JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 853-863

Application of Microorganisms for Induced Resistance in Jerusalem Artichoke (*Helianthus tuberosus* L.) against Stem Rot Caused by *Sclerotium rolfsii* Sacc.

Punyisa Charirak[1], Weerasak Saksirirat[1,2]*, Sanun Jogloy[3] and Suwita Saepaisan[1,2]

[1]Section of Plant Pathology, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Khon Kaen - 40002, Thailand.
[2]Agricultural Biotechnology Research Center for Sustainable Economy, Khon Kaen University, Khon Kaen - 40002, Thailand.
[3]Plant Breeding Research Center for Sustainable Agriculture, Khon Kaen University, Khon Kaen - 40002, Thailand.

(Received: 13 February 2016; accepted: 09 March 2016)

Jerusalem artichoke (*Helianthus tuberosus* L.) is a multipurpose crop grown for bio-fuel, feed additive and functional food products. Stem rot disease caused by *Sclerotium rolfsii* Sacc. is an important threat for Jerusalem artichoke production in the tropics, and disease control using induced resistance strategy is worth exploring. The objective of this study were to investigate the induction of chitinase and β-1,3-glucanase activities in leaves of *H. tuberosus* after treated with microorganisms, *Bacillus firmus* BSR 032, *Trichoderma harzianum* T9 and mycorrhizal fungus, *Glomus clarum* and to evaluate their efficacy on controlling the disease under green house condition. Increase in chitinase and β-1,3-glucanase activity and reduction in disease incidence were observed for the plants treated with these antagonistic microorganisms. The highest and most consistent increase in chitinase and β-1,3-glucanase was observed in the plants treated with *T. harzianum* T9 followed by *T. harzianum* T9+*G. clarum*, and β-1,3-glucanase was found at higher activity than chitinase. The *T. harzianum* T9 could reduced the disease incidence of 44.4%, followed by *T. harzianum* T9+*G. clarum* (22.8%), *B. firmus* BSR 032+*G. clarum* (15%), *B. firmus* BSR032 (8.6%) and *G. clarum* (4.2%), respectively. This *T. harzianum* T9 is a promising antagonistic microorganism for controlling stem rot disease.

Keywords: Antagonistic microorganisms, chitinase, disease control, disease incidence, 1,3-glucanase.

Jerusalem artichoke (*Helianthus tuberosus* L.) is native plant of North America and known as a source of inulin, a dietary fiber that is beneficial to health[1]. Consuming Jerusalem artichoke tubers helps reduce human lipid levels in blood and risks of diabetes and heart disease[2,3]. As a source of carbohydrate, the plant can be also used in production of acetone, butanol and ethanol[4,5]. Jerusalem artichoke is used as a food for human consumption and also feed for animals[6,7,8].

Sugar yield of Jerusalem artichoke under Mediterranean conditions could be as high as 7.48 to 10.82 tons ha^{-1} depending on years[9]. Yield in the tropical regions is rather low because shorter crop cycle, and the crop can be produced all year with irrigation. Stem rot disease caused by *Sclerotium rolfsii* is a devastating threat to Jerusalem artichoke production in the tropics and can cause severe

* To whom all correspondence should be addressed.
E-mail: weerasak@kku.ac.th

yield reduction. The fungus is able to infect both tubers and stems of Jerusalem artichoke[10]. Besides, *S. rolfsii* was reported to infect seed tubers and cause disease outbreak in the next planting season when the environmental conditions favor the growth of fungus[11].

S. rolfsii spreads all over the world both in tropical or temperate areas, and can widely destroy up to 500 species of epiphytes[12]. The disease outbreak of *S. rolfsii* in Jerusalem artichoke was reported in California, USA[10]. Control of the disease can be applied, based on chemicals or antagonistic fungi. Antagonistic fungi such as *Trichoderma* spp. are widely applied as beneficial microorganisms for biological control of various plant diseases. Their modes of action for suppress plant pathogens comprise competition, parasitism and antibiosis[13]. These fungi have been reported to be a high potential in substitution to chemical control[14,15]. Using *T. harzianum* T9 for controlling stem rot caused by *S. rolfsii* in Jerusalem artichoke previously studied[16]. The *T. harzianum* has also extensively studied on control of *Fusarium oxysporum* f.sp. *phaseoli* by seed treatment[14,17]. This fungus showed also the enhancement of been seedling development[18]. The use of antagonistic fungi is not only nontoxic to human beings and environment, but it can also stimulate in induced resistance in other plants[19]. The *Trichoderma* spp. have been investigated to induce resistance against root and foliar diseases of various crops[20,21].

Induced resistance in plants is a procedure occurring after the plant obtains signal chemicals derived from hypersensitive response (HR) or oxidative burst. Plant cells in the area synthesize signal chemicals to stimulate synthesis of secondary metabolite which results in preventing causes of disease. Induced resistance mechanisms are determined by a so called *R* gene (resistant gene). This gene has a role in controlling response of plant to pathogen and synthesize pathogenesis-related (PR) protein[22].

