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REACTION OF *Musa balbisiana* TO BANANA BACTERIAL WILT INFECTION

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ABSTRACT

Banana bacterial wilt (*Xanthomonas campestris)* is an emerging disease of bananas in Uganda. All banana cultivars grown are susceptible. *Musa balbisiana*, a wild banana relative exhibits a progenitor like resistance type reaction to banana bacterial wilt infection. The negative *M. balbisiana* disease reaction suggests it could be used to improve banana resistance to banana bacterial wilt disease. Screenhouse and field experiments were used to characterise the reaction of *M. balbisiana* to banana bacterial wilt infetion. *Psang awak*, a reference susceptible cultivar was used as a positive control. No disease development on *M. balbisiana* under field and controlled conditions was observed. In general, as early as two weeks after inoculation, significant differences ($P<0.05$) were observed between *M. balbisiana* and the susceptible banana cultivar, *Psang awak* (ABB). Time course analysis of the possible role of Pathogenesis related protein 2 (PR-2), Phenyl alanine ammonia lyase (PAL), Non-expressor of pathogenesis related gene (NPR1) genes in *Musa balbisiana* resistance reactions, revealed that only NPR1 was expressed 15 days after inoculation with *X. campestris* at 10^8 dosage. The expression of NPR1, a marker gene of the systemic acquired resistance plant defence system provides preliminary evidence that this may be the major form of resistance in *Musa balbisiana* to bacterial wilt infection.

Key Words: NPR1, PR proteins, Uganda, *Xanthomonas campestris*

RÉSUMÉ

Le flétrissement bactérien du bananier (*Xanthomonas campestris)* est une maladie émergente en Uganda, où tous les cultivars en sont susceptibles. *Musa balbisiana*, un bananier relatif sauvage manifeste un type de réaction de résistance progénitrice à l'infection due au flétrissement bactérien. La réaction négative de *M. balbisiana* à la maladie suggère que ceci pourrait être utilisé pour améliorer la résistance du bananier cette maladie. Des essais en serre et en champs étaient conduits pour caractériser la réaction de *M. balbisiana* à l'infection du bananier par le flétrissement bactérien. Le *Psang awak,* un cultivar susceptible de réference était utilsé comme témoins positif. Aucun signe de développement de la maladie n'était observé sur *M. balbisiana* dans les deux milieux en champs et en serre. En général, des différences significatives ($P<0.05$) étaient observées plus tôt avant deux semaines après inoculation entre *M. balbisiana* et le cultivar de bananier susceptible, *Psang awak* (ABB). L'analyse du temps de déroulement du rôle possible de la pathogenèse liée à la protéine 2 (PR-2), à la Phényl alanine ammonia lyase (PAL), à la non-expression de la pathogenèse liée au géne (NPR1) de résistance exprimée par *Musa balbisiana*, a révélé que seulement NPR1 s'était exprimé 15 jours après inoculation du *X. campestris* au dosage de 108. L'expression de NPR1, un marqueur de gène de résistance acquis du système de défence des plantes fournit une évidence que ceci serait une forme majeur de résistance du *Musa balbisiana* à l'infection du flétrissement bactérien.

Mots Clés: NPR1, protéines PR, Uganda, *Xanthomonas campestris*

INTRODUCTION

Banana bacterial wilt caused by *Xanthomonas campestris* pv.*musacearum* is an emerging disease of bananas in East and Central Africa. It poses one of the greatest threats to banana production, thus increasing food insecurity in East Africa where banana is a staple food. The disease threatens livelihoods of millions of farmers in the Great Lakes region of eastern Africa. Banana bacterial wilt was first identified in Uganda in 2001 and has now spread to epiphytotic proportions to almost all major banana producing districts of the country (Tushemereirwe *et al*., 2004).

Given the rapid spread of the pathogen in east Africa, and concomitant increase in inoculum load in regional locations, the epiphytotic threat of banana bacterial wilt requires application of stopgap measures. The most attractive strategy for bacterial disease control is to improve plant defense mechanism against the pathogens. Recent advances in genetic engineering offer ways to transfer resistance genes found in other plants, microbes, insects and animals into crop varieties without changing other favourable traits (Tripathi *et al*., 2004).

