Acta Physiol Plant (2010) 32:1177–1187
DOI 10.1007/s11738-010-0513-y

ORIGINAL PAPER

Induction of systemic resistance by mixtures of antagonist bacteria for the management of crown rot complex on banana

G. Sangeetha · R. Thangavelu · S. Usha Rani · A. Muthukumar · R. Udayakumar

Received: 21 October 2009 / Revised: 1 April 2010 / Accepted: 16 April 2010 / Published online: 13 May 2010

Abstract Among nine native bacterial strains isolated from banana fruit surface and rhizosphere and six bacterial strains introduced from the culture collection, three native strains viz., non-fluorescent *Pseudomonas* (NFP_6), *Pseudomonas fluorescens* (Pf3a), and *Bacillus subtilis* (BS_1); and two bacterial strains from culture collection viz., *Azospirillum* (AS1) and *Azotobacter* (AZ1) have recorded maximum inhibition of mycelial growth of crown rot pathogens (*Lasiodiplodia theobromae* and *Colletotrichum musae*) under in vitro condition. When these effective bacterial strains were treated on banana fruits under in vivo, significant reduction of crown rot disease and increased shelf life of banana was observed. However, bacterial strains applied as three way combinations (NFP_6 + Pf3a + BS_1) had greater effect compared with individual and two way combination of bacterial antagonist treatments. The effect of crown rot disease reduction was also comparable to that of fungicide Benomyl (0.1%) both under cold and room temperature storage conditions. Besides, the induction of defense-related enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (PO), polyphenoloxidase (PPO), and the accumulation of phenolics in banana fruit due to the application of bacterial antagonists were also studied at five different time intervals viz. 0th, 1st, 3rd, 5th and 7th days after treatment. When banana fruits treated with bacterial antagonists (individually and also in different combinations) and challenge-inoculated with crown rot pathogens, up to fourfold increase in defense-related enzymes and 3.6 fold increase in phenolic content was observed compared with control. The activity of these defense-related enzymes and phenolic content had gradually increased from 1st day after treatment to 3rd after treatment and reached their peak on 5th day after treatment. Among the bacterial antagonists which have been applied individually and in different combinations, the banana fruits treated with three-way antagonist mixture, i.e., NFP_6 + Pf3a + BS_1 recorded maximum induction of defense-related enzymes and accumulation of phenolics compared with individual and two-way combination of antagonist mixtures. This study suggest that the increased induction of defense-related enzymes and phenolic content due to the treatment of banana fruits with bacterial antagonists might have involved in the reduction of crown rot severity and in turn increased the shelf life of banana fruits.

Keywords Banana · Crown rot complex · *Lasiodiplodia theobromae* · *Colletotrichum musae* · Bacterial antagonist mixture · Induced systemic resistance · Shelf life

List of abbreviations

NFP	Non-fluorescent Pseudomonas
Pf	*Pseudomonas fluorescens*
BS	*Bacillus subtilis*
AS	*Azospirillum*
AZ	*Azotobacter*

Communicated by P. Wojtaszek.

G. Sangeetha (✉) · S. Usha Rani · A. Muthukumar · R. Udayakumar
Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram 608002, Tamil Nadu, India
e-mail: sangeethaau@hotmail.com

R. Thangavelu
National Research Centre for Banana, Thiruchirapalli 620 102, Tamil Nadu, India

Introduction

Anthracnose caused by *Colletotrichum musae* (Berk and Curtis) Arx and crown rot disease complex caused by *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl and *C. musae* are the main causatives of postharvest losses in banana (Persely 1993; Ploetz et al. 1994). Bananas are universally treated with Benzimidazoles such as Benomyl, a possible carcinogen and teratogen (http://www.pan-uk.org/pestnews/actives/benomyl.htm) or with thiabendazole (TBZ) or imazalil (Krauss et al. 2001). With public pressure to restrict the use of synthetic fungicides for the control of post harvest diseases of fruits and vegetables, a need exists for safe and effective alternatives.

Biological control through the use of antagonistic microorganisms has recently emerged as a viable disease management strategy (Wilson and Wisniewski 1989; Janisiewicz et al. 1994), and it is a promising option for the control of postharvest diseases of fruits and vegetables (Pusey and Wilson 1984; Wilson and Wisniewski 1989; Chalutz and Wilson 1990; Wisniewski and Wilson 1992; Janisiewicz et al. 1994; Korsten et al. 1997; Hong et al. 1998). So far, numbers of antagonistic bacterial species, which effectively control the post harvest pathogens, have been identified for post harvest disease control (Janisiewicz et al. 1991; Smilanick and Dennis-Arrue 1992; Mari et al. 1996a, b). However, use of single antagonists consistently does not offer an economically sufficient level of disease control when disease is caused by different strains of single pathogen or complex of pathogens (Krauss et al. 2001). Under this circumstance, mixed bioinocula of antagonists have been successfully employed on several pathogens infecting fruit crops by several researchers (Backman et al. 1997; Guetsky et al. 2001). Therefore use of antagonist mixtures would be more effective (Krauss et al. 2001; Janisiewicz and Korsten 2002), especially in case of crown rot disease control, because the composition of the complex varies considerably, and a single strain cannot be expected to perform equally against all possible compositions of the fungal complex. More over, use of antagonists which are isolated from the surface of plants or plant parts or fruits (Smilanick 1994) are more effective on post harvest pathogens because of their colonization ability and environmental adaptation (Wilson and Wisniewski 1989).

