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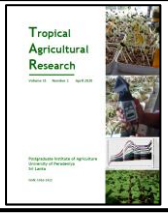

Field Performance of Mixtures of *Pseudomonas* and *Bacillus* spp. in Managing Papaya Ringspot Virus Disease and their Effect on Plant Defense Enzyme Activity

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ABSTRACT

The present study was conducted to determine the field efficacy of *Pseudomonas* and *Bacillus* spp., isolated from the rhizosphere of healthy papaya to manage papaya ringspot virus disease (PRSVD), when applied as isolate-mixtures along with their effect on plant defense enzyme activity. Mixtures of bacterial isolates were applied by two methods, namely seed soak (SS) and soil drench (SD) to field-grown and naturally-infected papaya plants (var. Red Lady). Disease severity and fruit yield were quantified over a period of six months and defense-related enzyme activity in leaves was quantified with spectrophotometry. At the sixth month after transplanting, SS-treated plants with the mixture of *Bacillus* isolates and the combined mixture of *Pseudomonas* and *Bacillus* isolates showed a significantly lower % disease index on foliage than the plants under control and the *Pseudomonas* mixture treatments. A significantly lower % disease index was shown on the fruits treated with all the bacterial treatments applied by both methods, in comparison to the untreated control. Mixture of *Pseudomonas* isolates and the combined treatment with *Pseudomonas* and *Bacillus* isolates, when applied as the SS method, gave significantly higher fruit yield than that of the control treatment. Activities of peroxidase and phenylalanine ammonia lyase were significantly higher in plants treated with the mixture of *Pseudomonas* isolates by SS method while β-1,3-glucanase activity was significantly higher when applied the same treatment by SD method. Findings revealed the ability to reduce severity of PRSVD and induce defense-related enzymes when mixtures of *Pseudomonas* and *Bacillus* spp. were applied under field conditions.

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INTRODUCTION

Papaya ringspot virus diseases (PRSVD) caused by papaya ringspot virus (PRSV) is a major disease in papaya cultivations around the globe. The disease is characterized by leaf mosaic and chlorosis, water soaked oily streaks on petioles and upper part of the trunk, distortion of young leaves leading to shoestring appearance and yield loss (Purcifull *et al*., 1984). Plants infected by PRSV result in qualitative yield reductions where sugar level of the fruits gets reduced by 50 % or more (Purcifull *et al*., 1984). PRSV is a member of the genus *Potyvirus* and the family *Potyviridae* and transmitted by many species of aphids (mainly *Myzus persicae* and *Aphis gossypii*) in a non-persistent manner. Management of PRSV has been successful with transgenic papaya varieties, cross protection and tolerant varieties (Tripathi *et al*., 2008).

Other than the use of germplasm having genetically-controlled resistance, plant diseases can be managed through the induction of host plant resistance mediated by various biotic and abiotic agents (i.e. virulent or avirulent pathogens, nonpathogens, cell wall fragments, plant extracts and synthetic chemicals) (Walters *et al*., 2005). Induced resistance in plants is of two types, namely systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR develops locally or systemically in response to, for example, pathogen infection or treatment with certain chemicals (e.g., 2,6-dichloroisonicotinic acid [INA]) which is effective against a wide range of pathogens and is mediated by a salicylic acid [SA]-dependent process (Walters *et al*., 2005). In contrast, ISR develops as a result of colonization of plant roots by plant-growth-promoting rhizobacteria (PGPR) and is mediated by a jasmonate- or ethylene-sensitive pathway (Pieterse *et al*., 1998).

Induced systemic resistance (ISR) by PGPR is effective against fungal, bacterial, viral, insect and nematode pests (Van Loon *et al*., 1998; Ramamoorthy *et al*., 2001) as an eco-friendly plant protection approach. Induced systemic resistance promoted through PGPR provides physical and mechanical strength to the cell walls of the plants and changes physiological and biochemical status of the host leading to the synthesis of defense chemicals against the incoming pathogen (Ramamoorthy *et al*., 2001). Seed treatment using *Pseudomonas fluorescens* strain 89B-27 and *Serratia marcescens* strain 90-166 have reduced incidence and delayed the symptom development of cucumber mosaic virus in cucumber and tomato (Raupach *et al*., 1996). Further, soil application of *P. fluorescens* strain P3 has induced host plant resistance against tobacco necrosis virus disease in tobacco (Maurhofer *et al*., 1998).

