UTILIZATION OF ROOT-COLONIZING BACTERIA TO PROTECT HOT-PEPPER AGAINST *TOBACCO MOSAIC VIRUS*

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ABSTRACT

Tobacco Mosaic Tobamovirus (TMV) is one of important constraint infecting Solanaceous plants including hot pepper in Indonesia. To accomplish and improve the effectiveness of virus management, we used root-colonizing bacteria (rhizobacteria) which isolated from healthy hot pepper. Eight rhizobacteria isolates were selected and were evaluated their capacity in enhancing plant growth and induce systemic resistance (ISR) against TMV in greenhouse trials. The rhizobacteria was applied as seed treatment and soil drench. Bacterized-seedling showed better growth vigor, fitness and milder symptom than non-bacterized control plants. It suggested that the effect of growth promotion and protection of rhizobacteria against TMV. The protection effect of rhizobacteria was more pronounced after challenged inoculation by TMV, especially for plant treated by isolates I-6, I-16 and I-35. However, the viral accumulation was slightly affected by bacterial treatment. The rhizobacteria treatment elicited ISR might be by increasing peroxidase enzyme activity or not depends on the species. Based on whole results, isolate I-35 was the potential plant growth promotion rhizobacteria (PGPR). The I-35 was identified as *Bacillus cereus* based on morphological characteristics and nucleotide sequences of 16S r-RNA.

Key words: Root-colonizing bacteria, TMV, ISR

Running title : Root-colonizing bacteria to protect hot pepper against TMV

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INTRODUCTION

Hot-pepper is one of the important crops in Indonesia and also several countries in Asia such Malaysia, India, Pakistan, Bangladesh, China and Singapore. One of the production constraint factor is pests and diseases. The main viral disease infecting hot-pepper are *Chilli Veinal mottle Virus* (ChiVMV), *Pepper Veinal Mottle Virus* (PVMV), *Pepper Mottle Virus* (PeMV), *Pepper Severe Mosaic Virus* (PeSMV) and *Cucumber Mosaic Virus* (CMV) (Dolores 1996). In Indonesia, ChiVMV, CMV, TMV and recently *Geminivirus* are important viruses infecting hot-pepper (Sulyo *et al* 1995, Duriat *et al* 1996, Sulandari 2004). Duriat *et al* (1996) reported that TMV infected not only on hot pepper, but also infect tomato, tobacco and egg plant in Indonesia.

TMV is a plant virus which is spread worldwide and infects many horticulture crops. As a member of *Tobamovirus*, TMV genome contains a single-stranded RNA (ssRNA) with rod-shaped and fairly uniformly sized particles. TMV caused heavy yield losses for tobacco, tomato and pepper worldwide (CABI 2005; Sutic *et al*., 1999).

Studies in controlling the TMV infection were conducted intensively on tobacco, by using resistant cultivars, cultural control, sanitary method and biological control

by using satellite TMV pepper or by cross protection using avirulent or attenuated strain of TMV (CABI 2005). Recently, Shin *et al* (2002) reported that they constructed transgenic pepper successfully by transferring the coat protein (CP) gene of ToMV (*Tomato Mosaic virus*) into pepper plant to develop virus-resistant hot-pepper.

Management strategies to control plant viruses in Indonesia were limited on the use of resistant cultivars, culture practices methods. Most farmers rely on chemical insecticides to control the insect vectors. To minimize the use of pesticides and to improve the effectiveness of virus disease control, utilizing of beneficial microbes isolated from plant rhizosphere referred as Plant Growth Promoting Rhizobacteria (PGPR) might offer a promising viral diseases control method. PGPR is defined as root colonizing-bacteria living in the rhizosphere, and distributes on plant root or its close vicinity. Some of these rhizobacteria is beneficial that influence the plant in direct or indirect way, such resulting in a stimulation of plant growth (Bloemberg and Lugtenberg 2001).

