1. Introduction

Arbuscular mycorrhiza is soil fungi forming a symbiosis with widespread plant families. AM fungi have a potential to improve plant productivity by providing a plant with nutrient elements as well as by increasing the resistance to abiotic and biotic stresses while AM fungi receive photo-assimilate and some other vital factors from the host plant (Smith and Read 2010). Therefore, the increase of symbiotic interaction between plant and AM fungi would provide more benefits to agriculture. Interestingly, some bacteria have been found to promote a better symbiotic interaction of AM fungi with the host plant. Tyllka et al. (1991) reported that volatile compounds produced by Streptomyces promoted the germination of Glomus mosseae (Now Funnelliformis mosseae). Schüßler and Walker (2010) spores, while the contact between AM spores and this bacterial cell could induce the germination of G. clarum (Now Rhizophagus clarus). Schüßler and Walker (2010) spores (Xavier and Germida 2003). Paenibacillus validus (Now Rhizophagus clarus) also stimulated the mycelial growth of G. intraradices (Now R. intraradices). Schüßler and Walker (2010) (Hildebrandt et al. 2006). Some plant growth promoting rhizobacteria (PGPR) strains, such as Azospirillum sp., Azotobacter chroococcum, Pseudomonas fluorescens, and P. striata, have been reported to enhance mycorrhizal colonization and spore number (Bhowmik and Singh 2004). Vivas et al. (2003) also reported that gram positive bacterium, Bacillus sp. supported R. intraradices for root colonization and arbuscule formation when plants encountered drought stress condition. However, it is still unclear why these bacteria could improve AM fungi colonization with plant. In terms of plant-microbe interaction, although AM fungi can colonize with different plant species (Barker et al. 1998), the AM fungi colonization may be controlled by the host plant defense mechanisms (Gianinazzi-Pearson 1996).

It has been reported that pathogen infection leads to enhance the generation of reactive oxygen species (ROS), which are the molecules involved in signal transduction cascades in plant-invader interaction that activates the defense response genes (Torres et al. 2006). ROS include free radicals, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen (O$_2^-$). Although AM fungi are not classified as plant pathogen, an inducible defense reactions have been found to be activated during the plant-AM interaction, which is varied in each stage of symbiotic interaction and occurred in some parts of the colonized root. For example, Salzer et al. (1999) reported the accumulation of H$_2$O$_2$, a compound generated during the oxidative burst which is one of plant defenses against microbes, in Medicago truncatula roots colonized by the R. intraradices. The accumulation presented within cortical root cells in the space occupied by arbuscules and also in hyphal tips attempting to penetrate a host cell (Lamb and Dixon 1997). However, no H$_2$O$_2$ accumulated in hyphal tips growing along the middle lamella or in appressoria or vesicles. The hypersensitive-like reactions were also observed in Ri T-DNA transformed roots of M. sativa when colonized by Gigaspora margarita (Douds et al. 1998). Not only the oxidative burst was found in the mycorrhizal roots, but also the alteration of ROS-scavenging or antioxidative enzyme activities has also been reported in mycorrhizal root at different stages of symbiosis development. The induction of ROS-scavenging enzymes, such as superoxide dismutases (SODs), peroxidases (PODs), and...
catalases (CATs), is known to be a mechanism to detoxify ROS synthesized in the cell (Wojtaszek 1997; Mittler 2002). The SOD was induced in Phaseolus vulgaris roots colonized by R. clarus at a late stage of the symbiosis under low phosphate (P) concentration and at early stage under high P. However, other antioxidative enzymes including guaiacol peroxidase (GPX) and CAT were induced in mycorrhizal roots at early time point under low P condition, and it was found that the GPX suppresses later (Lambaïs et al. 2003).

In mycorrhizal tomato roots, the Mn-SOD was also observed (Pozo et al. 2002). Therefore, the induction of ROS-scavenging enzymes in plant may be one of the mechanisms to alleviate the defense response and allow the compatible AM fungi to colonize the roots. Nevertheless, alteration of plant defense levels and ROS-scavenging enzyme activities could occur in each symbiotic stage to control the excessive colonization by AM fungi (Breuillín et al. 2010).

In this study, three potential PGPR that have been reported to support growth forage corn were used to investigate the promoting of mycorrhization abilities. Since Brevibacillus sp. strain SUT47 and Acaulospora tuberculata showed a positive relationship, the aim of this research is to investigate the alteration of plant proteins when interacting with AM fungi and SUT47. Moreover, the associated activities of some plant defense-related enzymes change in mycorrhizal roots co-inoculated with and without SUT47 were also determined. The analyses of these differential protein expressions and enzyme activities provide an appearance of which proteins may be involved in the maize roots response to AM co-inoculation with SUT47.

2. Materials and methods

This research was divided into two main parts of the investigation. The first part, we screened three different bacterium isolates and two different mycorrhizal fungi for a positive relationship with respect to maize root colonization in terms of percentage root length colonization and sporulation. The experiment for estimating the levels of mycorrhizal colonization was also performed in this part. The second part was to examine protein production changes using 2D-Gel Electrophoresis technique and also determine the enzyme activity changes for any positive associations found in the first part of this study. Preparation of materials and details of each methodology are described below.