Induced systemic resistance by PGPR has been used effectively to manage a range of plant diseases under field conditions. Field application of *P. putida* strain 89B-27, *S. marcescens* strain 90-166 and *Favomonas oryzihabitans* strain INR-5 have reduced angular leaf spot and anthracnose of cucumber under field conditions along with growth promotion and enhancement of yield (Wei *et al*., 1995). Instead of using individual strains, use of mixtures of PGPR strains and the better performance of such strain mixtures on reduction of plant diseases and enhanced growth performances under field conditions have been reported (Raupach and Kloepper, 1998).

Ranasinghe *et al*. (2018) isolated 20*Pseudomonas* and four *Bacillus* isolates from rhizospheric soil of papaya plantations in Sri Lanka. Application of these strains individually as seed and soil drench treatments, in a pot experiment has reported plant growth promotion, increase of yield and reduction of symptom development of PRSVD in papaya foliage.

Considering the beneficial effects of the isolated *Pseudomonas* and *Bacillus* spp. on management of PRSVD, plant growth promotion and better yield performances, this study was conducted to evaluate the field efficacy of the bacterial strains when applied as mixtures on the reduction of PRSVD and growth and yield performances. Further, the influence of bacterial isolates on the activity of selected defense-related enzymes was quantified when the mixtures of bacterial isolates were applied.

MATERIALS AND METHODS

Determination of field efficacy of PGPR mixtures in managing PRSVD

Experimental site

A field experiment was conducted at Grain Legume and Oil Crop Research and Development Center, Department of Agriculture (DOA), Angunakolapelessa (DL1b agro-ecological region of Sri Lanka). Experimental period was from November 2017 – July 2018. The location was selected as this region has been identified by the DOA as a potential area for commercial papaya cultivation.

Establishment and management of papaya plants

Papaya (variety Red Lady) was transplanted with 3 x 3 m^2 spacing when the seedlings were 45 days old. Each planting pit had the dimensions of 0.5 x 0.5 x 0.5 m^3 and was filled with compost: top soil: cow dung mixture of 1:1:1 ratio. Fertilizer application (basal and top dressings and Borax and $ZnSO_4$) was done according to DOA recommendations.

Bacterial isolates

Five *Pseudomonas* isolates (one *P. putida* and four *Pseudomonas* spp.) and four *Bacillus* isolates (*B. cereus*) isolated from rhizosphere of healthy papaya plants and identified by Ranasinghe *et al*. (2018) were used for field and planthouse experiments. Identity of the bacterial isolates was confirmed through molecular methods (i.e. PCR amplification of the rRNA region and subsequent DNA sequencing and homology search by BLAST, NCBI).

Treatments and method of application of the treatments

Treatments were applied by two application methods, namely seed soak (SS) and soil drench (SD). The four treatments used were; T1- Mixture of five *Pseudomonas* isolates, T2 – Mixture of four *Bacillus* isolates, T3- Mixture of five *Pseudomonas* isolates and four *Bacillus* isolates and T4- control (no application of any bacterial isolate). In SS treatment, papaya seeds were soaked overnight in bacterial suspensions having a cell concentration of 1 x 10^8 cfu/ml of each isolate, before planting. In SD method 45 days old papaya plants were applied as a soil drench (100 ml/ plant) with above bacterial treatments at the time of transplanting. Thereafter, the plants under SD treatment were applied with the freshly-prepared suspensions of the same bacterial isolates at one month intervals (250 ml/ plant). Plants were allowed to get naturally-infected with the PRSV. Each treatment was replicated three times in a randomized complete block design and each replicate contained 12 plants.

Data collection and analysis

Disease severity of the foliage was recorded at monthly intervals according to the following scale developed in the present study (Table 1).

Disease severity of the fruits was recorded according the following scale developed for the present study based on the % of fruit surface area having ringspot symptoms. **0**- 0%, **1**- less than 25 %, **2**- 26-50 %, **3**- 51-75 % and **4**- more than 76 %.

Fruit yield was collected for 10 picks. Disease severity of foliage and fruits was calculated by the following formula (McKinney, 1923).