Signal chemicals commonly found are salicylic acid (SA) and jasmonic acid (JA). The SA has a significant role in creating resistance, either specifically or wholly, after plants are attacked by fungi. About 69% of SA found is created and delivered from tissues of the fungus-attacked plants especially from those causing necrosis symptom. Plants produce a large volume of SA from Shikimate pathway and phenylpropanoid pathway procedures and deliver to other parts via phloem[23]. On the other hand, the JA is a signal chemical created by plants that are attacked by insects or pests. When plant cell walls are destroyed or wounded, linolinic acid will be produced by the lipid membrane of cell membranes and converted into JA which is delivered to all parts of plants via phloem. JA will then stimulate *R* gene operations[24]. Some microorganisms such as *Trichoderma* spp. can colonize on root plants and cause induced systemic resistance by trigger the plant to produce PR proteins. These proteins were classified in to various groups. However, a group of them comprise chitinase and β-1,3-glucanase, which able to degrade hyphal wall of fungal pathogens[22]. Normally, the antagonistic microorganisms are not only affected directly to the plant pathogens, but also induce the plant to produce PR protein for defense pathogen invasion. Preliminary test to control stem rot disease of *H. tuberosus* by using soil microorganisms was previously reported by Sennoi *et al*.[25]. However, there is no report on induced resistance in *H. tuberosus* against stem rot disease. Therefore, the objective of this study were to investigate the induction of chitinase and β-1,3-glucanase activities in leaves of *H. tuberosus* after treated with microorganisms, *Bacillus firmus* BSR 032, *T. harzianum* T9 and mycorrhizal fungus *Glomus clarum* and to evaluate their efficacy on controlling the disease under greenhouse condition.

MATERIALS AND METHODS

Plant materials and experimental design

Jerusalem artichoke variety JA 38 was used in the experiment. The variety was recommended for commercial production in Thailand because of high and stable tuber yield and good eating quality as vegetable. The micro-organisms used in the experiment included *G. clarum, B. firmus* BSR 032 and *T. harzianum* T9 were derived from the collection of Plant Pathology Section, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Thailand. *G. clarum* is a species of mycorrhizal fungi found in cultivated and uncultivated soils. *B. firmus* is a species of

bacteria commonly found in soil with ability to produce antimicrobial substances, and *T. harzianum* T9 an effective isolate against fungal plant pathogens[21]. It is used for foliar application, seed treatment and soil treatment for suppression of various disease causing fungal pathogens[20].

Six treatments consisting of *G. clarum, B. firmus* BSR 032, *T. harzianum* T9, *G. clarum+B. firmus* BSR 032, *G. clarum+T. harzianum* T9 and untreated control were assigned in a completely randomized design with three replications, and each replication consisted of one pot or plant. Therefore, there were 18 pots altogether. The experiment was duplicated for four sets to facilitate the sampling of leaves for extraction of proteins at 0, 5, 10 and 15 days after inoculation of the fungi, and, therefore, total experiment had 92 pots or plants.

Cultured microorganisms

Two-week old seedlings were used in this study, and the preparation of the seedlings was described previously[16]. The seedlings were planted in containers with 6 inches in diameter and filled with steamed-sterilized soil.

G. clarum was inoculated in to the soil by placing the inoculum at the bottoms of the pots at the rate of 100 spores/pots, and *G. Clarum* was allowed to grow in the soil for three weeks to enable *G.clarum* to permeate the seedlings. The suspension of *B. firmus* BSR 032 was applied to the soil surface at the optical density (OD) of 0.1 and the suspension of *T. harzianum* T9 was also applied to the soil surface at the concentration of 10^8spores/ml in a volume of 20 ml/pot.

Leaf samples of Jerusalem artichoke were harvested at 0, 5, 10 and 15 days after inoculation of *T. harzianum* T9. Six to seven foliage leaves were harvested from each plant, labeled, placed in a sealable polyethylene bag in an ice box. The samples were further stored in a freezer at-20°C. Harvest of leaf samples for each sampling date was carried out on each set of plants to avoid over harvest. The samples were extracted for crude enzymes and the extracts were analyzed for activity of Chitinase and β-1,3-glucanase.

Determination of proteins in leaf

Leaf samples of 0.5 g were ground in a mortar in liquid nitrogen and filled with 0.1 M of sodium acetate buffer pH 5.0 (sodium acetate trihydrate 13.6 g/ H_2O 1 liter adjusting pH level by1 N acetic acid) in a volume of 1,200 µl. The ground samples were loaded in 1.5 ml microtubes and centrifuged at 4°C and 14,000 rpm for 20 minutes. The supernatants were stored at -20°C until analysis. Analysis of protein concentration was performed according to the method presented by Bradford[26] using bovine serum albumin (BSA) as a standard protein.

Chitinase enzyme assay

Colloidal chitin was used as substrate. The reaction mixture containing 0.1 ml of crude protein, 1% colloidal chitin 1 in 0.1 M acetate buffer pH5.0 in a volume of 0.9 ml was incubated at a temperature of 37°C for 1 hour according to the method described previously[27] with some modifications. The reaction was then stopped by heating with water at 100°C for 20 minutes. Reducing sugar in the supernatant was determined according to the method of Somogyi[28]. One unit of enzyme activity was evaluated by amount of 1 µmol of N-acetylglucosamine equivalent released from colloidal chitin within 1 hour per mg of protein in the reaction mixture.

β -1,3-glucanaseenzyme assay

β -1,3-glucanase activity was evaluated by incubation 0.09% laminarin (β -1,3-glucan, Sigma Co.) solution in 0.1 M acetate buffer, pH 5.0 in a volume of 0.9 ml with 0.1 ml of crude protein at a temperature of 37 ºC for 1 hour as described previously[27]. Enzyme activity was assayed by analyzing reducing sugar (glucose) released from laminarin according to the method of Somogyi[28]. One unit of enzyme activity was evaluated by amount of 1 µmol of glucose equivalent released from colloidal chitin within 1 hour per mg of protein in the reaction mixture.

Data analysis

The enzyme activities of the two enzymes were analyzed statistically with the analysis of variance (ANOVA) and compared means with Duncan's multiple range test (DMRT).

