INDUCED SYSTEMIC RESISTANCE (ISR): AN EFFICIENT TOOL TO CONTROL BACTERIAL TRANSMITTED PAPAYA DIEBACK AND BANANA BLOOD DISEASES

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ABSTRACT

The Malaysian Government is focused on increasing the production of fruit types that can contribute economically to the industry. Among the fruits, are papaya and banana, which are important for local consumption and export. However, the goal to increase the production and export has been hampered by the emergence of invasive alien diseases namely papaya bacterial dieback and banana blood disease, which are transmitted by bacterial pathogens. Various approaches including chemical and biological controls have been attempted but none were successful in controlling these diseases. One of the techniques that has not been fully explored is the enhancement of plant resistance against diseases known as the Induced systemic resistance (ISR). The aim of this study was to induce systemic resistance in plants against both bacterial diseases using Plant growth-promoting rhizobacteria (PGPR). ISR technology to control both papaya bacterial dieback and banana blood disease was developed by bio-prospection and manipulation of ISR inducing PGPR from soil. For papaya, the ISR seedlings showed total recovery against the dieback infection while the Control seedlings (non-ISR) totally succumbed to the disease. When papaya seedlings (ISR and non-ISR) were produced and tested in a hotspot for dieback disease, for one crop cycle (2 years), all treatments except one survived, compared to the control where none of the plants survived. In another trial, one of the ISR treatment was selected and up-scaled in a famer's plot (hotspot). 95% of resistance against the dieback disease was recorded. This success enabled the technology to be commercialized and the product made available in the market for farmers to adopt. This success on papaya, compelled a repetition of the same approach to develop ISR technology against blood disease of banana. When the ISR banana seedlings were challenged to the pathogen, three treatments had recorded 100% suppression and another two had 75% suppression against the disease, whereas all the control plants did not survive. All these five treatments were tested in a hotspot area (area of elevated disease occurrence) where disease development was observed. During the first crop harvested from the mother plants, the ISR treatment recorded 0-8% disease infection compared to the Control which recorded three-fold higher incidences of infection than the ISR treatment (24%).

Keywords: papaya bacteria dieback, blood disease of banana, induced systemic resistance (ISR), plant growth-promoting rhizobacteria (PGPR)

1. INTRODUCTION

Papaya and banana are important Malaysian fruits grown for the domestic and export markets. Both fruits have been devastated by the emergence of bacterial transmitted diseases, specifically the papaya bacterial dieback (also known as crown rot of papaya) and blood disease of banana.

The causal bacterial pathogens for both diseases have been listed under invasive alien species.

Various approaches including chemical and biological controls have been attempted but none were successful in controlling these diseases. The possible strategy to control these diseases is through the development of resistant varieties which is time consuming and cannot offer a faster disease control solution. One technique that has never been tested for this papaya and banana diseases is the enhancement of plant resistance against the pathogens. This process of plant resistance enhancement, known as induced systemic resistance (ISR), systematically activates the plant disease resistance in the roots which then extends it to above-ground plants parts. The ISR can be activated by inoculating the plant growth-promoting rhizobacteria (PGPR) on the roots. PGPR mediated ISR has been reported on a wide range of crops, such as *Arabidopsis*, bean, cucumber, carnation, radish, tobacco, tomato, and banana against various pests and pathogens, such as viruses, bacteria, nematode, fungi, and insects (van Loon, *et al*., 1998); therefore, it would be a worthwhile effort to study the effect of PGPR induced ISR as a possible mode of control for both bacterial diseases. *Bacillus* and *Pseudomonas* are among the PGPR genera that are commonly used for ISR induction in plants (Liu, *et al*., 1995; Pieterse, *et al*., 1998; Ramamoorthy, *et al*., 2001). Therefore, this study was carried out to isolate such promising bacilli and pseudomonads from healthy papaya and banana grown soils, screening them for ISR induction against papaya bacterial dieback and blood disease before their efficacies were evaluated in infected field sites (hotspot- area of elevated disease prevalence).

2. MATERIALS & METHODS

2.1. Induced Systemic Resistance for Papaya

2.1.1. Source of Erwinia mallotivora and 'Eksotika I' papaya seeds

The pathogen, *E. mallotivora*, culture used in this study was kindly provided by Ms. Noriha Mat Amin from the Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI) Headquarters. The 'Eksotika I' papaya seeds were purchased from the Seed and Planting Materials Unit, MARDI, Serdang.