Percentage Disease Index (PDI)

$$\text{PDI} = \frac{\text{Sum of all numerical ratings x 100}}{\text{Total number of observations x Maximum rating}}$$

Treatment variation was determined by ANOVA and the mean separation was done by Duncan's multiple range test using SAS 9.1 software.

Confirmation of PRSV infection by RT-PCR

As plants were allowed to be infected by PSRV naturally, infection was confirmed by RT-PCR using PRSV coat protein specific primers, MB 11 and MB 12 (Temaja *et al*., 2015). RNA was extracted from immature young leaves of plants at the flowering stage by silica RNA extraction protocol (Gunasinghe *et al*., 2009). Extracted total RNA was used for cDNA synthesis using Maxima reverse transcriptase according to manufacturer's protocol (Thermo Fisher Scientific, USA). cDNA mixture was used to amplify with MB 11 and MB 12 primers and a PCR product of 905 bp was expected (Temaja *et al*., 2015). PCR products were DNA sequenced and subjected to DNA homology search (BLAST, NCBI).

Table 1: Details of the symptoms used to develop the scale for disease severity of foliage

Scale	Description of the severity of symptoms
0	No symptoms
1	Less than 25 % of the canopy having mild symptoms and 0 % moderate symptoms
2	26-50 % of the canopy having mild symptoms and 0% moderate symptoms
3	51-75 % of the canopy having mild symptoms and less than 25 % of the canopy having moderate symptoms
4	more than 75 % of the canopy having mild symptoms and 26-50 % of the canopy having moderate symptoms
5	Less than 25 % of the canopy having severe symptoms and 51-75% of the canopy having moderate symptoms
6	26-50 % of the canopy having severe symptoms
7	more than 76 % of the canopy having moderate symptoms
8	51-75 % of the canopy having severe symptoms
9	100 % of the canopy has severe symptoms

Note: Mild, moderate and severe symptom categories on leaves were defined as follows: mild symptoms – < 25 % of the leaf area showing mosaic symptoms and reduction of leaf lamina in one lobe, moderate symptoms – 26 – 50% of the leaf area showing mosaic symptoms and reduction of leaf lamina in 2-3 lobes and severe symptoms – > 51% of the leaf area showing mosaic symptoms and reduction of leaf lamina in more than three lobes with shoe string appearance

Determination of the defense enzyme activity due to bacterial treatments

Experimental site and treatment structure

An open field pot experiment was established at Plant Virus Indexing Center, Homagama. Papaya (variety Red Lady) seeds and plants were treated with six different treatments by SS and SD methods as described in the field experiment elsewhere in the text. The treatment details are given in Table 2.

Each treatment was replicated three times and maintained in a completely randomized design. Mechanical inoculation of PRSV was done for plants under T1, T3 and T5 treatments at the age of 45 days after seeding.

Quantification of defense enzymes

Leaf samples were collected from plants treated with treatments (T1-T6), before inoculation of PRSV and 1, 24, 48, 72, 96, 120 and 144 hours after inoculation of PRSV. These leaf samples were subjected to quantification of peroxidase (POX), phenylalanine ammonia lyase (PAL) and β-1,3- glucanase activity by methods described by Hammerschmidt *et al*. (1982), Dickerson *et al*. (1984) and Pan *et al*. (1991), respectively. Treatment variation on defense enzymes was determined by ANOVA and the mean separation was done by Duncan's multiple range test using SAS 9.1 software.

Table 2. Details of the treatments used to determine the activity of defense enzymes

Treatment	Description of the treatment
T1	Positive control (not treated with any bacterial suspension and inoculated with PRSV)
T2	Negative control (not treated with any bacterial suspension and not inoculated with PRSV)
T3	Mixture of five *Pseudomonas* isolates treated by SS and inoculated with PRSV
T4	Mixture of five *Pseudomonas* isolates treated by SS and not inoculated with PRSV
T5	Mixture of five *Pseudomonas* isolates treated by SD and inoculated with PRSV
T6	Mixture of five *Pseudomonas* isolates treated by SD and not inoculated with PRSV

RESULTS AND DISCUSSION

Field efficiency of bacterial mixtures on management of PRSV

Disease severity of plant canopy

Plants treated with the bacterial mixtures showed no significant difference between the methods of applications of the bacterial mixtures with reference to the disease severity (percentage disease index PDI) of plant canopy. PDIs of leaves exhibited by SS and SD-treated plants using four different bacterial treatments, over a period of six months are shown in Figures 1a and 1b, respectively.