Efficacy test of microorganisms for induced resistance

Preparation of Jerusalem artichoke pot plants

The three-week old seedlings of JA38 were used in the experiment. The seedlings were grafted as described by Yogev *et al*.[29]. Briefly, two seedlings were sliced with a sharp razor in different directions. Care must be taken when cut the stems of the seedlings to avoid damage of the seedlings. The wounds of the two plants were jointed together

and fastened with cellophane tape (Fig. 1). The grafted seedlings were grown in separated pots. After 7 days of grafting, the cellophane tape was removed and the grafted seedlings were ready for the experiment.

Experimental design

The experiment was set up in a completely randomized design with three replications in the greenhouse. Seven treatments consisted of *G. clarum*+*S. rolfsii*, *G. clarum*+*B. firmus* BSR 032+*S. rolfsii*, *G. clarum*+*T. harzianum* T9+*S. rolfsii*, *B. firmus* BSR 032+S. *rolfsii*, *T. harzianum* T9+S. *rolfsii*, *S. rolfsii* and blank control. The experimental unit comprised three grafted plants (grafted plants and stock plants) and there were 63 grafted plants altogether.

Preparation of *B. firmus* BSR 032, *T. harzianum* T9, *G.clarum* and *S. rolfsii*

B. firmus BSR032 was cultured in nutrient agar (PDA) medium and incubated for 48 hours. Bacterial cells were separated from the medium culture with sterilized distilled water to prepare bacterial suspension. Optical density was measured at a wave length of 600 nm and optical density of 0.1 was used in this study.

T. harzianum T9 was cultured in potato dextrose agar (PDA) medium for three days. The fungus was cut into small pieces using cork borer with the diameter of 0.7 cm. Five pieces of the fungus were inserted into polypropylene bag containing boiled sorghum grains. The bag was sealed and stored at room temperature of (28 ±2 ºC) for two days. After 2 days of incubation, the bag was gently shaken to separate the mycelium in the bag, the fungus was further incubated for 7 days until green spores cover all the bag, and the fungus was then ready for the experiment. The preparation of *S. rolfsii* was similar to this method.

G.clarum spore not prepared in the laboratory, but it was kindly provided by the Department of microbiology, Faculty of science, Khon Kaen University. Host plant for propagation of the fungi was corn (*Zea mays*).

Inoculation of *B. firmus* BSR 032, *T. harzianum* T9, *G. clarum* and *S. rolfsii*

For the treatments that involved the inoculation of *G. clarum*, 100 spores of *G. clarum* per pot were inoculated into the soil at the bottom of the pots, and the pots were incubated at room temperature for three weeks. For the inoculation of *S. rolfsii* and *B. firmus* BSR032 and *T. harzianum* T9, these microorganisms were inoculated into the soil surface on the separated pots of pathogen and antagonistic microorganisms, which were separated by a plastic sheet (Fig. 2). *S. rolfsii* was inoculated after 7 days of inoculation of antagonistic microorganisms. Stem rot disease was evaluated and leaf samples from five plants in each plot were separately harvested for extraction of chitinase and β-1,3-glucanase enzymes at 0, 5, 10 and 15 days after inoculation of *S. rolfsii*. The experiment was replicated three times.

Measurement of chitinase and β-1,3-glucanase enzyme activity

Enzyme activity of chitinase and β-1,3-glucanase was determined from 0.5 g of leaf samples, and the method for analysis was described in the above subtitle.

RESULTS

Chitinase activity

Significant differences (P≤0.05) among treatments were observed for chitinase activity at 0, 5, 10 and 15 days after inoculation (DAI) (Table 1). The range of chitinase activity values was between 6.68 units for *B. firmus* BSR 032 at 0 DAI and 13.58 units for *T. harzianum* T9 at 15 DAI. *T. harzianum* T9 and *T. harzianum* T9+*G. clarum* (11.34 units) were significantly (P≤0.05) higher than control (8.76 units) at 0 DAI, and *G. clarum*(9.15 units) and *B. firmus* BSR032+*G.clarum* (8.28 units) were not statistically different from control, whereas *B. firmus* BSR 032(6.68 units) was significantly (P≤0.05) lower than control.

At 5 DAI, all antagonistic microorganism treatments (7.387 units for *B. firmus* BSR 032 to 13.01 units for *T. harzianum* T9+*G. clarum*) were significantly higher (P≤0.05) than control (7.09 units). At 10 DAI, three treatments consisting of *T. harzianum* T9 (11.54 units), *G. clarum* (9.95 units) and *T. harzianum* T9+*G. clarum* (12.73 units) were significantly (P≤0.05) higher than control (8.77 units), whereas *B. firmus* BSR 032(7.74 units) and *B. firmus* BSR032+*G.clarum* (7.42 units) were significantly lower (P≤0.05) than control. At 15 DAI, all antagonistic microorganism treatments (10.07 units for *B. firmus* BSR032+*G. clarum* to 13.58 units for *T. harzianum* T9) were significantly (P≤0.05) higher than control (7.99 units).

It should be noted here that *T. harzianum* T9, *G. clarum* and *T. harzianum* T9+*G. clarum* were consistently higher than control for most evaluation times except at 0 DAI, whereas *B. firmus* BSR 032 and *B. firmus* BSR 032+*G.clarum* dropped at 10 DAI.