In the case of banana bacterial wilt, there is little information on nature of disease resistance, with all east African highland bananas (EA-AAA) being susceptible (Ssekiwoko *et al*., 2006). However, *M. balbisiana*, a wild banana relative and exhibits a progenitor like resistance type reaction characterised by recovery from infection despite development of leaf wilt like symptoms and internal vascular streaking (Ssekiwoko *et al*., 2006). The negative X*anthomonas campestris* pv. *musacearum- Musa balbisiana* disease interaction suggests it could be used to improve banana resistance to banana bacterial wilt. Little is known about the molecular nature of reaction of *M. balbisiana* to banana bacterial wilt infection.

Plants in nature are able to defend themselves against pathogen attack by stimulating multiple defence signalling pathways. Systemically acquired resistance (SAR) is one of the best described defense responses in plants. Systemic acquired resistance is characterised by broad spectrum resistance to viral, bacterial and oomycete pathogens and also, is associated with localised necrosis (Ryals *et al*., 1996). NPR1 is an essential positive regulator of SAR-induced PR gene expression and SAR. The PR proteins are expressed in plants in response to infection by pathogens. These include PR-1, whose biological activity is still unknown, but seemingly has antifungal activity, and PR-3, which consists of various chitinases and lysozymes. NPR1 is localised in the cytoplasm and exists as an inactive oligomer (Mou *et al*., 2003). The oligomer has to be activated by perception of SAR. In addition to its role in regulating SAR, a further function of NPR1 in cross-communication between SAR and Jesmonic acid-dependent defence signaling pathways has been found (Pieterse *et al*., 2004).

Several studies have shown that over expression of NPR1 provides resistance to a variety of bacterial and fungal pathogens. Also, there is evidence from transgenic plants that SAR and NPR1 are required in *Arabidopsis* for resistance against pathogen infection (Thomma *et al*., 2001. However, transgenic *Oryza sativa* (rice) plants, expressing an Arabidopsis NPR1 gene displayed a lesion mimic cell death phenotype; while rice plants over-expressing a rice NPR1 homologue (NH1) had increased SA levels and were more sensitive to light, resulting in a dwarf phenotype (Pieterse *et al*., 2004). NPR1 is further functionally conserved in diverse plant species and full length NPR1 sequences from some of these have been deposited in the NCBI Genbank (www.ncbi.nlm.nih.gov).

In the *Arabidopsis* genome, six NPR1-related genes have been identified (Liu *et al*., 2005). In addition, MpNPR1-1 has been recently cloned from *Malus domestica* (Malnoy *et al*., 2007). In rice, three homologous NPR1-like genes, OsNPR1/NH1, OsNPR2/NH2 and OsNPR3, have been isolated (Yuan *et al*., 2007). OsNPR1 is induced not only after treatment with the rice pathogens bacterial blight *Xanthomonas oryzae* pv. *oryzae*rice and blast *Magnaporthe grisea*, but also by benzothiadiazole. Despite these reports, little information exists in literature about existence and expression of NPR1-like genes in monocot plants.

The objective of this study was to characterise reaction of *Musa balbisiana* to *Xanthomonas campestris* pv. *musacearum* infection.

MATERIALS AND METHODS

Reaction of *Musa balbisiana* to *Xanthomonas campestris*. Two cultivars of *Musa, balbisiana* and *Psang awak* (ABB) (locally called Kayinja) were selected from the core collection at National Agricultural Research Laboratories Institute-Kawanda in Uganda for this study based on the reported susceptibility or resistance to *Xanthomonas* infection. *Psang awak* is an introduced banana cultivar that shows a highly susceptible reaction, whereas *M. balbisiana* shows resistance (Ssekiwoko *et al*., 2006). Disease free *M. balbisiana* and *Psang awak* were introduced as corms into pots containing sterile soil and allowed to establish for four months before inoculation.

