the isolates from Zingiberaceae plants and the host range of the types (Yano et al. 2011). Among zingiberaceous strains, there appears to be existed strains with different virulence and pathogenic specialization. Biovar 4 was the cause of a severe and rapid wilt distinct from a less common slow wilt, with which biovar 3 was associated in Australia (Queensland) (Hayward et al. 1967, Pegg and Moffet 1971). Some similarities or differences of infection between the results of cross-inoculation studies carried in several countries.

**Disease management strategies for control of bacterial wilt**

Despite decades of efforts by many national and international organizations, bacterial wilt has continued to be a considerable problem throughout the world. The variability of both pathogen and the agroecosystems has undoubtedly hampered progress in controlling the disease. As control strategies of bacterial wilt, there are cultural, chemical, and biological controls as well as host resistance for the combination of these strategies forming the integrated disease management.

Various cultural practices, whether deliberate or not, have been effective in reducing the occurrence of bacterial wilt. Rotation to a non-host crop forces pathogens to persist as survival structures and/or as saprophytes. Starvation of the pathogen is a key mechanism of crop rotation. Rotation with maize, okra, cowpea, French marigolds, Chinese chive, rice and so on have been examples of cropping systems that have reduced the incidence of wilt in infested potato, groundnuts, eggplant and tomato fields in Nepal, Taiwan, China, India and other Asian countries (Saddler, 2005). Solarization assays have demonstrated some efficacy against *Rs* biovar 2, but not against other biovars (French 1994).

Chemicals and other forms of treatments, in particular treatment of soil infestations have been investigated in addition to cultural methods. Chemicals, including antibiotics, fertilizers and fungicides, have been tested without much success. A number of soil fumigants like chloropicrin and methyl bromide have been tested for control to some extent for many crops, but in general fumigation is not economically feasible over large areas. There are increasing concerns about applicator hazards and environmental problems, including destruction of the protective ozone layer by methyl bromide and groundwater contamination with toxic compounds. Some soil fumigants have been and will be banned. On the other hand, products called “plant activators” that induce SAR (systemic acquired resistance) in plants were identified, and have been shown to induce host resistance in tomato to bacterial wilt (Qui et al. 1997).

Biological control is based on microbial antagonism, which can be direct (competition, antibiosis) or indirect (induced resistance of the host). Bacteria (*Pseudomonas fluorescens, Pseudomonas glumae* etc.) (Keime and sequeira 1983, Wakimoto 1987) which are antagonistic towards *R. solanacearum* have been isolated from various sources, e.g. suppressive soils and rhizosphere of host plants. In addition, spontaneous avirulent mutants of *R. solanacearum*, deficient in EPS production that are able to colonize the host, have long been reported as potential antagonists towards virulent strains of the pathogen (Tanaka et al. 1990, McLaughlin and Sequeira 1988). Biocontrol strategies developed in the laboratory often fail under natural conditions; thus a correlation between antagonism as demonstrated in the laboratory and biocontrol is lacking. Numerous authors have proposed that direct antagonism may not be involved in planta and that there are other mechanisms, such as induced resistance of host plants (Keime and sequeira 1983, Furuya et al. 1991, Wakimoto 1987). These suggestions are based on observations that avirulent mutants devoid of any in vitro antagonism are nevertheless effective in reducing the disease severity in greenhouse and field tests. Recently, a number of plant growth-promoting (PGP) endophytic bacteria (EB), *Pseudomonas* spp., has been isolated from surface-sterilized leaves, stems and roots of tomato (Aino et al. 1997), *Solanum nigrum*, a wild solanaceous plant species (Hoang et al. 2010, 2011) in Japan. They exerted with/without strong in vitro antagonism against *Ralstonia solanacearum* and
significantly reduced the wilt incidence in tomato or tobacco. It is indicated that resistance in tobacco against *R. solanacearum* induced by endophytic *Pseudomonas* spp. is associated with the systemic induction of PR proteins in the SA-dependent pathway Hoang et al. (2011).

It is universally recognized that the most effective and practical way to control bacterial wilt is to plant cultivars showing durable resistance. Thus, bacterial wilt-resistant varieties have been in use for disease control for over 90 years. Worldwide, breeding for resistance to bacterial wilt has been concentrated on crops of wide economic importance such as tomato, potato, tobacco, eggplant, peppers and peanut. In several crops, generating effective resistance has proven to be difficult while in certain crops, the development of useful resistant cultivars has been quite successful (Boshou 2005). So far, the use of cultivar resistance is the most effective means for controlling bacterial wilt. In tomato, however, resistance to bacterial wilt is controlled by many genes. No fresh market tomato cultivar possesses both high resistance and high fruit quality. In Japan, therefore, highly resistant rootstocks as well as grafting have been investigated and developing for eggplant and tomato. Susceptible but high-quality tomato cultivars onto these resistant rootstocks have been adopted to manage bacterial wilt (Nakaho and Takaya 1993). However, recently, bacterial wilt has been reported in commercial tomato cultivars grafted onto resistant rootstocks. Nakaho and his colleagues examined and clarified that the resistance in tomato cultivars is due to reduced horizontal and vertical movement of *R. solanacearum* in the xylem tissues, which may limit pathogen density in infected tissues, and bacterial wilt resistance in rootstocks is due to bacterial movement and multiplication in plants. Based on the results they have developed newly high grafting (grafting at a higher position) method, which provide more effective bacterial wilt protection than usual (Nakaho et al. unpublished).

**DISCUSSION**

In the studies on genetic diversity of *R. solanacearum*, the use of multiplex PCR and partial endoglucanase gene sequences has enabled to define the phylootypes and rep-PCR fingerprint enabled to define the existence of variability among strains from Asian countries. This finding contributes to update the existing information on *Rs* strains in Asia, and can help us to discriminate and assess emerging pathogens or strains that could be potentially introduced into the country. It will also be useful in the development of molecular methods for practical diagnosis and establishing new strategies for disease control.

Concerning the emerging occurrence of diseases such as the ginger bacterial wilt in Japan, the movement of infected, generally asymptomatic, planting material represents the most significant route by which the disease has spread on not only on global but also on a domestic scale. Seed and plants for planting are generally the subject of rigorous inspection to ensure material is pathogen-free. Methods range from visual inspection of material, to sampling backed-up with laboratory diagnosis or the imposition of post-entry quarantine measures to ensure freedom from disease. The imposition of measures to prevent spread and eradicate the outbreak involve the enforcement of quarantine measures on infected fields and farms, crop rotation, control of weed hosts and volunteer plants, avoidance of surface water for irrigation, and education. This will be followed up by extensive surveys and the site of importation over an extensive period to ensure that the pathogen will not emerge.

**CONCLUSION**

Effective control of bacterial wilt requires an integrated management program. High-quality pathogen-free seed and plant materials with an acceptable level of resistance, combined with multi-year rotational cropping schemes, should be effective in limiting pathogen population. Fumigants and chemical treatments are still used in some situations, but increasing public concern may limit their use in the future, and biological control agents might be the alternative. Information on resistance mechanisms is also essential for breeding cultivars with reliable bacterial wilt resistance, or for effective use of grafting cultivation to increase the resistance.

**REFERENCES**


Kempe, J. and Sequeira, L. 1983. Biological control


Villa, J. E., Tsuchiya, K., Horita, M., Opina, N and Hyakumachi, M. 2005. Phylogenetic relationships of *Ralstonia solanacearum*
species complex strains from Asia and other continents based on 16S rDNA, endoglucanase, and hrpB gene sequences. *Journal of General Plant Pathology* 71, 39–46.