β-1,3-glucanase activity

Treatments were also significantly different (P≤0.5) for β-1,3-glucanase activity at 0, 5, 10 and 15 DAI (Table 2). The range of β-1,3-glucanase activity values was from 11.25 units for *B. firmus* BSR 032 at 5 DAI to 42.13 units for *T. harzianum* T9 at 15 DAI. At 0 DAI, *G. clarum* (18.11 units) and *B. firmus* BSR 032+*G.clarum* (17.96 units) were significantly higher (P≤0.05) than control (13.57 units), whereas *T. harzianum* T9 (11.90 units), *B. firmus* BSR 032 (12.76 units) and *T. harzianum* T9 + *G. clarum* (14.59 units) were significantly lower (P≤0.05) than control. At 5 DAI, *B. firmus* BSR 032 (11.25 units) was still lower than control (17.22 units). *G. clarum* (17.46 units) and *harzianum* T9 + *G. clarum* (17.36 units) were similar to control, whereas *T. harzianum* T9 (20.18 units) and *B. firmus* BSR 032+*G.clarum* (19.44 units) were

Table 1. Chitinolytic activity of Jerusalem artichoke in 0, 5, 10 and 15 days after inoculation with *T. harzianum* T9, *Bacillus firmus* BSR 032 and *Glomus clarum*

Treatment	Chitinase activitiy [µmol(GlcNAc)/mg protein/hr] [1/]			
	0 DAI[2/]	5 DAI[2/]	10 DAI[2/]	15 DAI[2/]
T. harzianum T9	10.57 a	10.48 a	11.54 a	13.58 a
B. firmus BSR 032	6.68 c	7.87 ab	7.74 c	11.59 b
G. clarum	9.15 b	10.65 a	9.95 a	10.76 b
T. harzianum T9+*G. clarum*	11.34 a	13.01 a	12.73 a	13.27 a
B. firmus BSR032+*G.clarum*	8.28 ab	8.18 ab	7.42 c	10.07 b
Control	8.76 b	7.09 c	8.77 b	7.99 c
C.V. (%)	27.23	34.42	44.56	30.81

Means in the same column with the same letter are not significantly different (P> 0.05, DMRT).
[1/] enzyme activity 1 unit was defined as amount of reducing sugar 1 µmol (*N*-acetylglucosamine equivalent) released from colloidal chitin per mg protein in one hour.
[2/]DAI = Days after inoculation with concentration of T9 (1×10^8 spores/ml) and BSR 032 (OD 0.1) 50 ml/plant

Table 2. β-1,3-glucanolytic activity of Jerusalem artichoke in 0, 5, 10 and 15 days after inoculation with *Trichoderma harzianum* T9, *Bacillus firmus* BSR 032 and *Glomus clarum*

Treatment	β-1,3-glucanase activity [µmol(Glc)/mg protein/hr] [1/]			
	0DAI[2/]	5DAI[2/]	10DAI[2/]	15DAI[2/]
T. harzianum T9	11.90 c	20.18 a	39.01 a	42.13 a
B. firmus BSR 032	12.76 c	11.25 c	28.77 b	30.22 ab
G. clarum	18.11 a	17.46 b	19.34 c	21.37 bc
T. harzianum T9+*G. clarum*	14.59 c	17.36 b	22.74 b	31.60 ab
B. firmus BSR032+*G.clarum*	17.96 a	19.44 a	19.78 c	25.12 b
control	13.57 b	17.22 b	15.82 c	14.33 c
C.V. (%)	28.46	19.05	13.16	21.22

Means in the same column with the same letter are not significantly different (P> 0.05, DMRT).
[1/] enzyme activity 1 unit was defined as amount of reducing sugar 1 µmol (glucose equivalent) released from laminarin per mg protein in one hour.
[2/] DAI= Days after inoculation with concentration of T9 (1×10^8 spores/ml) and BSR 032 (OD 0.1) 50 ml/plant.

significantly higher (P≤0.5) than control.

At 10 DAI, *T. harzianum* T9 (39.01 units), *B. firmus* BSR 032 (28.77 units) and *T. harzianum* T9+*G.clarum* (22.74 units) were significantly (P≤0.05) higher than control, whereas *G. clarum* (19.34 units) and *B. firmus* BSR 032+*G. clarum* (19.78 units) were similar to control. At 15 DAI, most antagonistic microorganism treatments (from 25.12 units for *B. firmus* BSR 032+*G.clarum* to 42.13 units for *T. harzianum* T9) were significantly higher (P≤0.05) than control (14.33 units) except for *G. clarum* (21.37 units), which was similar to control.

It should be noted here that the values of β-1,3-glucanase activity increased with DAI and antagonistic microorganism treatments had a tendency to increase β-1,3-glucanase activity although it was not clear-cut for some treatments. *T. harzianum* T9 showed consistently higher than control across evaluation times except at the initiation of the experiment (0 DAI).

Effect of antagonistic microorganisms on disease incidence

Significant differences (P≤0.05) among the treatments were observed for disease index, and all antagonistic microorganism treatments were significantly lower (P≤0.05) than control (Table 3). The results indicated that all antagonistic microorganism treatment were effective in reducing disease incidence of *S. rolfsii* in Jerusalem artichoke and the efficacies in reducing disease incidence were also different among these treatments.

T. harzianum T9 was the best treatments for low disease index of 2.78 compared to control (5.0) followed by *T. harzianum* T9+*G. clarum* (3.86), *B. firmus* BSR 032+*G.clarum* (4.25), *B. firmus* BSR 032(4.57) and *G. clarum* (4.79), respectively. *T. harzianum* T9 also had the highest reduction in disease incidence (44.4%) followed by *T. harzianum* T9+*G. clarum* (22.8%), *B. firmus* BSR 032+*G.clarum* (15%), *B. firmus* BSR 032(8.6%) and *G. clarum* (4%), respectively.