2.1.2. Isolation of bacilli and pseudomonads

The soil from healthy papaya plants were sampled from three locations *viz* Serdang, Selangor and Lunas and Keladi in Kedah. The soil beneath the papaya canopy was sampled by scooping. The soil samples were placed in a sterile Falcon tube and transferred to the MARDI Headquarters, Serdang in a cooler box for bacilli and pseudomonads isolation.

The bacilli were isolated using mannitol egg yolk polymyxin agar (MEYP) and polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA); and the pseudomonads were isolated using *Pseudomonas* F agar and King's media B (Atlas, 1997). Bacterial cultures with different morphology, size and color were purified. The bacilli will form red culture on MEYP and blue culture on PEMBA. The pseudomonads grown on the King's B will produce fluorescence surrounding the colony mainly for *Pseudomonas fluorescens* and *Pseudomonas* F agar does not exhibit any special feature for the pseudomonads grown on it. The pure culture of the isolates were grown on slant nutrient agar and kept stored in a refrigerator for a few months before sub-cultured again.

2.1.3. Screening of bacilli and pseudomonads for ISR induction

The 'Eksotika' papaya seeds were soaked overnight in a container containing distilled water. Floating seeds were discarded as they were considered non-viable. The submerged seeds were sown into germination trays filled with peat moss. Two weeks after that, young seedlings (3-4 leaves stage) were transferred into 15 x 23 cm perforated polybags containing a soil mixture of 2 top soil: 1 organic matter: 1 sand and kept under 50% shade in the nursery.

Two-week old seedlings were then transferred to soil media. The treatments were treated with the PGPR bacterial suspension while the control seedlings were applied with sterile distilled water. Each bacterial treatment and control had five replicates. The second application of bacterial suspension for treatments and sterile distilled water for control was conducted two weeks after the first application. The seedlings were maintained for a further two weeks before being challenged with the papaya bacterial dieback (BDB) pathogen, *E. mallotivora*. This experiment was conducted at a glasshouse in MARDI, Serdang.

The pathogen was grown in a nutrient broth with agitation for 48 hours at room temperature. The treated and control seedlings were pricked (20 pricks/seedling) with a sterile needle on the stem close to the crown. Ten mL of the *E. mallotivora* suspension was sprayed onto the pricked area using a hand held sprayer. Then, the pathogen-sprayed seedlings were covered with a plastic bag and left for two weeks. The disease symptom development was observed at 4, 7, and 14 days after pathogen application and the rate of the plants dying due to the dieback infection was recorded.

2.1.4. Evaluation of ISR seedling for disease resistance in hotspot

The best performing ISR treatments (with 100% disease resistance) were selected and tested for disease infection in a hotspot (area of elevated disease occurrence) in MARDI. Briefly, ISR seedling were prepared as mentioned above and transferred to a hotspot in MARDI and maintained for a crop cycle (24 months). After the seedlings were transferred, a routine monthly application of ISR inoculant was conducted throughout the crop cycle. Control plants were also prepared and cultivated as comparison to the ISR plants, and to evaluate the effects of disease infection. Fertilizer application and other pest and control measures were according to (Chan, *et al*., 1994). Good Agriculture Practice (GAP) was employed during the whole crop cycle. Symptoms of disease infection and plant death caused by the dieback disease were recorded.

2.1.5. Up-scaling of ISR technology for disease resistance in farmer's plot (hotspot)

One of the best performing PGPR treatment from the field study above was selected and tested in the farmer plot (also a hotspot) as up-scaling activity at Ampang Tinggi, Kuala Pilah, Negeri Sembilan. Two thousand eight hundred and fifty (2850) ISR papaya seedlings were prepared and cultivated in the field. A routine application of ISR inoculant was conducted once a month throughout the crop cycle. Fertilizer application and pest and disease management were followed according to common farmer's practices. GAP was employed during the whole crop cycle. The disease infection and plant death caused by the dieback disease was recorded.

2.2. Induced Systemic Resistance for Banana

2.2.1. Source of Ralstonia syzygii and banana plantlets (var. 'Berangan')

The pathogen, *Ralstonia syzygii* culture used in this study was kindly provided by Ms. Nursulastri Jaffar from the Horticulture Research Centre, MARDI Head Quarters. The 'Berangan' banana plantlets were purchased from Exotic Biotech Sdn. Bhd., Ampang Tinggi, Kuala Pilah, Negeri Sembilan.