Xanthomonas campestris pv. *musacearum* was obtained from a stock culture stored in glycerol (50%) at -20 ^{o}C. The bacteria was cultured on yeast peptone glucose agar (YPGA) (5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose and 15 g L^{-1} agar) for 72 hours at 25 ^{o}C, sub-cultured on YPGA, grown overnight at 25 ^{o}C and suspended in sterile distilled water. Cell suspension was adjusted to~10^8 colony forming units per ml, with sterile distilled water and serially diluted to 10^6, 10^4 and 10^2. Ten microlitres of 10^8, 10^6,10^4 and 10^2 dilutions were plated on yeast peptone glucose agar grown at 25 ^{o}C for five days and the colony forming units counted to verify inoculation concentration.

Experimental set up. The experiments were set up in the screenhouse and field. The field experiment was set up on a farmer field in Mukono district in central Uganda (0^{o} 30'N-1^{o} 00'N latitude and 32^{o} 30'E-33^{o} 00'E longtitude; 1160 masl). Mukono is a disease hotspot and was the first district where banana *bacterial* wilt was reported. Screenhouse experiments were conducted at National Agricultural Research Laboratories Institute at Kawanda.

Screenhouse experiment. A screenhouse experiment was used to characterise disease reactions of *M. balbisiana* and *Psang awak* to *Xanthomonas campestris*. Four months old plantlets derived from corms and established in pots were used for the study. The experiment was established following a randomised complete block design with eight replicates per treatment level. Plantlets were inoculated by infusing the petiole of the youngest open leaf with 1 ml of innoculum containing 10^8, 10^6, 10^4 and 10^2 cfus ml^{-1} using a hypodermmic syringe and needle. The inoculum used was prepared as described earlier. In addition, control plants were mock-inoculated with sterile distilled water. Weekly disease severity recordings were taken using a scale of 1 to 7 and used to compute the area under disease progress curves (Campbell and Madden, 1990). Up to six severity ratings were recorded and used to compute area under disease progress curve using Equation 1.

$$A = \sum_{i=1}^{n} [(x_i + x_i+1)/2] t_i \quad \text{..... Equation 1}$$

Where: xi = the number of wilted leaves on datei;
n= the number of assessments; and
ti = the time in weeks between disease assessment xi and xi+1.

Severity ratings and AUDPC were subjected to analysis of variance (ANOVA) and means were compared using Fisher's protected least significant difference at $P<0.05$. *Musa balbisiana* was also subjected to repeated infection after the first six weeks. Four inoculum levels were used. Weekly disease severity ratings were taken using a scale of 1 to 7 and ratings analysed as with single inoculation.

Field experiments. Four months old plantlets of *Musa.balbisiana* and *Psang awak* genotypes derived from corms and established in pots filled with farm soil were used for the study. The experiment was established following a randomised complete block design, with eight replicates per treatment. Plantlets were inoculated

as described earlier. Weekly disease severity recordings (wilted leaves) were taken and used to compute the area under disease progress curves, analysed by ANOVA and means compared as described for screeenhouse experiment.

Putative host defence signaling in *Musa balbisiana to X. campestris*

Plant material and treatment. *Musa balbisiana* and *Psang awak* were introduced as corms into pots containing sterile soil and allowed to establish for four months before inoculation. Inoculum preparation and inoculation followed procedures described earlier. Leaves were harvested at 12, 24, 48, 72 hours and 7, 15, 30 days post-inoculation, quickly frozen in liquid nitrogen, and stored at -80 °C using aluminium foil. Leaves were also harvested from non inoculated and mock inoculated plantlets.

RNA extraction. Total RNA was extracted from leaf petiole material using the Nucleospin® RNA Plant (Machery-Negel Germany) according to the manufucture's instructions. Traces of DNA in the RNA samples were eliminated by treating each RNA sample with DNase 1 (Fermentas Life Science, Hanover, MD).

cDNA synthesis. First strand cDNA was subsequently synhesised from the DNA-free RNA samples by random hexamer primering (Fermentas Life Science, Hanover, MD) using first strand cDNA synthesis kit, according to the manufucture's instruction (Promega,USA). The quality of cDNA was verified by amplifying a 100bp actin with banana actin forward 5'-AACTTCGTGTTGCTCCTGAAAGAG-3'and 5'CAGTAGTACGACCACTGGCATAGAG-3' primers.