Induced resistance to *S. rolfsii* in Jerusalem artichoke

The plants in separated containers were inoculated with antagonistic microorganism and *S. rolfsii* in graft experiment (Fig.1 and Fig.2). The plants inoculated with antagonistic microorganism did not showed disease symptoms of *S. rolfsii*, whereas the plants inoculated with *S. rolfsii* showed wilting. In grating experiment, the planted inoculated with antagonistic microorganisms and those inoculated with *S. rolfsii* were evaluated for β-1,3-glucanase activity. Significant different among the treatments were observed for plant inoculated with antagonistic microorganism and those inoculated with *S. rolfsii* evaluated at 0, 5, 10 and 15 DAI (Table 4). At 0 and 5 DAI, similar results were obtained for plants inoculated with antagonistic microorganisms and those inoculated with *S. rolfsii*. *G. clarum*, *T. harzianum* T9+*G. clarum* and *B. firmus* BSR 032+*G. clarum* were significantly higher (P≤0.05) than control (11.63

Table 3. Disease index in grafting Jerusalem artichoke plant when inoculated with microorganism and *Sclerotium rolfsii*

Treatment	Microorganism[1/]		*S. rolfsii*	
	Di[2/]	Reduction (%)	Di[2/]	Reduction (%)
T. harzianum T9	0	-	2.78 c	44.4
B. firmus BSR 032	0	-	4.57 b	8.6
G. clarum	0	-	4.79 b	4.2
T. harzianum T9+*G. clarum*	0	-	3.86 bc	22.8
B. firmus BSR032+*G.clarum*	0	-	4.25 b	15
control	0	-	5.00 a	-
C.V.(%)	0		37.16	

Means in the same column with the same letter are not significantly different (P> 0.05, DMRT).

[1/] Inoculation with antagonistic before *S. rolfsii* 7 days and after inoculated with *G. clarum* three weeks.

[2/] disease incident rating by rating score 0, 1, 2, 3, 4 and 5 with no symptom to the most severity.

Table 4. β-1,3-glucanase activity (µmol(Glc)/mg protein/hr)[1/] in grafting Jerusalem artichoke when inoculated with microorganism and *Sclerotium rolfsii*

Treatment	0 DAI[2/]		5 DAI[2/]		10 DAI[2/]		15 DAI[2/]	
	Antagonistic microorganism[2/]	*S. rolfsii*	Antagonistic microorganism	*S. rolfsii*	Antagonistic microorganism	*S. rolfsii*	Antagonistic microorganism	*S. rolfsii*
*T. harzianum*T9	11.29 c	12.06 b	13.36 c	14.15 d	21.35 b	21.27 b	38.13 a	40.11 a
B. firmus BSR032	8.56 d	8.746 c	8.046 d	11.14 e	16.31 cd	17.79 c	36.93 a	26.59 b
G. clarum	20.89 a	18.39 a	21.69 a	25.76 a	17.83 c	20.04 b	29.58 b	29.42 b
*T. harzianum*T9+*G. clarum*	20.00 a	19.51 a	19.70 b	17.22 c	26.85 a	26.41 a	40.76 a	41.93a
B. firmus BSR032+*G. clarum*	16.28 b	19.92 a	20.35 ab	20.87 b	17.03 c	16.43 c	25.17 b	29.11 b
control	11.63 c	11.26 b	12.91 c	13.15 d	15.43 d	15.28 c	15.63 c	15.13 c
C.V.(%)	6.59	7.11	27.23	32.86	15.23	20.2	8.17	9.92

Means in the same column with the same letter are not significantly different (P> 0.05, DMRT).
[1/] enzyme activity 1 unit was defined as amount of reducing sugar 1 µmol (glucose equivalent) released from laminarin per mg protein in one hour.
[2/]DAI = day after inoculation with concentration of T9 (1×10^8 spores/ml) and BSR 032 (OD 0.1) 50 ml/plant.

Table 5. Chitinase activity (µmol(GlcNAc)/mg protein/hr)[1/] in grafting Jerusalem artichoke when inoculated with microorganism and *S. rolfsii*

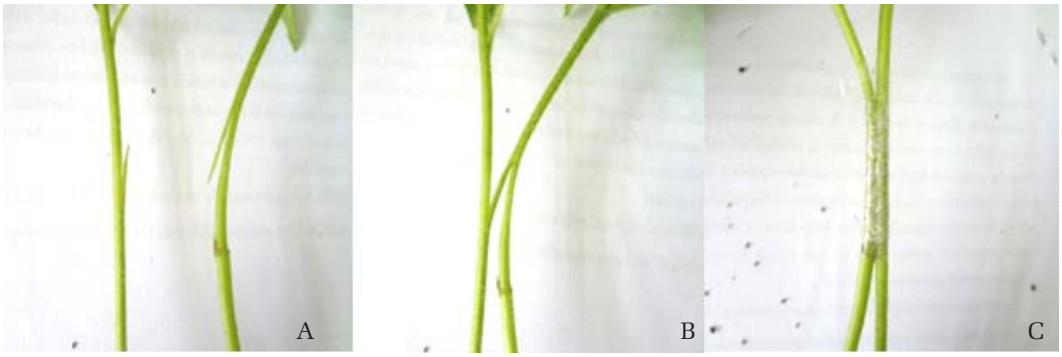
treatment	0 DAI[2/]		5 DAI[2/]		10 DAI[2/]		15 DAI[2/]	
	Antagonistic microorganism[2/]	*S. rolfsii*	Antagonistic microorganism	*S. rolfsii*	Antagonistic microorganism	*S. rolfsii*	Antagonistic microorganism	*S. rolfsii*
*T. harzianum*T9	8.44 b	8.32 c	14.13 b	15.42 c	27.87 a	28.88 a	38.69 a	38.73 a
*T. harzianum*T9+*G. clarum*	6.98 c	13.73 b	12.05 c	12.26 d	23.95 ab	23.84 b	30.85 b	33.66 b
G. clarum	16.78 a	15.54 ab	21.98 a	19.25 b	21.98 b	22.74 b	33.66 b	33.62 b
*T. harzianum*T9+*G. clarum*	17.04 a	16.73 a	21.15 a	23.18 a	20.91 b	22.03 b	37.40 a	38.86 a
B. firmus BSR 032+*G. clarum*	16.03 a	17.93 a	19.12 ab	18.44 b	19.96 b	20.78 b	33.07 b	35.88 b
control	15.30 ab	13.49 b	12.74 c	14.33 c	12.20 c	14.077c	18.417c	17.40 c
C.V.(%)	22.61	21.59	19.55	17.27	19.65	10.72	9.69	8.56