At the sixth month after transplanting, SS-treated plants with the mixture of *Bacillus* isolates and the combined mixture of *Pseudomonas* and *Bacillus* isolates showed a significantly lower % disease index than the control and the *Pseudomonas* mixture treatments (Figure 1a). The plants treated with SD treatment by the combined mixture of *Pseudomonas* and *Bacillus* isolates gave a lower % DI values than all the other treatments throughout the experimental period (Figure 1b).

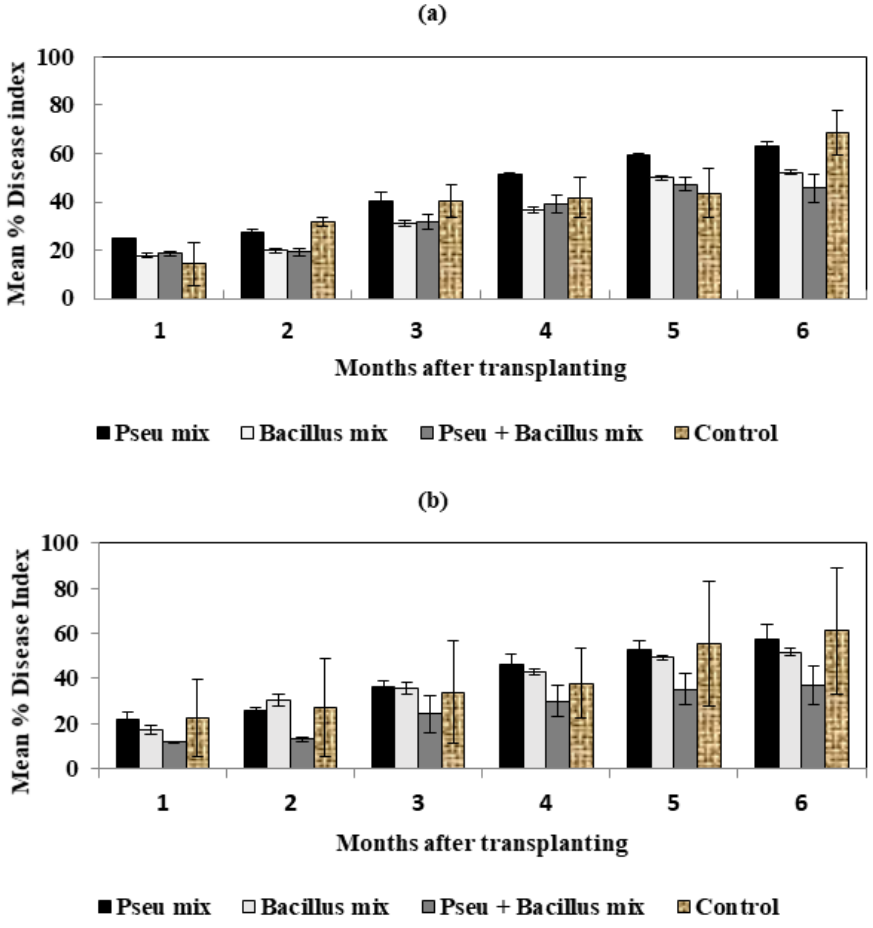

Figure 1: Disease severity of leaf symptoms (Mean % disease index) under field conditions when bacterial isolates were applied as mixtures by SS method (a) and SD method (b). Error bars indicate standard errors of means.

In vivo efficiency of controlling plant virus diseases by several plant growth promoting rhizobacteria (PGPR) has been well documented. Control of Tobacco necrosis virus in tobacco by *Pseudomonas fluorescens* strain CHAO (Maurhofer *et al*., 1998), Tobacco mosaic virus in tobacco by *P. aeruginosa* strain 7NSK2 (De Meyer *et al*. 1999), Tomato mottle virus and Cucumber mosaic virus in tomato by *Bacillus subtilis*

IN937b and *B. pumilus* strain SE34, respectively (Murphy *et al*. 2000; Murphy *et al*. 2003) are few examples.