2.1. Plant, AM fungi, and PGPR preparation

Maize (Zea mays Linn) seeds were surface sterilized and placed on sterilized Petri dishes containing moist filter paper. Plates were incubated in the dark at room temperature until the seeds germinated.

Pure culture spores of Claroideoglomus etunicatum (FJ687414) and A. tuberculata (FJ687415) were obtained from School of Biotechnology, Suranaree University of Technology, Thailand and propagated as described by Watanarojanaporn et al. (2011) using maize as the host. The spores were extracted from pot samples using the wet-sieving method at 90 days after inoculation (dai). Spores were rinsed with tap water in a small Petri dish, and healthy spores were collected. The spores were surface-sterilized by incubation for 10 s in 95% (v/v) ethanol, followed by 10 min in a solution containing (in w/v) 2% Chloramine T, 0.02% streptomycin, 0.01% gentamycin and 10 µL Tween 20, and then 6 min in solution containing 6% (w/v) calcium hypochlorite (Budi et al. 1999). Finally, the spores were rinsed 5 times with sterilized water before stored at 4°C.

PGPR including Brevibacillus sp. strain SUT47 (SUT47) (HM453885), Pseudomonas sp. strain SUT19 (SUT19) (HM446471), and Bacillus sp. strain SUT1 (SUT1) (JX198548) were obtained from School of Biotechnology, Suranaree University of Technology, Thailand, and these bacteria have been used to promote growth of forage corn (Pirmyou 2010; Pirmyou et al. 2011). Bacteria were grown in liquid LG medium (Lipman 1904) at 28°C for 2 days to obtain bacterial cell concentration around 10⁸ cells/ml.

2.2. Screening of different PGPR and AM fungi for a positive relationship

The germinated maize seedling was transferred to plastic pot (15 cm diameter and 15 cm high) containing 1:2 (v/v) of autoclaved vermiculite and sand mixture (1 plant per pot). For one seedling, two hundred spores of each AM fungal strain were inoculated on maize roots, and the seeding was also co-inoculated with 1 ml (10⁸ cells/ml) of each PGPR strain on the roots to compare with mycorrhizal plant without PGPR inoculation. Plants were grown in a growth room at 25°C under a 16-h-light/8-h-dark cycle with a light intensity of 150 µmol m⁻² s⁻¹ and fertilized with 50 mL Hoagland’s solution (Hoagland and Arnon 1950) twice a week. Then, the percentage root length colonization and spore number were investigated at 90 dai. The best AM and PGPR in co-inoculation on the promotion of percentage root length colonization and spore number was selected to test in next experiment.

2.3. Determination of percentage root length colonization and spore number

The maize roots were collected and washed with tap water. Roots were incubated in 10% (w/v) potassium hydroxide (KOH) for 1 h at 90 °C in a water bath, and rinsed with tap water three times. Then, roots were covered with 2% (v/v) hydrochloric acid (HCL) for 30 min and HCL were discarded. Trypan blue staining solution 0.05% (w/v) trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) was used to stain roots for 1 h at 90°C in water bath. Verification of AM presence was done on a stereo microscope. Quantification of percent root length colonization was done using the gridline intersect method described by Mycorrhizal associations: the web resource (https://mycorrhizas.info/method.html).

To determine the spore number, the method of International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http://invam.wvu.edu/methods/spores) was used. The AM spores were collected from the vermiculite and sand mixture through 500 and 38 µm pore sieves with running tap water. The content on the bottom of 38 µm sieved was collected in a 50 mL beaker and then around 20 mL was transferred into the 50 mL centrifuge tubes. After centrifugation at 4000 rpm for 10 min, the supernatant was discarded. Then, 20 mL of 60% (w/v) sucrose solution was added into the tube. The tube was vortexed and centrifuged again at 4000 rpm for 10 min. The supernatant containing AM fungal spores was decanted into 38 µm sieves and washed with
running tap water. Then, the spores were placed into a petri dish and viewed under a stereo microscope for counting.