PCR amplification. Four genes (NPR1, PAL, PR-2 and PR-5 were used for expression studies in *Musa balbisiana*. Nucleic acid sequences for the PR-proteins (PR2 and PR5), Phenyl alanine ammonia Lyase (PAL), Non-expressor of pathogenesis-related gene (NPR1) and actin genes were downloaded from in the Gene Bank (www.ncbi.nlm.nih.gov). Multiple sequence alignments were done using clustal w (Channa *et al*., 2003). Conserved regions especially among monocots were used to design PCR primers which were subsequently used to amplify the genes (Table 2).

The primers were used to amplify putative actin, PR2, PR5, NPR1 and PAL genes from *Musa balbisiana* and *Psang awak* genomic DNA and cDNA. Reactions were performed in a PTC-100 Programmable Themocycler (MJ Research, Inc., Water-town, USA). PCR mixtures (12.5 µl) consisted of 1x PCR Buffer, 1.5 Mm $MgCl_2$, 0.2mM dNTPs, 0.8µM of each primer pair, 1.25 U of Taq DNA Polymerase (www.fermentas.com), and 1.25 ng cDNA template. The thermo cycling parameters were 94 °C for 5 minutes followed by 35 cycles of 94 °C for 45 seconds; 58 °C (Actin and PAL), 60 °C for (PR2 and PR5) or 65 °C (NPR1) for 1 minute; and 72 °C for 1minute, followed by 72 °C for 7 minutes. These methods were based on the computed annealing temperatures of the templates and the optimised protocol for similar genes. PCR products (8µl) were separated on 2% agarose gel, run in 0.5x Tris-borate EDTA (TBE) buffer, stained with ethidium bromide, and visualised with UV light.

RESULT

Inoculum dosage. The disease developed progressively, with severity increasing up to 10 fold in *Psang awak* at 6 weeks after inoculation (Table 1). The data are based on single inoculations of 10^8 cfu mL^{-1}. No disease developed on *M. balbisiana* both under field and screenhouse conditions. As early as two weeks after inoculation, significant differences ($P< 0.05$) were observed between *M.balbisiana* and the susceptible banana cultivar *Psang awak*. Similar results were observed six weeks after inoculation.

In general, there was a relationship between concentration of inoculum and disease development in *Psang awak* ($r = 0.8$, $P=0.063$). More disease developed at higher dosage with mean severity of 0, 0.125, 0.25 and 0.5 for the 10^2, 10^4,10^6 and 10^8 inoculum dosages, respectively two weeks after inoculation (Fig. 1). No disease was recorded on *M. balbisiana* at two weeks after inoculation, irrespective of inoculum dosage with single inoculation (Fig. 2). Analysis of final

TABLE 1. Disease development on susceptible banana cultivar *Psang awak* and *Musa balbisiana* under controlled and field experiments based on single inoculum dosage of 10^8 cfu mL^{-1}

Disease incidence/crop	Location of experiment		CV%
	Field	Screenhouse	
Psang awak			
Severity 2WAI[a]	0.62	0.50	94.85
Severity 6WAI[a]	6.0	5.60	21.54
AUDPC[b]	17.78	15.82	
M. balbisiana			
Severity 2WAI[a]	0	0	
Severity 6WAI[a]	0	0	

a = Weeks after inoculation; b=Area under disease progress curve computed according to Cambell and Madden, 1990

TABLE 2. Sequences of degenerate primers used for polymerase chain reaction (PCR) amplification

Target gene	Primer sequence	Expected size (bp)
PR2-F	TACGTACAACCAGGGGCTGA	190
PR2-R	GACTTGTCCGGGTTGAACAG	
NPR1-F	CAGAGGCACTTATTGGACGTT	100
NPR2-R	TAACAAGCCTTCCGGCAAT	
PAL-F	TCAACTCGGTGAACGACAAC	220
PAL-R	ACATGAAGACCCATGCCTGT	
PR5-F	ACATGAAGACCCATGCCTGT	131
PR5-R	TGCAAATCCAAGCCTATTCC	
ACTIN-F	AACTTCGTGTTGCTCCTGAAAGAG	200
ACTIN-R	CAGTAGTACGACCACTGGCATAGAG	

severity (six weeks after inoculation) revealed a similar trend ($r = 0.9$, $P<0.001$). Highest disease severity was recorded on *Psang awak* plants inoculated with 10^8 bacterial cells (Fig. 3).