2.2.2. Isolation of bacilli

The soil from healthy banana plants were sampled from three different local varieties namely 'Berangan', 'Rastali' x 'Embun', and an unknown variety from MARDI's Integrated Organic Farm's banana plot. The soil beneath the banana canopy was sampled by scooping. The soil samples were placed in a sterile Falcon tube and transferred to the bioprocessing lab in a cooler box for bacilli isolation. Based on our previous experience with papaya, we found that only the bacilli can induce systemic resistance thus only the bacilli were isolated for banana ISR study. The bacilli were isolated as explained above for papaya.

2.2.3. Screening of bacilli for ISR induction

The banana plantlets were treated with PGPR inoculant and transferred into a germination tray filled with peat moss. For control seedlings, the PGPR inoculant application was omitted. Two weeks after that, both the control and inoculant treated plantlets were transferred into 25 x 30 cm perforated polybags containing a soil mixture of 3 top soil : 2 organic matter : 1 sand and kept under 50% shade in the nursery.

Two weeks after that, the seedling were transferred to soil media. Treatments were drenched with the PGPR bacterial suspension while for the control seedlings, sterile distilled water was applied instead. Each bacterial treatment and control had five replicates. The second and third application of bacterial suspension for Treatments and sterile distilled water for control were conducted every fortnight after the first application. The seedlings were maintained for a further two weeks before being challenged with the blood disease pathogen, *Ralstonia syzygii*. This experiment was conducted at a glasshouse in MARDI, Serdang.

The pathogen was grown in a nutrient broth with agitation for 48 hours at room temperature. Each treated and Control seedlings were injected with 5 mL pathogen suspension with density of 108 cfu/ml on the banana petiole close to the crown. The disease symptom development was observed at 2, 4, and 8 weeks after pathogen application and the disease severity was recorded. The severity index of 0 denoted a healthy plant while 100% indicated that the plant has died.

2.2.4. Evaluation of ISR seedlings for disease resistance in hotspot.

The best performing ISR treatments (with lowest disease severity: 0-25%) were selected and tested for disease infection in a hotspot in MARDI, Sintok. The plants were maintained for a crop cycle (18 months). One crop cycle of banana consists of banana production from mother plants and primary and secondary ratoons. After the seedlings were transferred, a routine monthly application of ISR inoculant was conducted throughout the crop cycle. Control plants were also prepared and cultivated as comparison to the ISR plants, to evaluate the effects of disease infection. Fertilizer application and other pest and control measures were as listed in the

following references by MARDI (1990) and Department of Agriculture (2009). GAP was employed during the whole crop cycle. Developments in disease infection and plant death caused by the blood disease were recorded.

3. RESULTS

3.1. Induced Systemic Resistance for Papaya

3.1.1. Isolation of bacilli and pseudomonads

Several bacilli and pseudomonads were isolated using selective media, MEYP and PEMBA for bacilli and King's B and *Pseudomonas* F for pseudomonads and the results obtained are shown in Table 1. Nine bacilli were isolated from the Serdang sample while 42 and 36 bacilli were isolated from Keladi and Lunas, respectively. Pseudomonads isolated from Serdang, Keladi and Lunas were 20, 19, and 13, respectively. The total number of bacilli and pseudomonad isolated from these three locations were 139.

Table 1: Bacilli and pseudomonads isolated from three different locations using selective media

Media	Location		
	Serdang	Keladi	Lunas
	Bacilli		
MEYP	7	23	23
PEMBA	2	19	13
	Pseudomonad		
King's B	10	16	6
Pseudomonas F	10	3	7
Total	29	61	49

3.1.2. Screening of bacilli and pseudomonads for ISR induction

All 139 isolates of bacilli and pseudomonads were tested for systemic resistance induction in papaya seedlings. All the treatments and control were challenged with *E. mallotivora*. Out of 139 isolates tested, only 31 shown systemic resistance induction with at least 20% control against dieback disease (Figure 1).