Means in the same column with the same letter are not significantly different (P> 0.05, DMRT).
[1/] enzyme activity 1 unit was defined as amount of reducing sugar 1 µmol (*N*-acetylglucosamine equivalent) released from colloidal chitin per mg protein in one hour.
[2/]DAI = day post inoculation with concentration of T9 (1×10^8 spores/ml) and BSR 032 (OD 0.1) 50 ml/plant

units for 0 DAI and 11.26 units for 5 DAI), and *B. firmus* BSR 032 was significantly lower (P≤0.05) than control, whereas *T. harzianum* T9 was similar to control.

At 10 DAI, the results were almost similar for plants inoculated with antagonistic microorganisms and *S. rolfsii* for β-1,3-glucanase activity. *T. harzianum* T9, *G. clarum* and *T. harzianum* T9+*G. clarum* were significantly higher (P≤0.05) than control, and *B. firmus* BSR 032 was similar to control, whereas *B. firmus* BSR 032+*G. clarum* was higher (P≤0.05) than control for plants inoculated with microorganisms but similar to control for plants inoculated with *S. rolfsii*. At 15 DAI, all antagonistic microorganism treatments were significantly higher (P≤0.05) than control for plants inoculated with antagonistic microorganisms and *S. rolfsii*.

The results were rather different between plants inoculated with antagonistic microorganisms and those inoculated with *S. rolfsii* for chitinase activity at 0 DAI (Table 5). Significant differences (P≤0.05) among treatments were observed for plants inoculated with antagonistic microorganisms. However, most antagonistic treatments (from 8.44 units for *T. harzianum* T9 to 17.04 units for *T. harzianum*T9+*G. clarum*) were similar to control (15.30 units), but *T. harzianum*T9+*G. clarum* (6.98 units) was significantly lower (P≤0.05) than control. For plants inoculated with *S. rolfsii*, *T. harzianum*T9+*G. clarum* (16.73 units) and *B. firmus* BSR 032+*G. clarum* (17.93 units) were significantly higher (P≤0.05) than control (13.49 units). *T. harzianum*T9+*G. clarum* (13.73 units) and *G. clarum* (15.54 units) were similar to control, whereas *harzianum* T9 (8.32 units) was significantly lower (P≤0.05) than control.



A: Inclined slicing the two seedlings in the opposite direction
B: Combine two seedlings by allowing the cut wounds to fit each other well
C: Tightly wrap with plastic sheet.

Fig. 1. Grafting Jerusalem artichoke seedlings

A: the stem rot symptom caused by *Sclerotium rolfsii*
B: healthy plant when inoculated with antagonistic microorganisms and *S. rolfsii*.

Fig. 2. Grafting Jerusalem artichoke inoculated with antagonistic microorganisms (*Trichoderma harzianum* T9, *Bacillus firmus* BSR 032 and *Glomus clarum*) and *S. rolfsii*;

At 5 DAI, there were two treatments what were different between plants inoculated with antagonistic microorganisms and plants inoculated with *S. rolfsii* in grating experiment. *T. harzianum* T9 (14.13 units) was significantly higher (P≤0.05) than control (12.74 units) for plants inoculated with antagonistic microorganism, but it was similar to control (14.33 units) for plants inoculated with *S. rolfsii* (15.42 units). *T. harzianum*T9+*G. clarum* (12.05 units) was similar to control for plants inoculated with antagonistic microorganism, but it

was significantly lower (P≤0.05) than control (14.33 units) for plants inoculated with *S. rolfsii* (12.26 units). Three treatments including *G. clarum*, *T. harzianum*T9+*G. clarum* and *B. firmus* BSR were significantly higher (P≤0.05) than control for both plants inoculated with antagonistic microorganisms and *S. rolfsii*. At 10 and 15 DAI, all antagonistic microorganism treatments were significantly higher (P≤0.05) than control for both plants inoculated with antagonistic microorganisms and *S. rolfsii*.

DISCUSSION

Stem rot disease caused by *S. rolfsii* poses a threat to Jerusalem artichoke production in the tropics. The disease caused yield loss of 60% and also reduces product quality[11]. Screening of Jerusalem artichoke accessions for resistance to the disease yielded unfavorable results as the accessions with high levels of resistance could not be clearly identified[16]. Chemical application is only a reliable means to control the disease. Jerusalem artichoke is known as an inulin containing crop that is beneficial to health[30]. However, this method cannot be used for Jerusalem artichoke production for use as a raw material for functional food products or use as a fresh vegetable as chemicals can be contaminated in the products. More effective methods for disease control of *S. rolfsii* are still required for Jerusalem artichoke production. Therefore, the disease control methods using antagonistic microorganisms to induce resistance to *S. rolfsii* in Jerusalem artichoke is worth exploring.