Of late, researchers have shown an interest in induced resistance as a method of controlling postharvest diseases. The investigation on mechanism of biological control by bacterial antagonists also revealed that biocontrol strains, which protect the plants from various pathogens in several crops, activate the defense-related enzymes including phenylalanine ammonia lyase (PAL), peroxidase (PO) etc., which are involved in synthesis of phytoalexins (Maurhofer et al. 1994; M'Piga et al. 1997). Besides, early and increased expression of defense-related genes in induced systemic resistance (ISR) is very important in protecting the crops against several pathogens (Ward et al. 1991). ISR, once expressed, activates multiple potential defense mechanisms that induce increased activity of defense-related enzymes which showed resistance to various plant pathogens (Xue et al. 1998). For the induction of ISR, strains of bacterial antagonists including *Pseudomonas* spp. appear to be promising. For example, increase in accumulation of phenolic compounds (M'Piga et al. 1997; Ramamoorthy et al. 2002) and increase in activities of peroxidase in bioagent-treated cucumber seedlings (Yedidia et al. 1999) increase of peroxidase (PO) and polyphenoloxidase (PPO) activities in cucumber roots treated with *Pseudomonas corrugata* against *Pythium aphanidermatum* (Chen et al. 2000), and accumulation of phenolics and increase in phenylalanine ammonia-lyase (PAL) in groundnut plants treated with *P. fluorescens* against *Cercospora personata* (Meena et al. 1999) were observed by different researchers. ISR against different pathogens by microbial strains has also been achieved in tobacco (Maurhofer et al. 1994), cucumber (Liu et al. 1995a, b), radish (Leeman et al. 1995), and sugarcane (Viswanathan and Samiyappan 1999). However, in the case of biological control of postharvest disease of banana, knowledge on the induced resistance for the management of crown rot disease is lacking.

Therefore, the present investigation was undertaken with an objective to identify the effective combination of antagonist mixture for the control of crown rot disease of banana both under in vitro and in vivo conditions and also to investigate the involvement of ISR for the control of crown rot disease complex and thereby increased shelf life of banana.

Materials and methods

Pathogen isolation and culture maintenance

Lasiodiplodia theobromae and *C. musae* were isolated separately from crown-rot-infected banana fruits. These cultures were maintained on Potato-Dextrose-Agar (PDA) at 4°C, and fresh cultures were grown on PDA plates before use. Spore suspension was prepared from 10-day-old culture of *L. theobromae* and *C. musae* grown on PDA plate (28 ± 2°C) by flooding the culture plate with sterile distilled water and scrapping the surface of the culture carefully with a sterile scalpel without disturbing the agar. The spore concentration of the fungus was adjusted with sterile distilled water to 10^6 spores mL^{-1} using a hemocytometer and the resulting spore suspension was used for inoculation experiments.

Isolation of bacterial strains and examination of their antagonism on L. theobromae and C. musae

Bacterial antagonists were isolated from fruit skin of banana cultivars namely Robusta (Cavendish-AAA) and Karpuravalli (Pisang Awak-ABB) as well as from rhizosphere soil samples collected from organically grown banana orchards in Thiruchirapalli district of Tamil Nadu, India. From rhizosphere soil, five bacterial strains were isolated by following serial dilution technique and the resulting bacteria were identified as non-fluorescent *Pseudomonas* and named as NFP_1, NFP_2, NFP_3; *Bacillus subtilis* as BS_1; *Pseudomonas fluorescens* as Pf2a. From the fruit surface of banana, four bacterial strains viz., non-fluorescent *Pseudomonas* NFP_4, NFP_5, NFP_6, and *P. fluorescens* Pf3a were isolated as per the procedure described by Zahavi et al. (2000). Characterization of different cultures of antagonistic bacteria was done as described by Schaad (1992). Besides, six bacterial strains viz., *Rhizobium* RH_1, *Bacillus subtilis* BS_2, *Azospirillum* AS_1; AS_2, *Azotobacter* AZ_1, and *P. fluorescens* Pf1a were collected form culture collection of Department of Agricultural Microbiology, Annamalai University and are designated as 'Introduced'.

Dual culture method was performed to know the antagonistic potential of all bacterial strains against both test pathogens (Anjaiah et al. 1998). Percent inhibition of mycelial growth of the pathogen and the zone of inhibition were measured. The bacterial strains which showed good performance on fruits when tested singly were further tested in vitro for their compatibility to evaluate them as mixture of bioinocula (Fukui et al. 1994).

Preparation of inoculum of bacterial antagonist

All the strains of Non-fluorescent *Pseudomonas*, *P. fluorescens* and *B. subtilis* strains (NFP_5, NFP_6, BS_1, AS_1, AZ_1, and Pf3a) which showed good antagonism over both *L. theobromae* and *C. musae* under in vitro condition alone were selected for in vivo studies. These bacterial strains were grown separately in conical flasks (250 mL) containing 100 mL of King's broth (KB) (for non-fluorescent *Pseudomonas* and *P. fluorescens*) and 100 mL of nutrient broth (NB) (for *B. subtilis*) and kept for 48 h on a rotary shaker (150 rpm) at 28 ± 2°C. Cells of individual bacterial strain were removed by centrifugation at 6,000×*g* for 10 min at 4°C and washed in sterile distilled water. The pellet was resuspended in a small quantity of sterile distilled water and brought to the concentration of 3×10^8 CFU mL^{-1} using a spectrophotometer ($OD_{595} = 0.3$) and used as bacterial inoculum for further experiments (Thompson 1996). For antagonist mixture studies, equal volumes of broth of respective strains were mixed and used for centrifugation (Fukui et al. 1994).