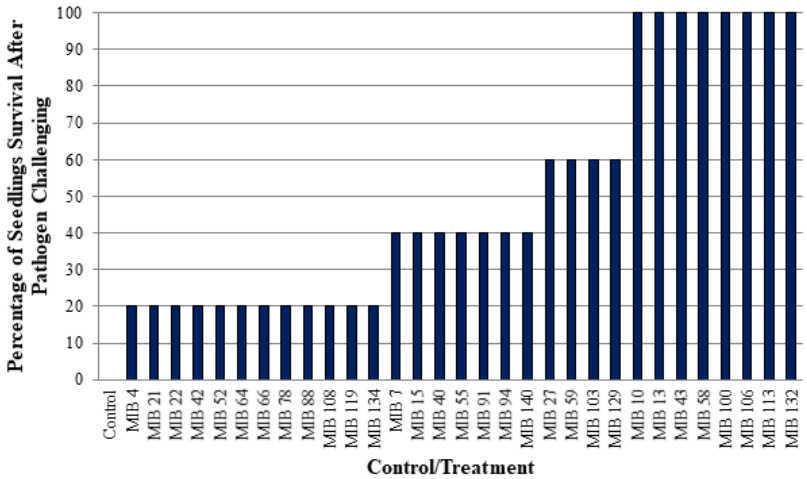

Figure 1: Effects of ISR treatment on survival against papaya bacterial dieback disease

Bacterial dieback symptom development was observed as early as 4 days after the *E. mallotivora* challenge. The seedling's shoot and stems showed watery lesions which are typical symptoms associated with papaya dieback disease. Generally, all the treatments and control exhibited these symptoms development. 108 treatments and control showed increased severity of disease symptoms after 7 days of challenging. The control and other treatments without ISR response finally started to die-off. One month after the pathogen challenge, plants with no resistance showed the crown either dried-off or snapped down. The remaining 31 treatments showed different levels of resistance against disease which ranged between 25-100% (Figure 1). The best treatment with total suppression (100%) against the disease were treatments inoculated with bacilli MIB 10, MIB 13, MIB 43, MIB 58, MIB 100, MIB 106, MIB 113, and MIB 132. The effects of pseudomonads used in this experiment were far inferior than the bacilli in resistance induction against papaya dieback disease.

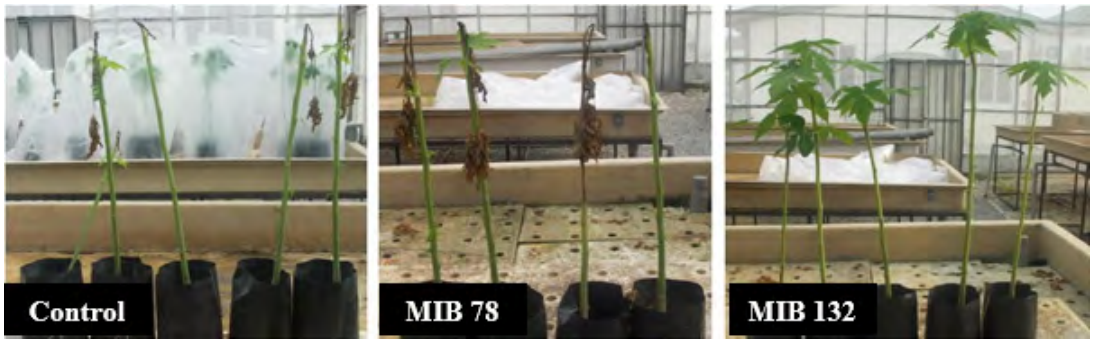

Figure 2: The effects of pathogen challenging on control, ISR-negative (MIB 78), and ISR-positive (MIB 132) plants

3.1.3. Evaluation of ISR seedling for disease resistance in hotspot

All the eight ISR positive PGPR isolates (with 100% suppression against dieback disease), MIB 10, MIB 13, MIB 43, MIB 58, MIB 100, MIB 106, MIB 113, and MIB 132 were used to produce ISR seedlings as mentioned above. Control (non-ISR) seedlings also were produced as mentioned above. Both the ISR and control seedlings performance against papaya BDB infection were evaluated in the open field in MARDI, Serdang which was considered as a hotspot (area of elevated disease prevalencee) were tested. After one crop cycle (2 years) in the hotspot and experiencing the second rainy season in Malaysia (3 months), all the control plants had died because of papaya BDB (Table 2). Almost all of the ISR treatments managed to survive 24 months without any casualty caused by the dieback disease, except one treatment (MIB 13) (Plate 2). MIB 13 had recorded 87.25% resistance against papaya bacterial dieback. All the control plants succumbed to dieback disease within 11 months after being transferred to the field.