In this study, three different antagonistic microorganisms could suppress the incidence of stem rot disease in Jerusalem artichoke, and the reduction in disease incidence resulted from the induction of degrading enzyme production in plant. It is well known that the chitinase and β-1,3-glucanase are involved with the resistant property in plants as a pathogenesis related protein (PR protein). These enzymes induced in plants play a role in plant defense mechanism against plant pathogens[22]. Inoculation of *T. harzianum* T9, *G. clarum* and BSR 032 increased activity β-1,3-glucanase and chitinase, and the increase in β-1,3-glucanase was higher than chitinase. However, when the plants were simultaneously inoculated with *S. rolfsii* and the antagonistic microorganisms, the rates of increase in activity of β-1,3-glucanase was higher than chitinase were similar because Jerusalem artichoke was also stimulated by *S. rolfsii*, and, therefore, the increases in enzyme activity of *T. harzianum* T9 and BSR032 at 15 and 5 days after inoculation of *S. rolfsii* were also high. *T. harzianum* T9, *T. harzianum*T9+*G. clarum* had the highest β-1,3-glucanase and chitinase activity, whereas *G. clarum* and *B. firmus* BSR 032 had low β-1,3-glucanase and chitinase activity.

The clear results in this study indicated that *T. harzianum* T9 could reduce the disease incidence of 44.4%, followed by *T. harzianum* T9+*G. clarum* (22.8%). Grafting Jerusalem artichoke could reflex the induced resistance mechanism of two plants as indicated by the increase in enzyme activity after inoculation of the antagonistic microorganisms. Enzyme activities in both grafted plants and inoculated plants increased at nearly the same rate and tended to increase in both plants, especially after of inoculation of antagonistic microorganisms. The increase in enzyme activity after inoculation *S. rolfsii* would be possibly due to the synergistic effect of the pathogenic fungi. Our result is similar to a report of grafting melons in a split-root system, based on side-grafted melon plants for induced resistance against melon with pathogen, *Fusarium oxysporum* f.sp. *cubensis* by using composed fertilizer which helped reduce *Fusarium* wilt in melons grown in soil with pathogenic fungi[29]. In field experiment of evaluation on bioagents for controlling of stem rot in Jerusalem artichoke, the bioagents, *T. viride*, *B. subtilis* and a chemical (sulphated canola oil, sulphex 0.5%) reduced disease incidence and disease severity more than 70.0 and 54.2%, respectively. In addition, yeast (*Saccharomyces cereviseae*), *T. viride*, *B. subtilis* and *Pseudomonas fluorescens* increased chitinase, peroxidase and polyphenol-oxidase activity in Jerusalem artichoke tubers under field conditions in Egypt[31]. This is evidently that the beneficial microorganisms can induce Jerusalem artichoke produces enzymes involving defense mechanism against *S. rolfsii*. In tomato, *T. harzianum* T9 stimulated activity of chitinase and β-1,3-glucanase enzymes at the higher rate than all isolates and the enzyme activity was likely to further increase after 15 days of inoculation of *T. harzianum* T9[21]. The result of the presented study suggests that the *T.*

harzianum T9 was able to continually stimulate the induced resistance, and suitable to be used in controlling of Southern blight (stem rot) of Jerusalem artichoke and other plants. In Jerusalem artichoke, *T. harzianum* T9+*G. clarum* could reduce stem rot incidence caused by *S. rolfsii* and *G. clarum* alone had lower disease incidence than did *T. harzianum* T9 alone[25].

ACKNOWLEDGEMENTS

This work was supported by The Higher Education Research Promotion and National Research University project of Thailand, Office of The Higher Education Commission, Through the Food and Functional Food Research Cluster of Khon Kean University. Thanks are also extended to Assoc. Prof. Dr. Sophon Boonlue, Dept. of Microbiology, Fac. of Science, KKU for supporting the culture of *Glomus clarum*. Mr.Thawan Kesmala for the manuscript preparation. For supply some equipments, the Cultivation and Product Development Research Group of Wild Silkmoths and Economic Insects for Value Added Creation, KKU, is also acknowledged.