Similar findings on reduction of disease severity of PRSVD have been observed when four *Pseudomonas* spp., *P. fluorescens* and a *Bacillus* sp. were applied as individual isolates through seed treatment and soil drench methods under field conditions in the Wet zone of Sri Lanka (WL3 agroecological zone) (Ranasinghe *et al*., 2018). Effectiveness of biological control using microorganism such as rhizobacteria depends on crucial factors such as environment condition and soil type (Damayanthi *et al*., 2007). However, results of present study and findings of Ranasinghe *et al*. (2018), confirmed the field performance of the indigenous *Pseudomonas* and *Bacillus* isolates as promising candidates in managing PRSVD, either as individual isolates or mixtures under different agroecological regions with contrasting environmental and climatic conditions. Because DL1b region which is located in the Dry zone of Sri Lanka, has been identified as an agroecological region which is slightly wet during *Maha* season (September to February) and a severe drought severity having agroecological region during the *Yala* (March to August) season (Chitranayana and Punyawardena, 2008).

Fruit Yield

Table 3. Average yield of 10 picks given by the plants under four different treatments at field conditions.

Treatment	Seed Soak method	Soil Drench method
Pseu mix	11.99[a]	8.27[a]
Bacillus mix	8.21[ab]	6.91[a]
Pse +Bacillus mix	10.18[a]	10.44[a]
Control	4.90[b]	4.62[a]
CV(%)	27.39	25.62

Values with the same letter along a column are not significantly different at (P=0.05)

Mixture of *Pseudomonas* isolates and the combined treatment with *Pseudomonas* and *Bacillus* isolates when applied as the SS method gave significantly higher fruit yield than that by the control treatment (Table 3). However, mixture of *Bacillus* isolates when applied by SS method did not give a significantly higher fruit yield than that in control plants. Fruit yield was not significantly different among the treatments when they were applied by SD method (Table 3). Better yield performances of papaya under field conditions have been reported by Ranasinghe *et a*l. (2018) when *P. fluorescens*, *Pseudomonas* spp. and a *Bacillus* sp. were applied as single isolates by seed treatment method.

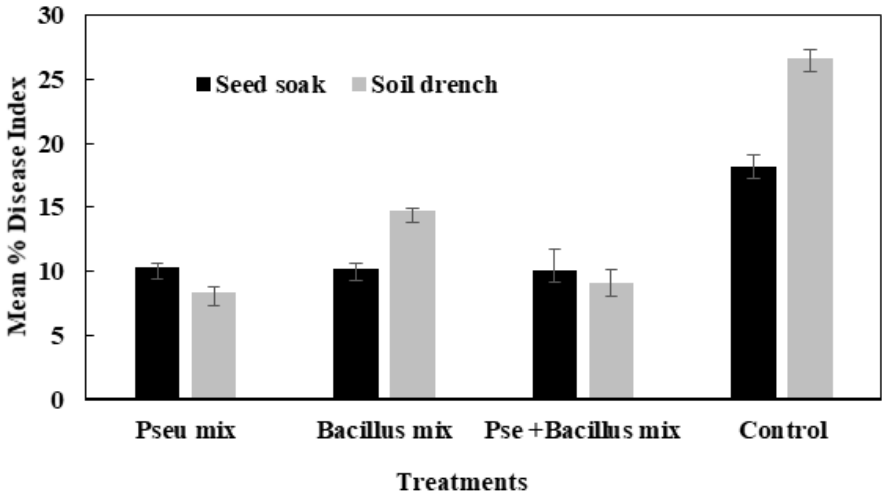

Figure 2: Percentage disease index of fruits due to different treatments of bacterial mixtures by seed soak and soil drench methods. Error bars indicate standard errors of means.

Disease severity of the fruits

Significantly lower percentage disease index was shown on the fruits treated with all the bacterial treatments applied by both methods in comparison to the untreated control (Figure 2). It is worth noting the absence of typical ring spot symptoms on fruits in some plants, even though PRSV symptoms were present on their foliage.

Molecular confirmation of the natural PRSV infection

Seventy-five % of the control-treated plants gave the expected PCR band size of 905 bp when amplified with MB11 and MB12 primers. Results in PCR products were subjected to DNA sequencing and subsequent homology search confirmed the presence of PRSV by giving the highest match with papaya ringspot isolate of Sri Lanka coat protein mRNA (U14741.1) with a 93 % query cover, an E value of 0.00 and a % identity of 93.08.