The ISR plants showed good vegetative growth without any changes against yield and quality of fruits. The quality of the fruits produced for ISR treatments were as good as control with TSS 13-14% and red intense flesh.

Table 2: Effects of ISR seedling on plant survival against dieback disease infection on papaya grown in hotspot.

Control/Treatment	Plant survival rate after 1 crop cycle (24 months) (%)
Control	0
MIB 10	100
MIB 13	87.5
MIB 43	100
MIB 58	100
MIB 100	100
MIB 106	100
MIB 113	100
MIB 132	100

Figure 3: The destruction of control plants (above) and survival of ISR (bottom) plants after 1 crop cycle grown in the hotspot for papaya bacterial dieback.

3.1.4. Up-scaling of ISR technology for disease resistance in farmer's plot (hotspot)

Two thousand eight hundred and fifty (2,850) ISR 'Eksotika I' papaya seedlings were produced at MARDI, Serdang and transferred to another locality at Ampang Tinggi farm. This site is considered as a hotspot because the previous papaya crop was totally infected by dieback disease. Two weeks after that, all the 2,850 ISR seedlings were transferred to the field. The first incidence of infection was observed 3 months after the seedling were transferred with 85 plants succumbing to the disease. Thirteen months after this, another 61 plants were infected by *E. mallotivora* and died. No further dieback infection was observed until the termination of the project. When the up-scaling activity concluded, the total number of papaya plants which died because of the dieback infection was 5% of total plants grown on the field. The yield and quality of fruits produced in this experiment were good.

3.2. Induced Systemic Resistance for Banana

3.2.1. Isolation of bacilli

Fifty-four bacilli were isolated from bananas grown in soil using two selective media MEYP and PEMBA from three varieties of banana (Table 3). Twenty-four bacilli were isolated from the

'Berangan' variety while 13 and 17 bacilli were isolated from 'Rastali' x 'Embun' and unknown varieties, respectively. The total number of bacilli isolated from these three varieties were 54.

Table 3: Bacilli isolated from soil of three different banana varieties

Media	Variety		
	Berangan	Rastali x Embun	Unknown
	Bacilli		
MEYP	10	6	8
PEMBA	14	7	9
Total	24	13	17

3.2.2. Screening of bacilli for ISR induction

All the 54 isolates of bacilli were tested for systemic resistance induction in banana seedlings. The control and all the treatments and were challenged with *Ralstonia syzygii*. Data of disease severity of 23 isolates are shown in Figure 2, and the remaining 31 are not shown because all of these recorded 100% severity and died off. Out of 54 isolates tested, only 5 showed systemic resistance induction with least severity of 25% and below against blood disease (Figure 2). Blood disease symptoms were observed as early as 14 days after the pathogen challenge. The leaves of seedlings turned yellowish and gradually the seedlings wilted and died-off in two months. Generally, all the treatments without ISR response and control exhibited these symptoms development. The best treatment with total suppression against the disease were treatments which were inoculated with bacilli BIB 10, IB 43, and IB 58. Another two treatments, BIB 23 and BIB 35 had recorded relatively lower severity of 25% than others.

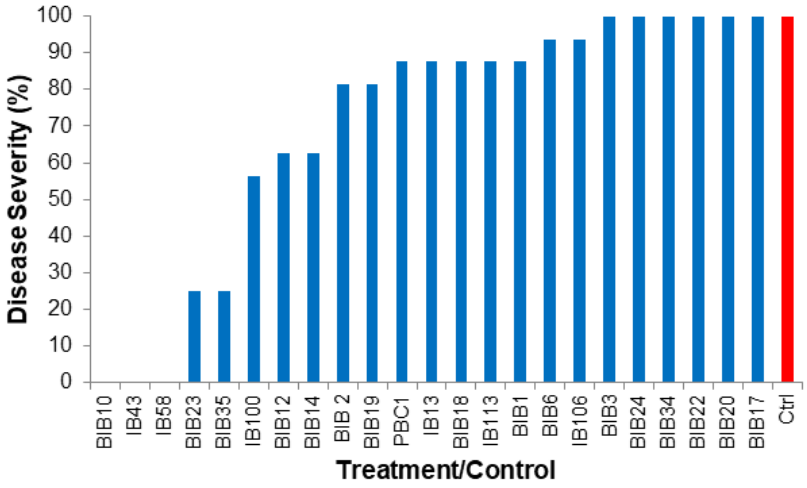

Figure 4: Effects of ISR treatment on disease severity survival against papaya bacterial dieback disease.