Efficacy of postharvest application of antagonistic bacteria to control crown rot disease of banana

Banana cv. Robusta (Cavendish-AAA) were hand harvested at 75% maturity. Hands free from damage and uniform sized hands were selected for the study. The bananas were transported to the laboratory immediately, dehanded along with crown portion, washed thoroughly in running tap water, then allowed to air dry for 3–4 h at a room temperature and used for inoculation experiments.

A uniform 5-mm-deep and 3-mm-wide cavity was made at the crown region of banana using the tip of sterile dissecting needle. This cavity was inoculated with 500 µL of spore suspension of each *L. theobromae* and *C. musae* (10^6 spores mL^{-1}) and kept for 2 h at 28 ± 2°C. The fruits were then sprayed separately with cell suspension of each individual strain of bacterial antagonist (3×10^8 CFU mL^{-1}). Fruits were then allowed to air dry in room condition for 12 h and placed in polythene bags. Two sets of identical experiment were carried out and one set of treatment was incubated at room temperature (28 ± 2°C), and another set of identical treatment was incubated under cold storage (14°C, 90% RH). Each poly bag contained one hand, loosely packed with mouth of the bag tied. Each treatment was replicated four times and scored as described below. The banana hands were then assessed for disease severity at regular intervals based on crown rot severity (0–5 scale), crown color (1–7 scale), and crown texture (0–4 scale) (Finlay and Brown 1993). The shelf life (green and yellow life) periods were also examined and recorded.

Efficacy of post harvest application of bacterial antagonistic mixture to control the crown rot disease of banana

The bacterial antagonists such as NFP_6, BS_1, and Pf3a which recorded significantly less crown rot severity, color and texture scores when applied singly, both under room and cold storage conditions, were selected to test them as mixture of bioinocula to control the crown rot disease complex. The compatibility among antagonists was ensured in vitro. The three bacterial strains were tested on fruits in all possible combinations such as $NFP_6 + BS_1$, NFP_6 + Pf3a, BS_1 + Pf3a, $NFP_6 + BS_1$ + Pf3a. The banana fruits were applied with cell suspension of aforementioned combination of bacterial antagonists (3×10^8 CFU mL^{-1}) and incubated under both room and cold storage conditions. The observation on crown rot severity was taken as per the method of Finlay and Brown (1993).

Besides, the shelf life (green and yellow life) periods were also recorded.

Induction of biochemical defense mechanisms

The selection of banana bunches var. Robusta, dehanding, and treatments were done as described above**.** The fruits were then sprayed separately with spore/cell suspensions of individual and combinations of bacterial antagonists (10^8 cells mL^{-1}) and allowed to dry for 2 h under room temperature condition. Then on the crown portion, a small cavity was made and 200 µL of spore suspension (10^6 spores mL^{-1}) of 10-day-old cultures of *L. theobromae* and *C. musae* was placed into this cavity and covered with cotton. Then the treated hands were placed in a polythene bag and incubated at 24± 2°C. Each treatment was replicated thrice. At different intervals, tissues from the crown portion of each treatment were taken and analyzed for biochemical changes viz., PAL (EC 4.3.1.5), peroxidase (EC 1.11.1.7), polyphenoloxidase (EC 1.14.18.1) activities, and total phenolic content. Samples from each treatment were analyzed thrice. The enzyme activities were determined on days 0, 1, 3, 5, and 7 of storage at 24 ± 1°C. Micro Soft excel program was used to create all graphical representations.

Determination of PAL activity

One gram of crown tissue was homogenized in 5 mL of 0.1 M sodium borate buffer, pH 7.0 containing 0.1 g insoluble polyvinyl pyrrolidone (PVP). The extract was filtered through muslin cloth and the filtrates were centrifuged at 12,000×*g* at 4°C for 20 min. The supernatant served as an enzyme source. Samples containing 0.4 mL of enzyme extract were incubated with 0.5 mL of 0.1 M borate buffer, pH 8.8, and 0.5 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm as described by Dickerson et al. (1984) and was expressed as nmoles of cinnamic acid min^{-1} g^{-1} of fresh tissue.

Assay of peroxidase

The peroxidase activity was assayed as described by Hammerschmidt and Kuc (1982). Extraction was carried out by homogenizing 1 g of the crown sample in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) using pre chilled pestle and mortar (4°C). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant served as enzyme source and the reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract, and 0.5 mL of 1% H_2O_2. The reaction mixture was incubated at 28 ± 2°C. At the start of enzyme reaction, the absorbance of the mixture was set to zero at 420 nm in the spectrophotometer and the change in the absorbance was recorded at 20 s interval for 3 min. Boiled enzyme preparation served as control. The peroxidase activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} of fresh tissue.