PGPR have various ability to induce systemic resistance in plant which provides protection against a broad spectrum of plant pathogens and is reffered as induce systemic resistance (ISR). ISR pathway is induced when plant is challenged by pathogenic organisms (Bloemberg and Lugtenberg 2001). Some PGPR such as *Pseudomonas fluorescens* strain CHAO effective to control *Tobacco necrosis virus* (TNV)

on tobacco (Maurhofer *et al*., 1994), *P. aeroginosa* strain 7NSK against TMV on tobacco (De Meyer *et al*., 1999), *Bacillus subtilis* IN937b and *B. pumilus* strain SE34 against *Tomato Mottle virus* (ToMoV) and against CMV on tomato (Murphy *et al*., 2000; Murphy *et al*., 2003). The resulting elevated resistance due to an inducing agent upon infection of pathogen; ISR is expressed upon subsequent or challenge inoculation with pathogen (Ramamoorthy *et al*, 2001; van Loon, 1997; van Loon *et al* 1998).

Mechanism of ISR mediated by PGPR was through the physical and mechanical strengt of the cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defence chemicals against the challenge pathogen (reveiwed by Ramamoorthy *et al* 2001). Further ISR by PGPR is associated with the pathogenesis-related (PR) proteins (Benhamou *et al*., 1996; Viswanathan and Samiyappan, 1999a), synthesis of phytoalexin and other secondary metabolites (Van Peer *et al* 1991), and increased the activity of pathogenesis-related peroxidase and chitinase protein (Viswanathan and Samiyappan 1999a, b; Ramamoorthy *et al* 2002). It was showed that the use of PGPR is one of promising approaches in controlling plant viruses. Thus, explorations of potential PGPR which is obtained from crop's rhizosphere are required to develop an integrated program for management of plant virus.

In Indonesia resistant cultivars of hot-pepper limited available commercially against either pest or disease. To improve the effectiveness of management of viral diseases, utilization of beneficial microorganism such as rhizobacteria needs to be explored extensively. Studies on PGPR as a bio-control agent to control plant pathogens especially plant virus was not explored very extensively in Indonesia. Exploration of beneficial rhizobacteria which elicit ISR and utilize them more frequent than chemicals, will be useful in Indonesia agriculture. Hence, the objective of this project was to select the ISR elicit rhizobacteria to protect hot pepper against TMV.

MATERIALS AND METHODS

Rhizobacteria Isolates

Rhizobacteria were isolated from healthy rhizosphere of hot pepper cultivated at Darmaga, Bogor, West Java, Indonesia and was cultured on Tryptic Soy Agar (TSA, Difco, USA**)**.Eight isolates rhizobacteria were used : I-1, I-6, I-8, I-16, I-25, I-35, II-5, II-10 and were evaluated based on their ability to enhance plant growth and their ability to protect hot-pepper against TMV infection.

Identification of rhizobacteria.

The potential candidate as a PGPR was identified using Microbact Kit (Medvet Science Pty, Ltd. Australia). Further identification was combined with

sequencing the 16S r-RNA. The primers were specific for prokaryote 16S-rRNA with the forward primer 63f (5'-CAGGCCTAACACATGCAAGTC-3') and the reverse primer 1387r (5'-GGGCGGWGTGTACAAGGC-3') as described previously (Marchesi *et al*., 1998).

The homology and similarity of the nucleotide sequences were analyzed usingWU-Blast2 software providing by EMBL-EBI (European Molecular Biology Laboratory-European Bio-informatics Institute).

TMV Inoculum.

The TMV was propagated on tobacco (*Nicotiana tabacum*). Tobacco was inoculated by infected pepper leaves sap prior gently dusted with Carborundum 600 mesh (Nacalai Tesque, Japan). Infected tobacco leaves were harvested at 10-14 day after infection, then stored in freezer at –80^{o}C for further experimental use.

Plant growth conditions and rhizobacteria treatment.

The experiments were conducted in greenhouse to evaluate the rhizobacteria ability as PGPR to protect hot pepper plants against TMV. Hot pepper seeds (*Capsicum annuum* L. var. TM 999) were soaked in rhizobacteria suspension (10^{9} cfu/ml) for 4 hours, and control seeds were soaked in sterile water. Seeds were then directly sown to sterile growth medium (soil type Latosol : cow dung manure = 2 : 1), without fertilizer application, and watered with tap water routinely.

Two weeks after seedling, plants were transplanted into pots. A week after transplanting, 1 ml (10^9 cfu/ml) of rhizobacteria suspension was added to pots as soil drench treatment.