3.2.3. Evaluation of ISR seedling for disease resistance in hotspot

All the five ISR-positive PGPR isolates (with least severity, 25% and below), BIB 10, IB 43, IB 58, BIB 23 and BIB 35 were used to produce ISR seedlings as mentioned above. Control (non-ISR) seedlings also were produced by omitting the PGPR inoculation. Both the ISR and control seedlings performance against blood disease infection were evaluated in the open field in MARDI, Sintok, Kedah that was considered a hotspot, where the blood disease incident was recorded in the adjacent plot (20 m away from the selected plot for this experiment). After the

completion of production from the mother plants in the hotspot, 24% of the control and 8% each for treatment BIB 23 and IB 43 (Figure 3) died because the blood disease infection. Other three treatments continue to be free from blood disease infection till to date. However, this observation may change during primary (going to be completed in 3 months) and secondary ratoon production of banana. The ISR plants showed good vegetative growth with shorter time for flower induction and higher yield. The quality of the fruits produced for ISR treatment was as good as the control.

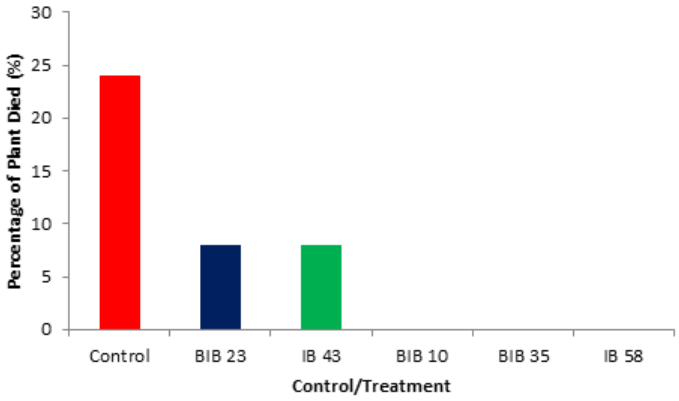

Figure 5: Percentage of plant died caused by blood disease in the hotspot after 1 year.

4. DISCUSSION

Bacilli and pseudomonads were successfully isolated from all three different locations for selection of strains that have capacities to induce systemic resistance in papaya seedlings against papaya bacterial dieback disease. Eighty-seven bacilli and 52 pseudomonads were isolated. Overall, the Keladi sample had the most diverse bacilli and pseudomonad populations followed by Lunas and Serdang. The Serdang sample however had more pseudomonads than bacilli. In contrast, Keladi and Lunas samples had relatively more bacilli than pseudomonads. Keladi has the highest number of bacilli and pseudomonads populations because it was a well managed papaya farm among the three farms, with better soil quality. In contrast, Serdang has the lowest number of bacilli and pseudomonas because the soil quality was very poor, degraded and had less organic matter. Generally, microorganisms including bacteria thrive in soil rich with organic matter.

Thirty-one isolates had shown some level of systemic resistance induction, which ranges between 20-100%. Among these, 8 isolates showed the best systemic resistance inducement in papaya seedlings with total suppression against disease infection. For all these 8 isolates, disease symptoms were observed only during the early period of pathogen challenge but totally healed after that. Although, some level of damage was observed on the plants, the seedlings survived and finally become healthy again. Molecular identification of all these 8 isolates revealed that they belong to the *Bacillus* genus and none were under the genus of *Pseudomonas*. Generally, Bacillus and Pseudomonas are among the genera that is commonly used for ISR induction in plants (Pieterse *et al.*, 1998; Ramamoorthy *et al.*, 2001; Kloepper, *et al.*). However, this study shows that only the *Bacillus* genus is involved in systemic resistance induction, possibly caused by its spore-forming ability that led to better survival capabilities compared to the non-spore forming *Pseudomonas*.