In all cases, analysis of variance revealed significant differences ($P<0.05$) in disease development between various inoculum dosages in *Psang awak*. - A comparison of the effect of inoculum on disease development revealed a strong positive linear correlation between inoculum dosage and severity ratings ($r = 0.98$, $P<0.01$). No disease developed on *M. balbisiana* with single inoculum dosages of 10^2, 10^4,10^6 and 10^8 cfu ml^{-1}. However, with double inoculations using the same dosages, disease symptoms were observed but only at high concentrations of 10^6 and 10^8 cfu ml^{-1} six weeks after the second inoculation (Fig. 4). Significant differences between single and double inoculations were also observed ($P=0.024$).

Molecular analysis of *M. balbisiana* and *Psang awak* response to *X. campestris* pv. *musacearum* infection. The Quality of cDNA was tested by amplifying a house keeping gene Actin (Fig. 5). Amplicons of the expected size were amplified from both genomic DNA and cDNA. A screen of genomic DNA for putative resistance gene analogues, orthologus to well known and characterised resistance genes such as PAL, PR2 and NPR1 were indicated the existence of these resistance gene analogues in *Musa balbisiana* (Fig. 6). Time course analysis of the possible role of these genes in *Musa balbisiana* resistance

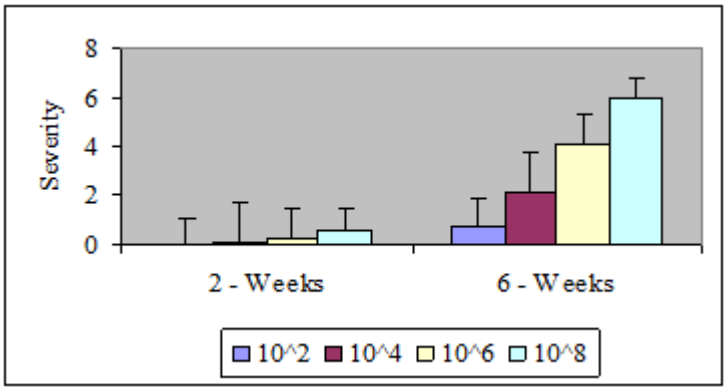

Figure 1. Effect of inoculation dose on disease development on susceptible banana variety *Psang awak*.

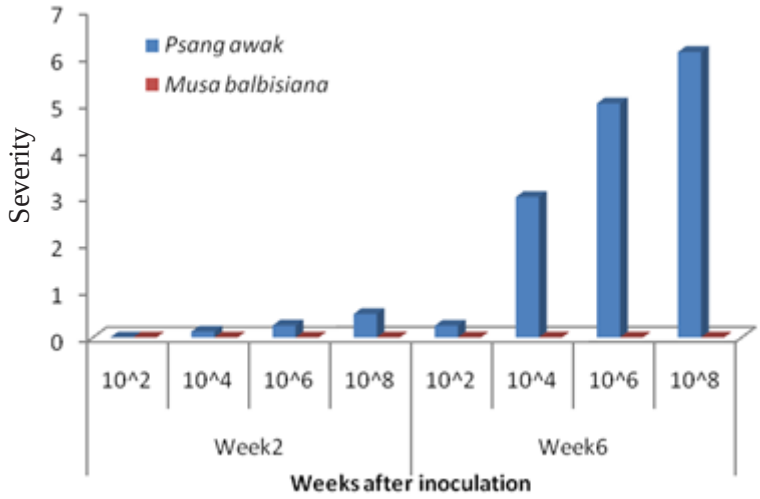

Figure 2. Inoculum dosage effect on disease severity in *Psang awak* and *Musa balbisiana* two and six weeks after inoculation.

reactions, revealed that only NPR1 was expressed 15 days after inoculation with 10^8 dosage (Fig. 7). No amplicons were detected prior to that period.