Assay of polyphenoloxidase

One gram of the sample was homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) in a pre chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant served as enzyme source. Polyphenoloxidase activity was determined as per the procedure given by Mayer et al. (1965). The reaction mixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µL of the enzyme extract. To start the reaction, 200 µL of 0.01 M catechol was added. The reaction mixture was incubated at room temperature and the absorbance was set to zero at 495 nm. The changes in absorbance were recorded at 30 s interval for 2 min and the activity was expressed as change in absorbance min^{-1} g^{-1} of fresh tissue.

Estimation of phenolic content

Banana crown tissues (1 g) were homogenized in 10 mL of 80% methanol and shaken for 15 min at 70°C. Phenol was assayed as described by Zieslin and Ben-Zaken (1993). One gram of crown tissue was homogenized in 10 mL of 80% methanol and agitated for 15 min at 70°C. Then 1 mL of the methanolic extract was added to 5 mL of distilled water and 250 µL of Folin–ciocalteau reagent (1 N) and incubated at 25°C. After 3 min 1 mL of the saturated solution of sodium carbonate and 1 mL of distilled water were added and the reaction mixtures were incubated further for 1 h at 25°C. The absorption of the developed blue color was measured using spectrophotometer at 725 nm. The total soluble phenol content was calculated according to a standard curve obtained from a Folin–Ciocalteau reagent with a phenol solution (C_6 G_6O) and expressed as catechol equivalent g^{-1} of fresh tissue.

Experimental design and statistical analysis

All the experiments were carried out in a completely randomized design (CRD). For the data on effect of bacterial strains on mycelial growth and spore germination of *L. theobromae* and *C. musae*, percent reduction over control was calculated. The data were analyzed using the

IRRISTAT version 92–1 program developed by the biometrics unit, International Rice Research Institute, Metro Manila, The Philippines. Data were subjected to analysis of variance (ANOVA). The treatment mean values were compared by Duncan's multiple range test (DMRT) at 5% significance level (Gomez and Gomez 1984).

Results

Antifungal activity of bacterial strains

All the antagonistic bacterial strains tested significantly reduced the mycelial growth of both *L. theobromae* and *C. musae*. However, among nine native bacterial strains tested on *L. theobromae*, strain NFP_6 recorded higher percent inhibition of mycelial growth, 65.1%, followed by the strains Pf3a and BS_1 which recorded the mycelial inhibition of 61.9 and 58.1%, respectively (Table 1). However, the inhibition zone recorded on *L. theobromae* was at a maximum (15.5 mm) in NFP_6 inoculated plates followed by Pf3a (12.5 mm). On *C. musae*, the native strain NFP_6 and the introduced bacterial strains AS_1, and AZ_1 achieved higher percent inhibition of 53.4, 52.3, and 52.3%, respectively (Table 2). However, the inhibition zone recorded was at maximum on *C. musae* in Pf3a and NFP_6 (14.5 and 14.3 mm respectively) inoculated plates followed by AZ_1 (11.8 mm) and AS_1 (11.3 mm).

Efficacy of individual bacterial antagonist strains on crown rot of banana

Those treatments that had been most effective in inhibiting the mycelial growth of both *L. theobromae* and *C. musae* were selected for in vivo evaluation on fruits. On both storage conditions, all the treatments reduced the crown rot severity and texture score significantly compared with pathogen alone inoculated control. At 10 days of storage under room condition, the strain NFP_6 recorded very low crown rot severity score of 1.5 followed by Pf3a (2.3) and BS_1 (2.5). In the case of pathogen alone inoculated hands, the rot severity was 4.5 (Table 3). The score for color and texture of the crown was also significantly less in fruits treated with NFP_6, BS_1, and Pf3a compared with other antagonists and pathogen alone inoculated control.

In cold storage (14°C), 24 days after storage, the crown rot severity was also significantly very less in NFP_6 applied hands (1.0) followed by strain Pf3a and BS_1 (2.0 each) treated hands, while pathogen alone inoculated control recorded the maximum severity score of 3.5. The texture of the crown was also found to be comparatively hard in NFP_6 bacterial strain treated hands followed by Pf3a and BS_1 treated hands as compared with little fibrous crown with decaying tissues in other treatments and complete tissue decay in pathogen-inoculated hands. On both storage conditions, some amount of infection was also noticed in the pathogen uninoculated control hands, indicating the latent infection by the crown rot pathogens.

Table 1 Effectiveness of native and introduced bacterial stains in inhibiting the radial mycelial growth of crown rot pathogen *L. theobromae*

Treatment strains[A]	Source	Mycelial growth of the pathogen[B] (mm)	Percent reduction over control	Inhibition zone (mm)
NFP_1	Rhizosphere soil of banana	67.0 hi	22.7	6.5 def
NFP_2	Rhizosphere soil of banana	69.3 jk	20.2	5.0 fgh
NFP_3	Rhizosphere soil of banana	68.7 ij	20.7	4.8 fgh
NFP_4	Fruit skin of banana cv. Robusta	70.0 jk	19.3	6.0 efg
NFP_5	Fruit skin of banana cv Karpuravalli	51.3 f	40.8	9.3 bc
NFP_6	Fruit skin of banana cv Robusta	30.3 a	65.1	15.5 a
BS_1	Rhizosphere soil of banana	36.3 c	58.1	9.0 bc
BS_2	Introduced	69.0 ij	20.4	4.3 gh
AS_1	Introduced	40.7 d	53.1	8.3 bcd
AS_2	Introduced	65.3 h	24.7	4.0 gh
AZ_1	Introduced	43.0 e	50.4	7.5 cde
RH_1	Introduced	56.0 g	35.4	6.0 efg
Pf1a	Introduced	73.0 l	15.8	3.5 h
Pf2a	Rhizosphere soil of banana	71.3 kl	17.7	7.3 cde
Pf3a	Fruit skin of banana cv Robusta	33.0 b	61.9	9.8 b
Control	–	86.7 m	–	–