Plants were grown in greenhouse with humidity and temperature depends on the natural condition. The experimental design used in the experiments were randomized complete design with six plants per treatment and three repeated experiments.

Virus inoculation.

Plants per treatment were mechanically inoculated with infected plant sap (1:10 w/v) in Phosphate buffer pH 7.0 (Merck, Germany) at 2 weeks post transplanting to the pots. The first two leaves on each plant were gently dusted with Carborundum 600 mesh (Nacalai Tesque, Japan) prior to rub-inoculation with sap containing TMV.

Evaluation of plant growth characters

To examine the effect of rhizobacteria on the plant growth characteristics, each plant height was measured from soil line to shoot apex taken 1 day prior to inoculation with TMV and 8 week post inoculation (wpi). Another growth characteristics were number of flowers/fruits (taken as single measure) at 6-8 wpi and fresh weight of above tissues were counted on each plant at the end of experiments. The growth characters data obtained from three repeated experiments.

Disease Assessments.

Disease severity rating was made by using the following rating scales on the leaves : 0 = no symptoms, 2 = mild mosaic symptoms, 4 = severe mosaic symptoms, 6 = mosaic and deformation, 8 = severe mosaic and severe deformation, and 10 = severe mosaic and deformation with stunted growth. Disease severity rating evaluation was performed with mock inoculated plants of treatment as a standard.

Accumulation of TMV in foliar tissues were determined by double antibody sandwich Enzyme-linked immunosorbent assay (DAS-ELISA). Sample leaves were taken at 2 and 4 wpi by collecting of the youngest leaflet from young non-inoculated leaves. ELISA procedure are carried out as manufacture's recommendation (DSMZ; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany).

TMV accumulation was quantitatively measured by using ELISA reader at 405 nm. Positive samples was considered for the presence of TMV when absorbance value was twice of accumulation of healthy control samples.

Extraction and quantification of peroxidase enzyme activities.

To test the effect of bacterized-treatment on plants, peroxidase (PO) enzyme activity was measured by using spectrophotometer method. Extraction and

quantification of PO enzyme activities were conducted at 1 week post-viral inoculation (wpi) according to method described previously (Hammerschmidt *et al*, 1982) with minor modification. Half gram of composite samples of each treatment was added with 1.5 ml of 0.1M phosphate buffer pH 7.0 (Merck, Germany) at 4^{o}C and ground in mortar. The sap was put in the 1.5 ml tubes, then centrifuged at 16.000 g for 15 minutes and the supernatant was used as the enzyme source.

The PO enzyme activity was quantified after addition of 1.5 ml of 5 molal pyrogallol and 0.5 ml of 1% hydrogen peroxide (H_2O_2) into the supernatant. The reaction mixture was incubated at room temperature and the absorbance was counted using spectrophotometer at 420 nm with interval of 30 second for 3 minutes. The enzyme activity was expressed as a change in absorbance $min^{-1}mg^{-1}$ protein. The total protein was measured by using Bradford reagent with bovine serum albumin (BSA; Sigma Aldrich, USA) as a standard. PO enzyme activity was extracted from leaf samples of each treatment as composite samples from three experiments.

Data Analysis

All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Duncan's Multiple Range test (DMRT) (P = 0.05) using SAS

software version 6.13 (SAS Institute, Gary, NC, USA).

RESULTS

Plant growth characteristics in response to Rhizobacteria and TMV

Four tested bacterial isolates (I-6, I-8, I-16 and I-35) showed their ability to enhance plant growth by improving seedling vigor and fitness greater than non-bacterized control (data not shown).

Plant height which measured at 1 day before viral inoculation was visible slightly difference in between bacterized-treated plants and non-bacterized (data not shown). However,bacterized-plants showed vigor, fitness and leaves size greater than non-bact erized control plants since seedling until the end of experiment (Table 1, healthy; data not shown). The differences were more visible when bacterized-plants challenge inoculated with TMV. At 8 wpi, plants treated with isolates I-16, I-25, and I-35 showed significantly different (P=0.05) in height and vigor than those of non-bacterized control plants, while plant treated with I-1, I-8 and II-10 did not showed any difference with non-bacterized control plants respectively (Table 1, infected with TMV).