DISCUSSION

The data from these studies confirm that in banana, the disease develops faster than in *M. balbisiana*. At all levels of inoculations disease developed on the susceptible cultivar *Psang awak* (Kayinja) and was affected by innoculum dosage (r =0.9). Conversely in *M. balbisiana* no disease developed on same dosages that caused disease on banana. However, with double inoculations at high inoculum dosages (10^8 and 10^6 cfu mL^{-1}) bacterial wilt disease developed on *M balbisiana*.

These findings confirm earlier reports that *M. balbisiana* is resistant to *X. campestris pv. musacearum* infection (Ssekiwoko *et al.*, 2006). The nature of resistance in *M. balbisiana*, however, suggests that it provides highly effective protection against *X campestris pv. musacearum* infection. The attributes of resistance are similar to those conditioned by major genes or vertical resistance. In general, vertical resistance is characterised by preventing epidemics exhibited as very low disease or prolongation of start of epidemics (Van der Plank, 1963; Campell and Madden, 1990; Agrios, 2005).

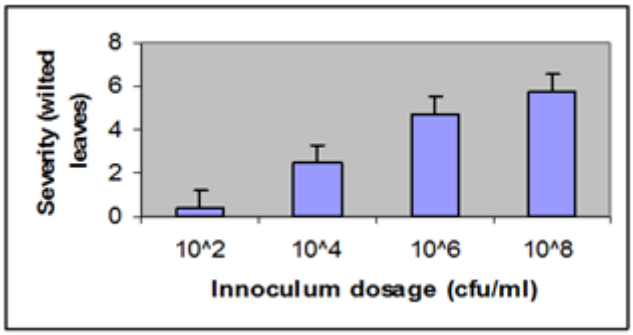

Figure 3. Disease severity ratings on banana variety *Pisang awak* six weeks after innoculation with different *X. campestris* dosages.

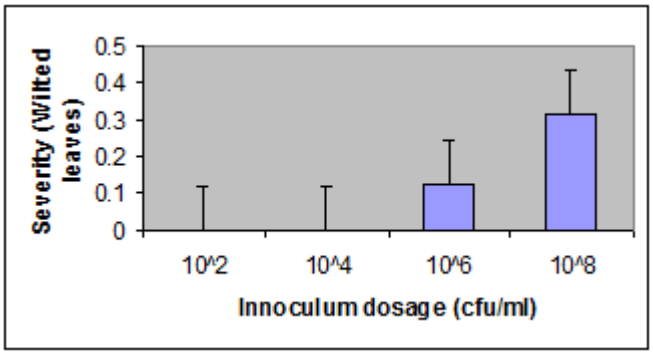

Figure 4. Effect of double inoculation on disease development in *M. balbisiana*. Data are based on means taken six weeks after double inoculation

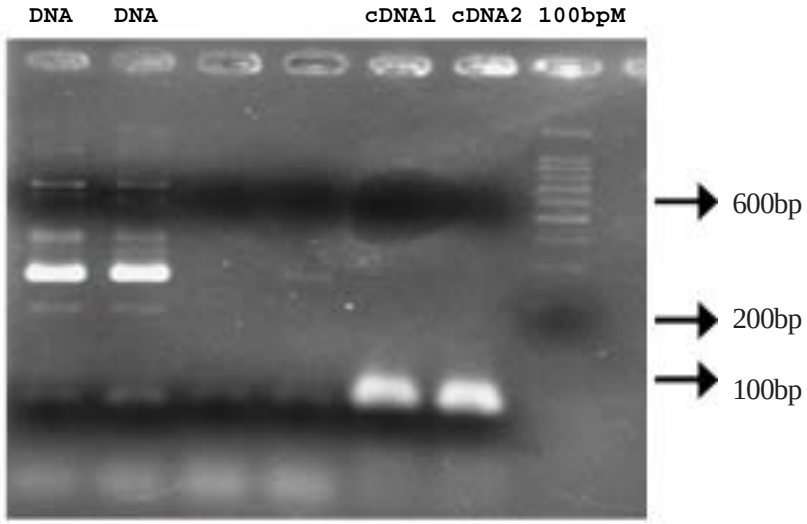

Figure 5. Quality of cDNA as detected by amplification of actin gene in cDNA of *Musa balbisiana* (cDNA1) and *Psang awak* (cDNA 2).