[A] NFP_1, NFP_2, NFP_3, NFP_4, NFP_5, and NFP_6 are strains of Non-Fluorescent Pseudomonads; BS_1, BS_2, are strains of *Bacillus subtilis*; Pf1a, Pf2a, and Pf3a are strains of *P. fluorescens,* AS_1, AS_2 are strains of *Azospirillum*, AZ_1 is the strain of *Azotobacter* and RH_1 is the strain of *Rhizobium;* BS_2, AS_1, AS_2, AZ_1, RH_1, and Pf1a are strains from culture collection are designated as Introduced

[B] Values are means of four replications. In a column means followed by a lower case letters are not significantly different at 5% ($P = 0.05$) level by DMRT

Table 2 Effectiveness of native and introduced bacterial strains in inhibiting the radial mycelial growth of crown rot pathogen *C. musae*

Treatment strains[A]	Mycelial growth of the pathogen[B] (mm)	Per cent reduction over control	Inhibition zone (mm)
NFP_1	71.0 de	20.0	6.5 fg
NFP_2	74.0 f	16.6	6.0 gh
NFP_3	69.0 cde	22.2	7.0 f
NFP_4	71.3 df	19.6	6.0 gh
NFP_5	50.7 bc	42.8	8.5 d
NFP_6	41.3 a	53.4	14.3 a
BS_1	50.3 b	43.3	9.3 c
BS_2	67.3 c	24.1	7.8 e
AS_1	42.3 a	52.3	11.3 b
AS_2	74.3 f	16.2	5.8 h
AZ_1	42.3 a	52.3	11.8 b
RH_1	77.7 g	12.4	4.5 i
Pf1a	79.3 g	10.6	4.3 i
Pf2a	67.0 c	24.5	9.3 c
Pf3a	51.3 b	42.2	14.5 a
Control	88.7 h	–	–

[A] For strain abbreviations see Table 1

[B] Values are means of four replications. In a column means followed by a lower case letters are not significantly different at 5% ($P = < 0.05$) level by DMRT

Table 3 Evaluation of selective bacterial antagonist strains against the crown rot complex of banana cv. Robusta (AAA)

Treatment[A]	Severity score at room storage (28 ± 2°C) after 10 days[B]			Shelf life at room storage (28 ± 2°C) (days)[B]	Severity score at cold storage (14°C) after 24 days[B]			Shelf life at cold storage 14°C (days)[B]
	Rot (0–5)	Color (1–7)	Texture (0–4)		Rot (0–5)	Color (1–7)	Texture (0–4)	
NFP_5	3.0 bc	6.0 c	2.5 d	12.7 b	3.5 e	6.3 c	2.3 c	38.0 d
NFP_6	1.5 a	4.5 a	1.5 b	13.0 b	1.0 a	5.0 ab	1.0 a	42.7 b
BS_1	2.5 b	5.8 bc	1.5 b	12.3 b	2.0 bc	5.3 ab	1.5 bc	40.0 c
AS_1	3.3 c	6.0 c	3.0 e	11.0 c	3.0 de	6.5 c	3.5 d	33.0 e
AZ_1	3.0 bc	5.3 bc	2.5 d	11.3 c	2.5 cd	5.5 bd	2.3 c	31.3 f
Pf3a	2.3 b	5.0 ab	1.5 b	13.0 b	2.0 bc	5.3 ab	2.0 bc	43.7 b
Benomyl (0.1%)	1.3 a	4.5 a	1.0 a	15.7 a	1.0 a	4.8 a	1.3 a	62.3 a
L. theobromae + *C. musae*	4.5 d	6.5 c	3.8 f	9.7 d	3.5 e	7.0 d	3.5 d	19.0 h
Control (no pathogen)	1.5 a	4.8 ab	2.0 c	13.7 b	2.3 cd	5.5 b	1.7 bc	27.7 g

[A] For strain abbreviations see Table 1

[B] Values are means of four replications. In a column means followed by a lower case letters are not significantly different at 5% ($P = 0.05$) level by DMRT

The assessment of total shelf life period (both green and yellow life) of banana indicated that all bacterial antagonists tested significantly increased the total shelf life of banana which ranged from 11.0 to 13.0 days in room storage and 31.3–43.7 days in cold storage compared with 9.7 and 19.0 days in pathogen alone inoculated hands on room and cold storage, respectively. However, under both storage conditions, the effect of increasing the total shelf life of banana by any bacterial antagonists was not as that of the fungicide Benomyl (0.1%) which increased the total shelf life to 15.7 and 62.3 days in room and cold storage, respectively.

Compatibility among biocontrol agents

Those antagonist strains that had shown most effective in reducing the crown rot and texture severity when tested singly on fruits viz. NFP_6, Pf3a, and BS_1 were alone selected for in vitro compatibility studies. In this, the strains that overgrew were considered as compatible with

each other, whereas strains that were separated by an inhibition zone were considered as incompatible. The results of the study showed that between NFP_6, Pf3a, and BS_1 strains, no inhibition zone was formed indicating that these strains were compatible with each other (data not shown).