Number of flower/fruits of healthy bacterized-plants fewer than control plants, however the flowers of control plants were fallen off severely lead the number of fruits fewer than bacterized plants. When plants challenge inoculated with TMV,

198 bacterized-plants still could produce more flowers/fruits greater than non-bacterized
199 control plants (Table1, see I-6, I-8, I-35 and II-5).

200 The aboveground fresh weight of healthy bacterized plants within
201 some treatment tend to be higher, however the difference was not significant (P=0.05). The fresh
202 weight difference was showed by plants treated with I-35
203 and I-16, respectively. Similar results were shown after plants challenge inoculated with TMV
204 (Table 1).

205 Taken together, above results showed that some bacterial treatments able to induce plant
206 growth (Table 1, healthy column). Furthermore, some of bacterial treatment could maintain
207 better plant growth characters than non-bacterized control plants even when infected by TMV
208 (Table 1, infected by TMV column).

209 **Diseases assessments**.

210 The incidence of TMV range from 66.7-100% with initial mosaic symptom presence in
211 control plants at 4-5 dpi, whereas bacterized-plants mostly remained
212 symtompless at that time especially plants treated with I-6, I-16
213 and I-35. The bacterized-plants exhibited phenotype mosaic symptom at 10-14 dpi with
214 symptom less severe than control plants (Table 2), indicating rhizobacteria treatment
215 delayed the incubation time and symptom expressions.

Furthermore, all bacterized-plants showed severity lower than control, especially plants treated by I-6, I-16 and I-35. In addition, some of plants treated with I-6, I-16 and I-35 treatment remained symptompless until the end of the experiment lead to lower incidence than non-bacterized control.

However, the symptom expression did not parallel with the TMV accumulation. The mean ELISA absorbance values for those plants infected with TMV was high at 2 wpi and decreased at 4 wpi. At 2 wpi, all ELISA absorbance of bacterized-plants except for plants treated with I-1, I-8, II-5 and II-10 different and the lowest absorbance value showed by plants treated with I-6 isolates. At 4 wpi showed the TMV accumulation decreased than non-bacterized control, even not different significantly, except absorbance value of plants treated with I-6 (Table 2).

The bacterial treatments increased the peroxidase (PO) enzyme activity in compared to non-bacterized control (Fig.1). After challenge inoculation with TMV some of bacterial treatment increased the PO activity higher than healthy plants (Fig 1).

Identification of Rhizobacteria

Based on the plant growth characters and disease assessments, the potential candidates as PGPR were the isolate I-6, I-16 and I-35. The I-6 and I-35 were gram-positive, whitish colony, produces spores in the center of the cell, and rod shape. The I-16

was gram-negative, whitish colony with rod shape. The nucleotide sequencing of the 16S r-RNA showed the I-6 has 99% nucleotide homology to *Bacillus sp*, the I-35 has 100% homology to *Bacillus cereus*, and I-16 has 99% homology to *Brevibacterium sanguinis*. The I-16 and I-35 were deposited in DDBJ (DNA Database of Japan) with accession no.AB288106 and AB288105.

DISCUSSION

Some of the rhizobacteria isolates used in this study could enhance growth of hot pepper TM-999 resulting of plants vigor and fitness greater than control treatment for some extend. However, the role of rhizobacteria either as growth promotor or as a plant systemic resistance inducer seemed affected by greenhouse environment condition. Since the humidity and temperature being uncontrolled and mostly extremely higher than compare to that of in nature. It affects to the biological activity of the rhizobacteria. The high temperature and humidity caused specific abiotic stress for either plants or rhizobacteria as seen on the blossom flowers. The optimum temperature for hot pepper growth is 24-28°C, while upper temperature affected to the blossom and fruit production (Warintek 2007). In these trials the average of daily temperature was above 32°C. Hence all blossom flowers could not develop into fruits, due to flowers fallen off soon

after the blossom especially for the non-bacterized control plants. However, many flowers from bacterized-plants produced more fruits than control plants even the flower numbers lower than control (Table 1, healthy column).

The effectiveness of biological control using microorganism such rhizobacteria depends on crucial factors such environment condition and soil type. However, some of isolates showed their ability to enhance plant growth subsequent to virus inoculation resulted in milder symptom and some of plants remained symptomless. The protection afforded rhizobacteria-treated plants resulted from the enhancement growth of hot pepper, thereby allowing them to respond to inoculation with TMV. This suggested that rhizobacteria treatment for some extend able to induced plant systemic resistance to overcome TMV infection on hot pepper TM-999.