Peroxidase activity

Peroxidase (POX) activity in leaf tissues was signficantly higher in the plants treated with the mixture of *Pseudomonas* isolates by SS method than that of the other treatments (Figure 3). Among the SS-treated plants, artificially-inoculated PRSV ones had the highest peroxidase activity and the enzyme level elevated significantly from 48 hr after the treatment. In contrast, the plants which were not artificially-inoculated with the virus but treated with the bacterial mixture by SS method, enhanced the peroxidase activity to a significantly higher level, starting from 72 hrs after treatment (Figure 3). By the 144 hr after treatment, SS treated plants which were inoculated with the pathogen and the plants under SS treatment but not inoculated with the pathogen showed 82 and 64 % increase of the peroxidase activity, respectively, in comparison to the rest of the treatments. Damayanthi *et al*., (2007) have observed higher peroxidase activity in pepper plants which were challenge-inocuated with tobacco mosaci virus than the plants which were not inoculated. Eventhough a significant increase of the peroxidase activity was shown by the SD method at 72 hr after treatment in both inoculated and non-inoculated plants (Figure 3), SD method has not shown a successful increase of peroxidase activity thereafter.

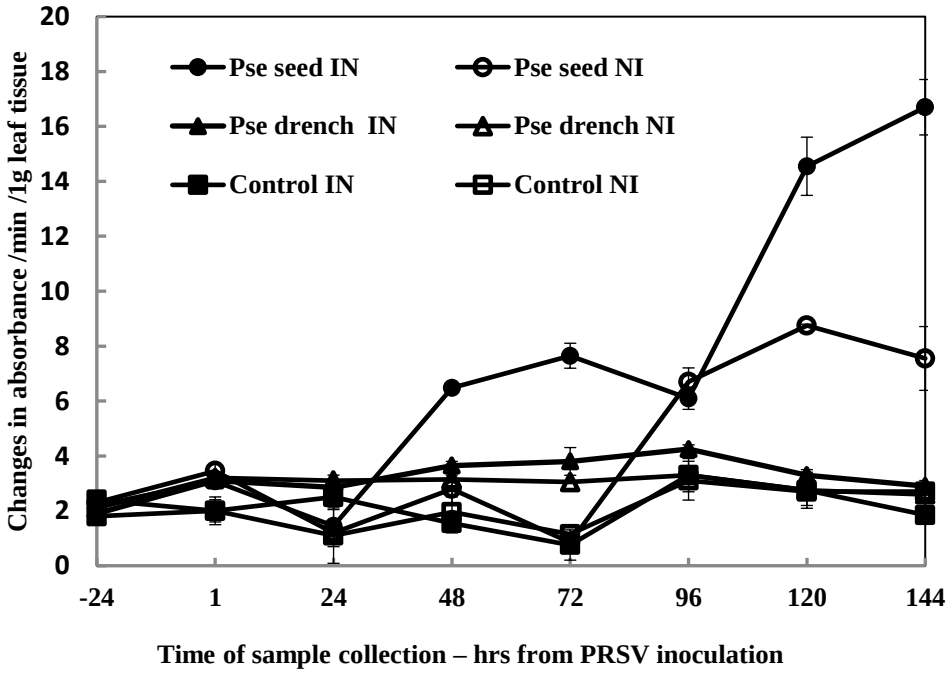

Figure 3: Changes in peroxidase activity in leaf tissues of papaya subjected to a mixture of *Pseudomonas* spp. by SS and SD methods, under challenged and non-challenged conditions by the viral pathogen. Error bars indicate standard errors of means.

POX is a distinguished class of Pathogenesis related (PR) protein and induced in host plant tissues by pathogen infection and reported to be a key enzyme involved in lignification, cross linking of cell wall polysaccharides, oxidation of indole-3-acetic acid, cell elongation regulation, healing of wounds, oxidation of phenolic compounds, and plant defense (Thakker *et al*., 2013). When POX level increases due to the induced systemic resistance, quick synthesis of reactive oxygen derivatives by oxidative burst leads to cell death and inhibition of pathogenic activities (Halfeld-Vieira *et al*., 2006). On par with the findings of the present study, application of *P. aeruginosa* increased the POX activity about 4.89 to 6.49 times when compared to untreated control plants against soya bean stunt virus in soyabean (Khalimi and Suprapta, 2011).