Effect of bacterial antagonistic mixture on crown rot of banana

In this study, the application of mixture of bacterial antagonists on banana fruits had more beneficial effect than the individual strains did in reducing the rot severity, crown firmness, and extending the total shelf life of banana under both storage conditions (Table 4). In room storage, the rot score of banana hands treated with combination of $NFP_6 + Pf3a + BS_1$ and other two way combination of antagonist were statistically on par with fungicide Benomyl (0.1%) treatment. Similarly, the three-way and all two-way combinations of antagonist mixture showed significant reduction in texture score as that of fungicidal treatment. In cold storage, the antagonist mixture consisting of $NFP_6 + Pf3a + BS_1$ greatly reduced the rot severity to 0.3 while it was 3.7 in pathogen alone inoculated hands. The effect of two-way antagonist mixture treatment was also statistically on par with three-way antagonist mixture treatment and also fungicide treatment. The texture of crown treated with $NFP_6 + Pf3a + BS_1$ and $NFP_6 + Pf3a$ was found to be hard with the score of 0.0 and 0.3, respectively, on 24 days of inoculation which is on par with the fungicide treatment.

Interestingly, in room storage, the effect of extending the total shelf life of banana by the combination of $NFP_6 + Pf3a + BS_1$ was significantly greater (16.3 days) than all two-way combination of antagonist treatment. The total shelf life period in pathogen alone inoculated hands was 9.6 days. In the case of cold storage, the maximum shelf life period of 61.7 days was achieved with antagonist mixture combination of $NFP_6 + Pf3a + BS_1$ which differed significantly from all other antagonist mixture treatments as well from uninoculated (50.3 days) and pathogen alone inoculated (28.3 days) controls. In addition, among two-way combination of antagonistic mixture tested, the combination of $NFP_6 + Pf3a$ bacterial strains performed better by in increasing the shelf life period to 15.3 days in room storage and 60.3 days in cold storage. On both storage conditions, effect of the three-way combination of bacterial strain mixture was comparable with those of fungicide Benomyl (0.1%) treatment in increasing the total shelf life of banana fruit.

Induction of biochemical defense mechanisms in banana fruits by bacterial antagonists

Levels of PAL were increased significantly with all biocontrol treatments up to 5 days, after which there was a decline (Fig. 1a). In general, three-way followed by two-way combination of bacterial antagonist mixture produced a greater PAL activity at all time intervals than did single antagonist and fungicide treatment. A similar increase in activity of PO (up to fourfold) and PPO (up to 2.55 fold) was recorded in all the hands treated with biocontrol agents as compared with untreated hands (Fig. 1b, c). The maximum activity of PO and PPO was observed till 5 days in all treatments and thereafter declined. Similarly, the three-way bacterial antagonist mixture recorded a greater PO and

Table 4 Effect of different combination of bacterial antagonists on crown rot complex of banana cv. Robusta (AAA)

Treatment[A]	Severity score at room storage (28 ± 2°C) after 10 days[B]			Shelf life at room storage (28 ± 2°C) (days)[B]	Severity score at cold storage (14°C) after 24 days[B]			Shelf life at cold storage (14°C) (days)[B]
	Rot (0–5)	Color (1–7)	Texture (0–4)		Rot (0–5)	Color (1–7)	Texture (0–4)	
$NFP_6 + BS_1$	1.0 a	5.3 b	1.7 a	14.3 c	1.0 a	5.0 b	1.3 b	54.3 c
$NFP_6 + Pf3a$	1.3 a	4.7 ab	1.3 a	15.3 bc	0.7 a	4.0 a	0.3 a	60.3 b
$BS_1 + Pf3a$	1.0 a	5.0 ab	1.7 a	14.7 c	1.0 a	5.3 b	1.0 b	53.3 d
$NFP_6 + BS_1 + Pf3a$	0.7 a	4.3 a	1.7 a	16.3 ab	0.3 a	4.0 a	0.0 a	61.7 a
Benomyl (0.1%)	1.0 a	4.3 a	1.0 a	16.7 a	0.3 a	4.0 a	0.0 a	62.0 a
L. theobromae + *C. musae*	4.3 b	6.3 c	1.0 a	9.6 d	3.7 b	6.3 c	3.0 c	28.3 f
Control (no pathogen)	1.3 a	5.0 ab	3.7 b	14.3 c	0.7 a	5.0 b	0.3 a	50.3 e

[A] For strain abbreviations see Table 1

[B] Values are means of four replications. In a column, means followed by lower case letters are not significantly different at 5% ($P = 0.05$) level by DMRT