REFERENCES

1. Swanton, C.J., Cavers, P.B., Clements, D.R., Mooret, M.L. The biology of Canadian weeds of *Helianthus tuberosus* L. *Canadian Journal of Plant Science*,1992; **72**:1367-1382.
2. Bakers, M., Grose, M., Upite, D., Kaminska, E., Lind, R., Scherbaka, R., Danilevich, A. Carbohydrates in Jerusalem Artichoke powder suspension. *Nutrition & Food Science*, 2007; **37**(1): 42-49.
3. Frese, L., Fuchs, A. Science and Technology of Fructans. Florida, USA: CRC Press, 1993.
4. Cervigni, T. The use of Jerusalem artichoke stalks for the production of fructose or ethanol. *Bioresource Technology*, 1991; **35**(3):247-250.
5. Kosaric, N., Cosentino, G.P., Wieczorek, A., Duvnjak, Z. The Jerusalem artichoke as an agricultural crop. *Biomass*,1984; **5**:1-36.
6. Long, X.H., Chi, J.H., Liu, L., Li, Q., Liu, Z.P. Effect of seawater stress on physiological and biochemical responses of five Jerusalem artichoke ecotypes. *Pedosphere*, 2009; **19**: 208-216.
7. Long, X.H., Mehta, S., Liu, Z.P. Effect of NO3 "N enrichment on seawater stress tolerance of Jerusalem artichoke (*Helianthus tuberosus*). *Pedosphere*, 2008; **18**:113-123.
8. Pilnik, W., Vervelde, G. Jerusalem artichoke (*Helianthus tuberosus* L.) as a source of fructose, a natural alternative sweetener. *Zeitschriftfür Acker-und Pflanzenbau*, 1976; **142**:153-162.
9. De Mastro, G., Manolio, G., Marzi, V. Jerusalem artichoke (*Helianthus tuberosus* L.) and Chicory (*Cichorium intybus* L.): potential crops for inulin production in the Mediterranean Area. *Acta Horticulturae*, 2004; **629**:365-374.
10. Koike, S.T. Southern blight of Jerusalem artichoke caused by *Sclerotium rofsii* in California.*Plant Disease*, 2004; **88**:769.
11. McCarter, S.M., Kays, S.J. Disease limiting production of Jerusalem artichokes in Georgia. *Plant Disease*, 1984; **68**:299-302.
12. Agrios, G.N. Plant Pahtology. 5th Ed. Amsterdam, The Netherlands, Elsevier Academic Press: 2005.
13. Mukherjee, P.K., Horwitz, B.A., Herrera-Estrella, A., Schmoll, M., Kenerley, C.M. *Trichoderma* research in the genome era. *Annual Review of Phytopathology*, 2013; **51**:105-129.
14. Calvalho, D.D.C., Mello, S.C.M., Lobo, Junior, M., Silva M.C. Control of *Fusarium oxysporum* f.sp. *phaseoliin vitro* and on seeds and growth promotion of common bean in early stages by *Trichoderma harzianum*. *Tropical Plant Pathology*, 2011; **36**(1): 028-034.
15. Maciel, C.G., Walker, C., Muniz, M.F.B., Araújo, M.M. Antagonismo de *Trichoderma* spp. *Bacillus subtilis* (UFV3918) a *Fusarium sambucinum* em *Pinusel liottii* Engelm. Revista Árvore, 2014; **38**:505-512.
16. Sennoi, R., Jogloy, S., Saksirirat, W., Kesmala, T., Singkham, N., Patanothai, A. Levels of *Sclerotium rolfsii* inoculum influence identification of resistant genotypes in Jerusalem artichoke. *African Journal of Microbiology Research*, 2012; **6**(38):6755-6760.
17. Calvalho, D.D.C., Lobo Junio, M., Martins, I., Inglis, P.W., Mello, S.C.M. Biological control of *Fusarium oxysporum* f.sp. *phaseoli* by *Trichoderma harzianum* and its use for common bean seed treatment. *Tropical Plant Pathology*, 2014; **39**(5): 384-391.
18. Guimaraees, G.R., Pereira, F.S., Matos, F.S., Mello, S.C.M., Calvanho, D.D.C. Suppression of seed borne *Cladosporium herbarum* on common bean seed by *Trichoderma harzianum* and promotion of seedling development. *Tropical Plant Pathology*, 2014; **39**(5): 401-406.
19. Brunner, K., Zeilinger, S., Ciliento, R., Woo, S.L., Lorito, M., Kubicek, C.P., Mach, R.L.

Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. *Applied Environmental Microbiology*, 2005; **71**(7):3959-3965.

20. Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M. *Trichoderma* species opportunistic, avirulent plant symbionts. *Microbiology*, 2004; **2**: 43-56.
21. Saksirirat, W., Chareerak, P., Bunyatrachata, W. Induced systemic resistance of biocontrol fungus, *Trichoderma spp*. against bacterial and gray leaf spot in tomatoes. *Asian Journal of Food and Agro-Industry*, 2009; **Special Issue**: 99-104.
22. Van Loon, L.C. Concurrent and properties of plant pathogenesis related proteins in plants. In: *Pathogenesis-related proteins in plants* (Datta SK and Muthurishman S, Eds.). Boca Raton, Florida, USA: CRC Press LLC, 1999; pp. 1-19.
23. Hurtado, O. Study and manipulation of the salicylic acid-dependent defense pathway in plants parasitized by *Orobanche aegyptiaca* Pers. M.Sc. Thesis, Virginia Polytechnic Institute and State University, Virginia, USA. 2004.
24. Wasternack, C., Stenzel, I., Hause, B., Hause, G., Kutter, C., Maucher, H., Neumerkel, J., Feussner, I., Miersch, O. The wound response in tomato – Role of jasmonic acid. *Journal of Plant Physiology*, 2006; **163**:297-306.
25. Sennoi, R., Singkham, N., Jogloy, S., Boonlue, S., Saksirirat, W., Kesmala, T., Patanothai, A. Biological control of southern stem rot caused by *Sclerotium rolfsii* using *Trichoderma harzianum* and arbuscular mycorrhizal fungi on Jerusalem artichoke (*Helianthus tuberosus* L.). *Crop Protection* 2013; **54**:148-153.
26. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 1976; **72**: 248-254.
27. Saksirirat, W., Hoppe, H.H. Secretion of extracellular enzyme by *Verticillium psalliotae* Treschow and *Verticillium lecanii* (Zimm,) Viegas during growth on uredospore of the soybean rust fungus (*Phakopsora pachyrhizi* Syd). in liquid cultures. *Journal of Phytopathology*, 1991; **131**:161-173.
28. Somogyi M. Notes on sugar determination. *Journal of Biological Chemistry*, 1952; **195**: 19-23.
29. Yogev, A., Raviv, M., Hadar, Y.,Cohen, R., Wolf, S., Gil, L., Katan, J. Induced resistance as a putative component of compost suppressiveness. *Biological Control*, 2010; **54**: 46-51.
30. Baldini, M., Danuso, F., Turi, M., Vannozzi, G.P. Evaluation of new clones of Jerusalem artichoke (*Helianthus tuberosus* L.) for inulin and sugar yield from stalks and tubers. *Industrial Crops & Products*, 2004; **19**(1):25-40.
31. Eid, K. Field application of some bioagents and safety chemicals to control stem rot disease of Jerusalem artichoke (*Helianthus tuberosus* L.). *Journal of Applied Sciences Research*, 2013; **9**(11): 5825-5834.