Zehnder *et al* (2000) previously evaluated the application of *B. subtilis* IN937b, *B. pumilus* SE34 and *B. amyloliquefaciens* IN937a against CMV on tomato. The treatment with those *Bacillus* strains resulted in reduction of severity even the virus titer in the plants was not affected by bacterial treatment; ELISA values as indication of viral titer within the plant was not changed by bacterial treatment. Similar results was shown on TMV in these experiments. It was indicated that rhizobacteria treatment

might not prevent TMV replication. Bacterial treatment might affect the movement of virus and/or the symptom expressions. Alternatively nutritional factors especially nitrogen le vels might serve to offset or mask the symptom. This masking symptom may play role during early stage of systemic infection of rhizobacteria treated plants by TMV when symptoms were delay or not apparent, even though virus accumulation was similar to that of control plants as previously reported by Murphy *et al* (2003) against CMV on tomato.

Some of bacterized-plants increased the PO activity after TMV inoculation, while others were not. It suggested that some of rhizobacteria able to enhanced plant's defense response through elevated PO activity (I-1, I-16, I-35, II-5), while others might PO-independent. The role of polyphenol oxidase enzyme and peroxidase oxidizes phenolics to quinones and generates hydrogen peroxide (H_2O_2). H_2O_2 is an antimicrobial, also releases highly reactive free radicals and further increases the rate of polymerization of phenolic compound into lignin-like substances. These substances are then deposited in cell walls and papillae and interfere with the further growth and development of pathogen (Agrios 2005; Hammond-Kosack & Jones 1996). The result was suggested that some of rhizobacteria isolates (I-16 and I-35)

288 are able to activate the plant's defense response of virus leads to the greater
289 degree of resistance might be by increasing the PO activities, while others might
290 be by PO-independent. However, the increasing of PO activities did not prevent the TMV
291 accumulation, suggested the PO elicit plant's defense response at the early of infection
292 stage rather than viral suppression. Alternatively, the disease suppression afforded
293 by rhizobacteria treatment might be caused by enhancement of plant growth which
294 made plants could increase plant resistance to overcome the virus infection by ISR with
295 PO-independent mechanism which was not covered from these experiments.

296 *Bacillus spp* was known can promote crop health and some strains expressed activities that
297 suppress pests and pathogens (Gardener 2004). In most cases, *Bacillus* spp. that
298 elicit ISR typically elicit plant growth promotion (Kloepper *et al*., 2004) and
299 our results also supported the previously reports (reviewed in Kloepper *et al*., 2004).

300 *B. cereus* was previously reported had
301 activities to suppress pests and pathogens or promote plant growth,
302 while *Brevibacterium* genera had not been reported yet
303 as PGPR. This finding extended the role of *Brevibacterium* in plant
304 disease suppression. Treatment hot pepper seeds and plants with these rhizobacteria might
305 improved the hot pepper health and its productivity might through the promotion

of host nutrition and growth and stimulation of plant host defenses rather than antagonism (Table 1 & 2). The *B. cereus* treatment was able to protect hot pepper and maintained plant growth and production even plants being infected by TMV. Among the three species, the *B. cereus* was the best potential candidate as PGPR for protecting hot pepper against TMV.

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FIGURE

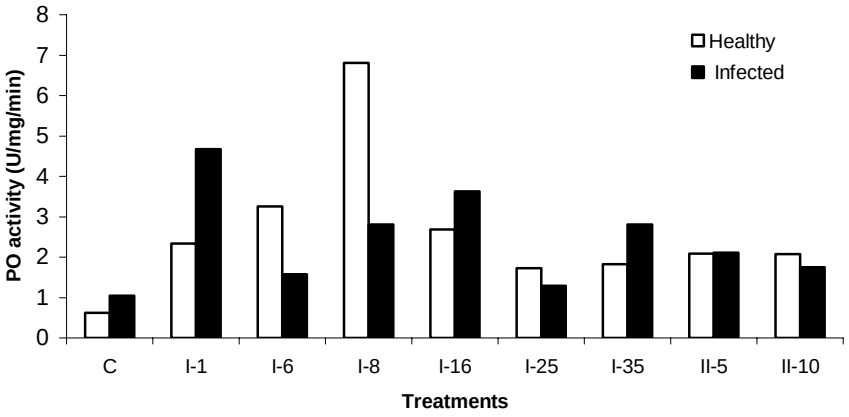

Figure 1. Peroxidase enzyme activity of bacterized-and non-bacterized plants either healthy (white boxes) or challenge inoculated with TMV (black boxes).