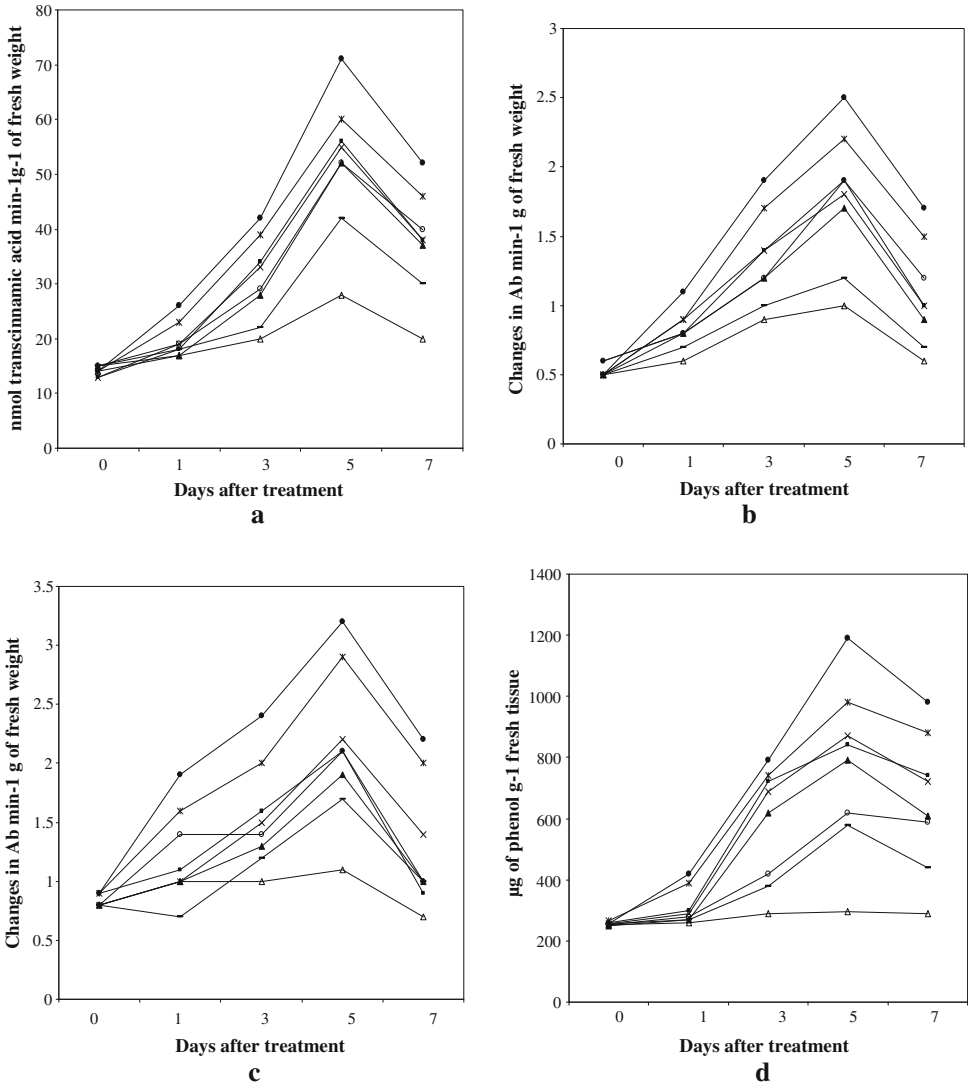

Fig. 1 Induction of defense enzymes and phenolic content in crown tissue of banana fruits var. Robusta (Cavendish -AAA) after treatment of potential bacterial antagonists and challenge inoculated with crown rot pathogens *L. theobromae* + *C. musae*. **a** Phenylalanine ammonia-lyase (PAL) activity. **b** Peroxidase (PO) activity. **c** Polyphenoloxidase (PPO) activity. **d** Phenol content

PPO activity than in untreated control, pathogen inoculated, as well as individual biocontrol agents or of fungicide treated hands. Phenolic content was increased significantly in crown tissue of banana fruit treated with bacterial antagonist 1 day after application and reached maximum after 5 days of treatment and then declined (Fig. 1d). However, at all time intervals, three-way mixture of bacterial antagonists recorded up to 3.66 fold increase in phenolic content compared with all other treatments.

Discussion

Crown rot of banana is the major factor contributing to decrease in quality of exported bananas and damage is more severe when transit exceeds 14 days. Losses also are substantial in local markets where fruits are displayed on hooks. Extension of total shelf life of banana fruit is a big problem for many of the traders who are involved in marketing of banana in local as well as distant markets. Microbial antagonists have been reported to protect a variety of harvested perishable commodities against a number of postharvest pathogens (Wisniewski et al. 2001). It is evident from the results of this investigation that the native strains from fruit skin of banana such as NFP_6 and Pf3a (from cv. Robusta), and BS_1 strain from rhizosphere soil of banana achieved higher antagonistic activity against *L. theobromae*, whereas the native strain NFP_6 and introduced strains AS_1 and AZ_1 recorded maximum mycelial growth inhibition against *C. musae*. Based on these in vitro screening, six bacterial strains (four native strains and two introduced strains) were tested on banana fruits in two storage conditions viz., room storage (28 ± 2°C, meant for local market) and cold storage (14°C, meant for distant transport). The results showed that no antagonists which have been applied individually could result in control of