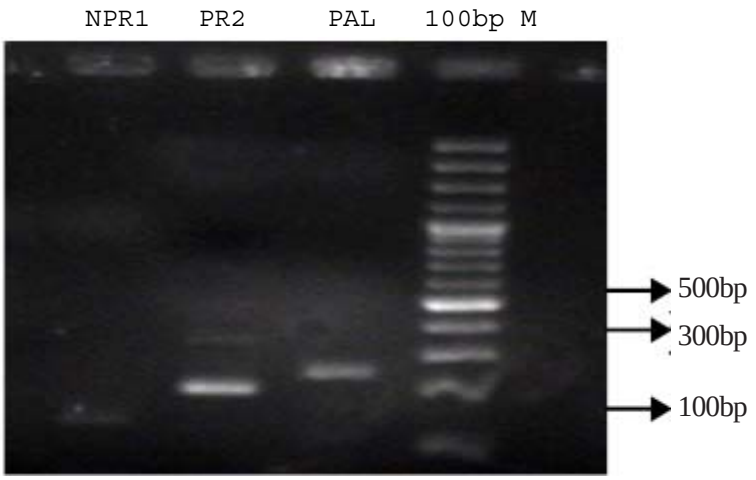

Figure 6. Amplicons of PAL, PR2 and NPRI genes amplified from *Musa balbisiana* genomic DNA with degenerate PAL, PR2 and NPR1 primer pairs.

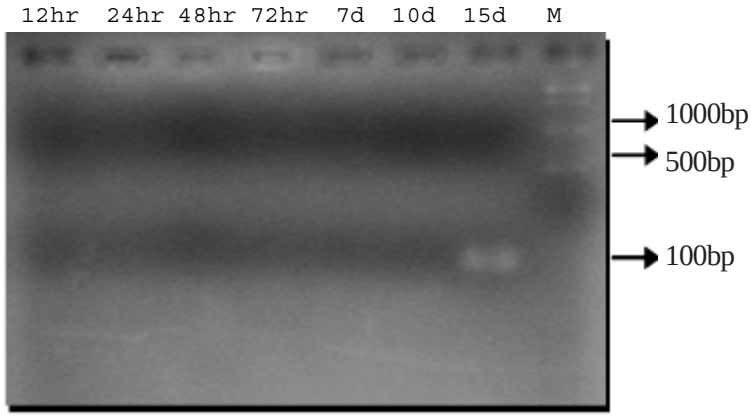

Figure 7. PCR amplification of *Musa balbisiana* cDNA template prepared 15 days after infection using NPR1 gene specific primer. M=Marker.

The development of disease at high doses with repeated infection suggests that *M. balbisiana* may employ mechanism that also limits movement of the pathogen and perhaps its reproduction in the host.

Molecular analysis further showed that indeed resistance is conditioned by pathways regulated by major genes. The expression of NPR1, a marker gene of the systemic acquired resistance plant defence system provides evidence for a major form of resistance in *M. balbisiana* to *X. campestris.* Systemic acquired resistance provides broad spectrum resistance to infection (Hunt and Ryals, 1996; Ryals *et al*., 1996). The capacity of NPR1 to participate in both systemic acid-dependent and systemic acid-independent processes points to a central signal modulating role in different systemic resistance pathways that is clearly influenced by the nature of input signal (Cao *et al*., 1997). In *Arabidopsis* for example, recruitment of NPR1 to systemic acquired resistance, induced systemic resistance and plant defensin induction pathways appears to depend on the nature of the input signal and

evidence points to NPR1 functioning as signal modulator, determining a particular signal output (Cao *et al*., 1997).

The fact that NPR1 an up-stream marker gene for systemic acquired resistance has been expressed suggests that possibly other downstream genes are induced as has been reported in other pathosystems (Ward *et al*., 1991; Uknes *et al*., 1993; Cao *et al*; 1997; 1998). The activation of a series of plant defence genes cannot therefore be preconcluded.

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