TABLES

Table 1. Effect of rhizobacteria treatment on plant growth characters

Treatment	Healthy[a]			Infected by TMV[a]		
	Height at 12 WAT (cm)	Flower/ fruit number	Fresh weight (g)	Height at 8 wpi (cm)	Flower/ fruit number	Fresh weight (g)
C	101.3 ± 6.8b	474.5 ± 4.9a	131.0 ± 3.5 bcd	68.3 ± 9.2e	185.0 ± 5.7 c	110.4 ± 3.4 b
I-1	110.3 ± 5.5ab	322.5 ± 3.5 c	135..4 ± 4.1bc	75.7 ± 12.7de	168.0 ± 8.5c	76.7 ± 5.9 c
I-6	108.7 ± 5.7ab	343.0 ± 32.5bc	119.8 ± 2.5e	82.7 ± 9.3bcd	185.0 ± 14.1c	106.0 ± 7.2 b
I-8	108.0 ± 4.4ab	239.5 ± 7.8d	124.6 ± 4.7cde	79.7 ± 1.5bcde	211.0 ± 2.8bc	105.0 ± 2.3b
I-16	111.0 ± 2.0ab	232.0 ±12.7d	141.1 ± 9.6b	91.0 ± 3.0ab	198.0 ± 14.1c	128.6 ± 3.6a
I-25	111.3 ± 7.2ab	215.0 ± 38.2d	134.3 ± 0.6bcd	90.3 ± 2.1abc	156.0 ± 4.2c	126.9 ± 4.7a
I-35	114.0 ± 5.3a	342.0 ± 2.8bc	153.9 ± 1.3a	97.7 ± 7.2a	314.0 ± 13.4a	134.5 ± 8.1a
II-5	102.0 ± 9.2b	375.0 ± 7.1b	100.9 ± 3.6f	84.0 ± 1.7bcd	248.5 ± 16.3b	112.8 ± 6.9b
II-10	104.0 ± 4.6ab	337.5 ± 23.3 bc	124.2 ± 4.3de	77.7 ± 2.5cde	172.0 ± 1.4c	105.6 ± 5.8 b

[a] Means followed by different letters within a column represent a significant different (α= 0.05) by DMRT

WAT = week after transplanting; 12 WAT = 8 wpi (week after inoculation for TMV infected plants)

Table 2. Enzyme-linked immunosorbent assay (ELISA) values, and severity of hot pepper treated with rhizobacteria and challenged with TMV.

Treatment	ELISA Values[a]		Severity[b]
	2 wpi	4 wpi	
Control	2.283 ± 0.004a	2.235 ± 0.088a	8.0 ± 0.0a
I-1	2.283 ± 0.004a	1.878 ± 0.361ab	6.0 ± 0.0ab
I-6	0.680 ± 0.014e	0.958 ± 0.495b	2.7 ± 3.1b
I-8	2.202 ± 0.005ab	1.589 ± 0.867ab	4.7 ± 1.2ab
I-16	2.005 ± 0.027d	1.550 ± 0.644ab	3.3 ± 3.1b
I-25	2.106 ± 0.057c	1.448 ± 0.931ab	4.7 ± 1.2ab
I-35	2.116 ± 0.035bc	1.592 ± 0.741ab	2.7 ± 2.3b
II-5	2.282 ± 0.010a	1.821 ± 0.653ab	5.3 ± 1.2ab
II-10	2.235 ± 0.088a	1.590 ± 0.908ab	4.7 ± 1.2ab

[a] Absorbance value of ELISA at wavelength 405 nm, Positive = twice of healthy absorbance value. The means of healthy absorbance at 2 wpi = 0.309; and at 4 wpi = 0.285

[b] Means followed by different letters within a column represent a significant different (α= 0.05) by DMRT