crown rot disease as that of the fungicide Benomyl (0.1%) under both storage conditions. It has been suggested by different researchers that antagonist mixture should be used to assure adequate disease control under various conditions. For example, a combination of two strains of *Aureobasidium pullulans* and a yeast, *Rhodotorula glutinans*, when applied simultaneously, controlled infection caused by *B. cinerea*, *P. expansum* and *Pezicula malicorticis* as effectively as that of commercial fungicide (Leibinger et al. 1997). More over, the antagonist mixtures may also help to achieve satisfying results under fluctuating environment when one or other mechanisms paralyzed (Leeman et al. 1996). Hence, when the antagonists were tested as mixture in two-way and three-way combinations, we could observe greater suppression of crown rot disease as that of fungicide Benomyl (0.1%) under both storage conditions. Besides, the three-way combination of antagonist (NFP_6 + Pf3a + BS_1) also increased total shelf life of banana fruits, especially in cold storage (14°C). These results are in accordance with studies made by earlier researchers (Duffy and Weller 1995; Pierson and Weller 1994) who have demonstrated that mixtures of Fluorescent pseudomonads were significantly more suppressive of take all disease in wheat than treatment with individual antagonist. Mixtures of biocontrol agents will also have the advantage of applying a broad-spectrum activity, enhancing the efficacy and reliability of biological control over individual strains. The maximum reduction of crown rot severity under cold storage compared with room temperature which has been observed in the present study might also be due to the inhibition of the growth of decay organisms under cold storage as reported by Aharoni et al. (1997).

Plants are endowed with defense genes, but they are quiescent in normal healthy plants. When these defense genes are activated by various factors, they induce systemic resistance against diseases. One of the factors is antagonistic microbes, which show direct antagonistic activity against pathogens not only by producing various metabolites, but also by inducing defense-related enzymes. These have recently been found to be a new way whereby plants defend themselves from pathogen attack (Bharathi et al. 2004). This phenomenon called "Induced Systemic Resistance" (ISR) has been demonstrated in several plants (Alstrom 1991; Van peer et al. 1991; Wei et al. 1991; Maurhofer et al. 1994; Leeman et al. 1995) and found to reduce disease symptoms of a wide range of pathogens (Wei et al. 1991; Liu et al. 1995b). This bacterial antagonist-mediated systemic resistance is generally associated with onset of defense mechanism including the early and increased expression of defense enzymes such as PO, PAL and accumulation of phenolics, phytoalexins, lignins etc. (Chen et al. 2000). In the present study, up to fourfold increase in PAL activity was observed in bioagent-treated fruit and the maximum was recorded in banana fruits treated with three-way combination of bacterial antagonists mixture, i.e., NFP_6 + Pf3a + BS_1. Generally, induction of PAL enzyme is correlated with increased resistance to pathogenic infection (Bell et al. 1984). PAL was reported to be induced in bean by fluorescent pseudomonads against *Botrytis cinerea* (Zdor and Anderson 1992). In citrus and in other commodities, ethylene induces activity of PAL, an enzyme which catalyzes the branch point step reaction of the shikimic acid pathway, leading to the biosynthesis of phenols, phytoalexins, and finally lignins with the help of peroxidases—all associated with ISR to diseases (Kuc and Rush 1985). In citrus fruit, PAL activity was induced after the application to the peel of an effective yeast antagonist, possibly leading to increased resistance (Wilson et al. 1991).

Peroxidase has been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxyl cinnamyl alcohols into free radical intermediates, which subsequently are coupled to lignin polymer (Gross 1980). Further more, peroxidase itself was found to inhibit the spore germination and mycelial growth of *Pseudocercospora abelmoschi* and *P. cruenta* (Joseph et al. 1998). In the present study, banana hands treated with the three-way combination of bacterial antagonist NFP_6 + Pf3a + BS_1 and followed by NFP6 + Pf3a had higher levels of peroxidase and polyphenoloxidase enzymes compared with hands treated with single biocontrol agents viz., NFP_6, Pf3a, and BS_1 and also hands treated with fungicide treatment. Several workers have reported the association between higher levels of defense-related enzymes and greater disease resistance. Increased PO and PPO activity was observed in a number of resistant interactions involving plant-pathogenic fungi and bacteria (Reimers et al. 1992; Deborah et al. 2001). Sariah et al. (2001) also reported that the increased peroxidase activity in banana was positively correlated with resistance to Fusarial wilt. Morpurgo et al. (1994) reported that the activity of peroxidase was at least five times higher in the roots and corm tissues of the *F. oxysporum*-resistant banana cultivar than in the susceptible cultivar. Inoculation of resistant cultivar with *F. oxysporum* resulted in tenfold increase in peroxidase activity after 7 days of inoculation, whereas, the susceptible cultivar exhibited only a slight increase in peroxidase activity.

In addition to PAL and PO, the presence of phenolic compounds in plants or their synthesis in response to infection has often been associated with resistance (Ingham 1972). The increase in phenolic content in plant system has been correlated with increased resistance to pathogens (Velazhahan and Vidhyasekaran 1994). It is also well

known that resistant plants contain more phenols or produce polyphenols more rapidly than susceptible ones. In the present study also, the treatment of three-way bacterial antagonist mixture which have recorded maximum reduction of crown rot disease also induced higher accumulation of phenolics and therefore this increased phenolic content might have contributed to increased resistance to crown rot pathogens.

To conclude, the increased activities of defense-related enzymes such as PAL, PO, and PPO and elevated accumulation of phenolics due to the treatment of native bacterial antagonistic mixtures in the banana fruits suggest that activation of host biochemical defense mechanism may be involved in the suppression of crown rot disease of banana and which in turn increased the shelf life of banana.

Acknowledgments Authors are grateful to Dr. A. Anandan, Sr. Lecturer, Department of Agricultural Botany, Annamalai University for his help rendered during statistical analysis.

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