β-1,3-glucanase activity

β-1,3-glucanase activity has increased significantly in plants inoculated with PRSV and treated with the mixture of *Pseudomonas* as a soil drench. In this treatment, enzyme activity has increased significantly compared to all the other treatments, though a reduction of the enzyme activity was observed at 24 hr after the treatment (Figure 4). A significant increase of the enzyme activity was reported in the plants inoculated and non-inoculated with the virus and treated the bacterial mixture as a seed treatment at 144 hr after the treatment (Figure 4). β-1,3-glucanase is a member of the pathogenesis-related protein (PR) family, known to directly destroy pathogen cell walls; its degradation products are oligosaccharides that may subsequently induce disease resistance-related enzymes such as Phenylalanine ammonia lyase (PAL) (Keen and Yoshikawa, 1983). On par with the findings of the present study, Edreva (2005) reported the highest β-1,3-glucanase and viral inhibitory activity in plants treated with *Pseudomonas fluorescens*. High β-1,3-glucanase and peroxidase activities in the leaves treated with *P. fluorescens* and *Bacillus globisporus* culture filtrates reduced the incidence of tobacco necrotic virus in bean plants (Shoman *et al*., 2003).

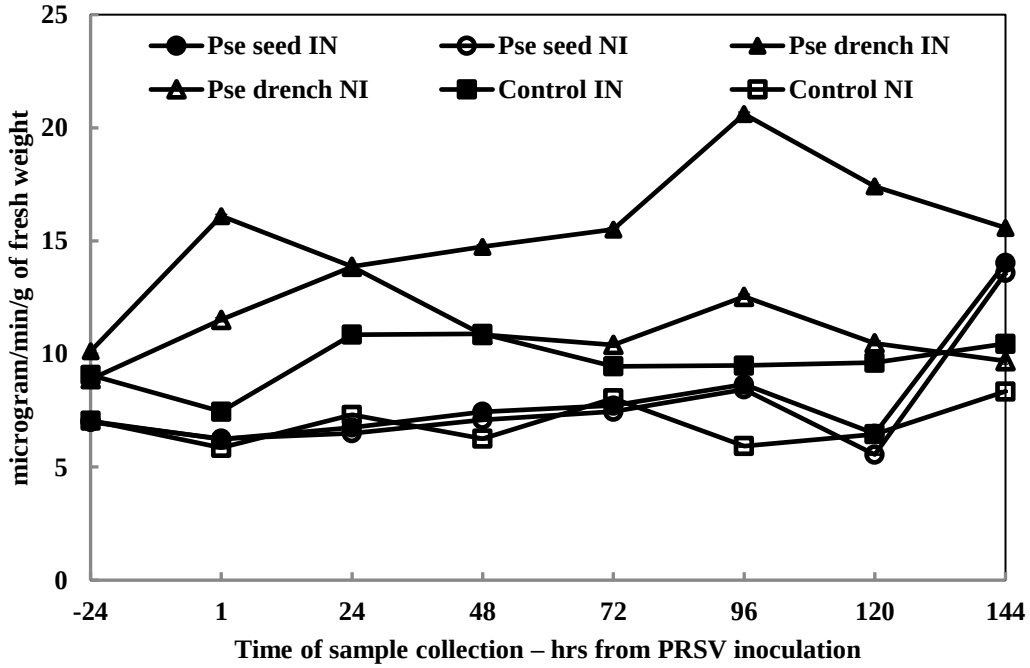

Figure 4: Changes in β-1,3-glucanase activity in leaf tissues of papaya subjected to a mixture of *Pseudomonas* spp. by SS and SD methods, under challenged and un-challenged conditions by the viral pathogen. Error bars indicate standard errors of means.

PAL activity

The highest PAL activity was reported in plants treated with *Pseudomonas* mixture as a seed treatment and inoculated with the virus, from 120 hr after the treatment (Figure 5) and it was significantly higher than the plants under the same treatment but non- challenged with the viral pathogen. The levels of PAL activity in leaves of the plants treated with seed treatment was higher than that of the plants under soil drench treatment (Figure 5). The enzyme PAL initiates the phenylpropanoid pathway, resulting in the biosynthesis of phytoalexins and/or phenolic compounds. Application of PGPR to plants have suppressed the early blight disease in tomato and rice blast pathogen by elevating the activity of PAL, PO, PPO, chitinase, β1,3-glucanase, superoxide dismutase, catalase, lipoxygenase, and phenolics in plant tissues treated with PGPR (Senthilraja *et al*., 2013; Raise *et al*., 2017).