All the ISR treatments except one had recorded excellent control against the dieback disease in the hotspot areas. All the control plants succumbed to disease infection and died within

one year after cultivated. The induction of systemic resistance from the seedling stage and maintained by a periodical ISR inoculant application on mature plants made it resistant against dieback disease. The PGPR mediated ISR has been reported in a wide range of crops such as Arabidopsis, bean, cucumber, carnation, radish, tobacco, tomato, and banana against various pests and pathogens such as viruses, bacteria, nematode, fungi, and insects (van Loon, 1998) in glasshouse studies; but there have not been many studies reported success on disease control by systemic resistance against disease in the open field where there is elevated disease prevelence (hotspots).

This technology was up-scaled in 1 ha of a farmer's plot where this technology can be adapted to commercial farm practices. MIB 106, the best performing treatment in disease control, vegetative growth and yield production from the hotspot study in MARDI Serdang was selected for this up-scaling activity. After one crop cycle, this technology managed to control the dieback disease up to 95%. The 5% losses due to the dieback infection occurred when the weeds were not managed properly in the up-scaling plot. Dense weed around the root region of the papaya hindered the penetration of the ISR inoculant to the root region, therefore the efficacy of systemic resistance in the plants reduced and consequently the disease incidence increased. GAP including weed management is crucial for optimal systemic resistance induction against disease.

4.1. Induced Systemic Resistance for Banana

Based on our previous experience with the papaya's ISR, we found that only *Bacillus* can induce systemic resistance. Therefore, for the banana ISR study, we only isolated bacilli from three varieties. Fifty-four isolates were isolated, where the 'Berangan' variety was found to harbor more bacilli than the 'Rastali' x 'Embun' and an unknown varieties. This possibly was caused by the root exudates generated by the 'Berangan' variety which were more attractive for growth and survival of the bacilli in the soil within the banana root zone.

All the 54 isolates were screened for systemic resistance induction in banana against blood disease. The first disease symptom was observed 14 days after the pathogen challenge and the severity gradually increased and plants would die if they did not have resistance. Treatment of banana seedlings with bacilli had elevated the resistance against blood disease in banana seedlings. Previously the PGPR mediated ISR has been reported on a wide range of crops such as Arabidopsis, bean, cucumber, carnation, radish, tobacco, tomato, and banana against various pests and pathogens such as viruses, bacteria, nematode, fungi, and insects (van Loon, 1998); but nothing much has been reported on blood disease control by using this mechanism.

The best five treatments with disease severity not exceeding 25% were tested in the hotspots for disease resistance. Among these, three recorded total suppression and other two 92% resistance against disease on the mother plants. However, the disease severity may increase in the primary and secondary ratoons of banana in the future. At the point of writing this article, there has been no disease incidence reported in the primary ratoon. Generally the ISR treatment has shown better vegetative growth compared to the control.

5. CONCLUSION

The ISR technology was developed to control papaya bacterial dieback through the bioprospection of PGPR. For this process, 139 bacilli and pseudomonads were isolated,screened for disease resistance inducement, and the eight best ISR inducing PGPR with total control (100%) against papaya bacterial dieback in seedling level were selected for field (hotspot) evaluation. All these

eight PGPR treatments were tested in a hotspot for disease resistance for one crop cycle (2 years), where seven of these had recorded a total suppression (100%) to papaya dieback infection. One of the best performing PGPR treatment (MIB 106) in disease suppression and growth was tested in an up-scaling activity in a 1 ha plot which managed to suppress the disease up to 95%. Development of this technology with a higher disease suppression rate is a boon for the ailing Malaysian's papaya industry.

Our success in controlling the papaya bacterial dieback by ISR technology, compelled us to develop a similar ISR technology to control the blood disease of banana. As for the papaya ISR, 54 bacilli were isolated from three banana varieties. All the isolates were screened for ISR induction, of which five of exhibited least disease severity (25% and below) and were shortlisted as potential candidates to control banana blood disease. Currently, all these five PGPR treatments are being evaluated in a hotspot and at the point of writing this article, all the treatments in mother plants have recorded 92-100% suppression against the blood disease.

The ISR technology to control the papaya BD is the first available and efficient technique in the world to control this disease; thus this technology has huge potential to boost local and international papaya production. Similarly, development of ISR technology to control blood disease will be a game changer in the Malaysian banana industry.

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