The results revealed that peroxidase and PAL activity was higher when the plants were treated by the SS method, but β-1,3-glucanase activity was higher in plant leaves when treated with the bacteria by SD method, indicating an effect of the method of application of bacterial consortia on enhancement activity of different enzymes. Variation in the disease reduction effects of PGPR have been reported in field trails at multiple locations and due to abiotic factors, such as soil fertility, temperature and moisture (Hol *et al*., 2013). Therefore, method of application of PGPR could have an indirect effect by the above ecological parameters.

In conformity with the present study, Srinivasan and Mathivanan (2011) have reported the efficiency of using consortia of PGPR (i.e. *B. licheniformis*, *Bacillus* spp., *P. aeruginosa* and *Streptomyces fradiae*) to reduce the sunflower necrosis virus disease under field conditions when applied as seed and soil treatments.

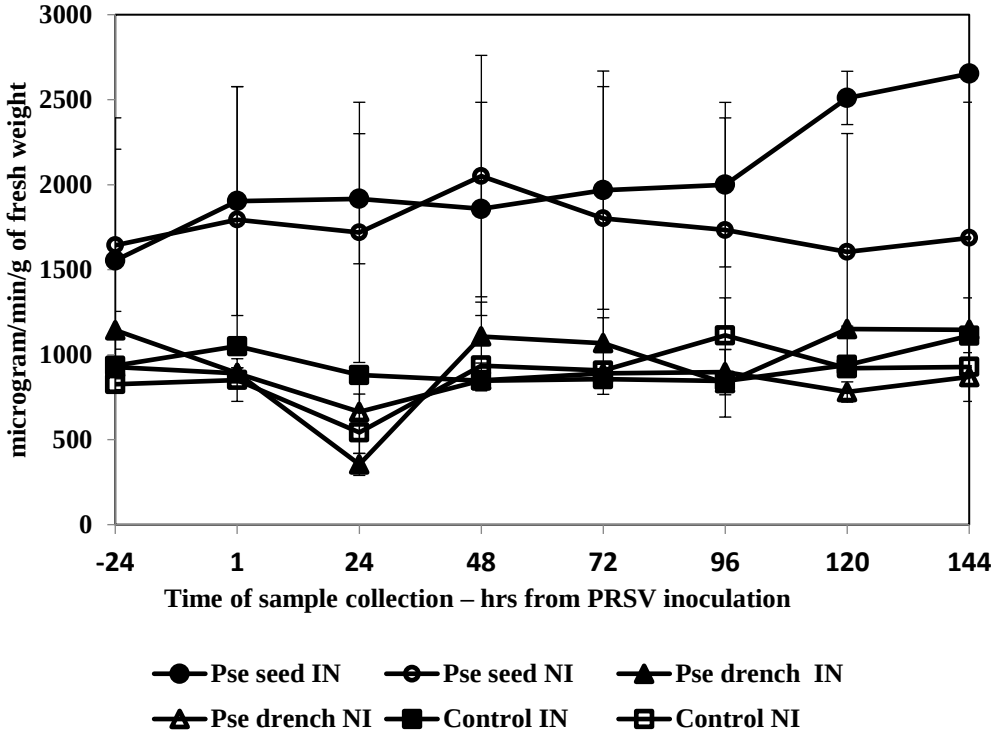

Figure 5: Changes in PAL activity in leaf tissues of papaya subjected to a mixture of *Pseudomonas* spp. by SS and SD methods, under challenged and non-challenged conditions by the viral pathogen. Error bars indicate standard errors of means.

CONCLUSIONS

Application of mixture of *B. cereus*, *Pseudomonas* spp., and *P. putida* by seed soak and soil drench methods reduced the percentage disease index of PRSVD under field conditions in DL1b agroecological zone of Sri Lanka. Soaking papaya seeds by a mixture of *Pseudomonas* spp. increased peroxidase and PAL activity in leaf tissues while drenching the soil with the same bacterial mixture enhanced the β-1,3-glucanase activity in leaves.

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