2.4. Estimating the levels of mycorrhizal colonization

In order to estimate the levels of mycorrhizal colonization, the selected AM fungus, *A. tuberculata* and the selected PGPR, SUT47 were used in this experiment. The experiment was designed into 2 treatments including (i) maize inoculated with *A. tuberculata* (AM) and (ii) maize co-inoculated *A. tuberculata* with SUT47 (AM+SUT47). In such a case, 100 spores of *A. tuberculata* and 10^8 cells/ml of SUT47 were used for inoculation into one seedling. Hoagland’s solution was used to water plant in each treatment twice a week. The plant growth condition was adjusted as described above. All treatments were performed in three different biological replicates growing under the same condition. Estimation of the levels of mycorrhizal colonization was investigated at 7, 14, 21 and 30 dai. The plants were harvested completely at each date. For plant at 14, 21, and 30 dai, the primary root was used to gather the 30 one-cm root pieces based on Trouvelot et al. (1986) method’s. All the root fragments (both primary and seminal roots) were gathered to 30 cm for observation at 7 dai due to the small size of roots. The mycorrhizal colonization parameters included frequency of mycorrhiza (F%), intensity of mycorrhizal colonization (M%), arbuscule abundance (A%) in the whole root system, and the intensity of mycorrhizal colonization and the arbuscule abundance in the root fragment as the m% and a%, respectively. The vesicular abundance in the whole root system (V%) and the vesicular abundance in the root fragment (v%) were carried out using the same method for determination the A% and a%. These parameters were calculated based on microscopic observation at the magnification of 200×. The observation of hyphae, vesicles, and arbuscules inside the root in each replication was assessed from 30 pieces of 1 cm root length sample. The mycorrhizal colonization was scored based on the technique of Trouvelot et al. (1986) and calculated by MYCOCALC software (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The scoring scale and calculating formulae were explained in Table S1.

2.5. 2D-Gel Electrophoresis

In order to investigate the plant protein expression changes associated with the co-inoculation of *A. tuberculata* and SUT47, the experiment was designed into 4 treatments with 3 biological replications. The 4 treatments were composed of (i) non-inoculated maize, (ii) maize inoculated with *A. tuberculata* (AM), (iii) maize co-inoculated with SUT47 (SUT47), and (iv) maize co-inoculated *A. tuberculata* with SUT47 (AM+SUT47). The plant growth condition was adjusted in the same way as described above. The plant roots in each treatment at 30 dai were used for analysis. The upper roots (10 cm depth from the crown) were collected for protein extraction. Total proteins were extracted from 3 independent biological replicates of each treatment using 10% (w/v) sucrose in SDS buffer, which modified from the protocol according to Wu, Xiong et al. (2014). The protein profile was analyzed by 2D-Gel Electrophoresis (2DE). The precast 13 cm linear pH 3–10 IPG strips (Immobiline DryStrip gels, GE Healthcare Life Sciences) were rehydrated for overnight with 250 μg/mL of each total protein sample in 250 μL of 8 M Urea, 20 mM DTT, 2% v/v CHAPS, 2% v/v IPG buffer pH 3–10 and Bromophenol blue for the first-dimension isoelectric focusing (IEF). IEF was performed automatically using Etan IPGPhor 3 Isoelectric Focusing System (GE Healthcare), and then IPG strips were immediately equilibrated (Görg et al. 1987). Then, proteins were separated in the second dimension on 12% SDS-polyacrylamide gels electrophoresis (PAGE). The gels were run at 25°C for 10 min at 40 V, and then at 80 V until the dye front reached the bottom of the gels using an Etan DALTSix electrophoresis (GE Healthcare). The gel was stained with 40 mL of Bio-Safe Coomassie G-250 stain (Bio-Rad). The stained gels were scanned with ImageQuant 5.2 software (GE Healthcare) on a Typhoon 9200 scanner (GE Healthcare) with a resolution of 300 dpi. Image analysis was carried out with ImageMaster 2D Platinum software version 7.0 (GE Healthcare, USA) which allows spot detection, quantification, background subtraction and spot matching among multiple gels. Quantification of intensity of each spot was performed in terms of spot volume (area intensity). The total spot volume normalization method was applied in which the percentage of each spot volume on a gel image is calculated relatively to the total volume of all spots on that image. Each analyzed gel was matched individually to the reference gel, and matched spots were grouped into subclasses. Finally, determination of differentially expressed proteins was conducted by comparing the ratio of average relative spot volume with non-inoculated maize. The protein spots were selected based on the following criteria: (i) the spots must appear or disappear in all 3 independent replicates; and (ii) the spot must the significantly different expressed in protein spots (p < .05) with an at least 2-fold change in intensity when compared with non-inoculated maize (control). Proteins of interest were manually excised from coomassie blue stained gel. The interesting spots were digested with the sequencing grade trypsin (20 ng/µl Trypsin; Promega, Madison, WI) as described by Gundry et al. (2009). Digested proteins were identified by the nano liquid chromatography-electrospray ionization quadrupole-time of flight MS (nano-LC–ESI–MS/MS) using an EASY-nLCII spectrometer coupled with a MicroTOF QII (Bruker, Germany). The tandem mass spectra of the tryptic peptides were searched from Mascot database (http://www.matrixscience.com/cgi/search_form.pl?FORMVER = 2&SEARCH = MIS). The precursor and MSMS tolerances were set to ±1.2 and ±0.6 Da, respectively (Niyomploy et al. 2014). The expressed proteins were classified and functionally searched on the PIR (Protein Information Resource) (http://pir.georgetown.edu) and PANTHER (ProteinANalysis Through Evolutionary Relationships) system (http://www.pantherdb.org).

2.6. Antioxidant enzyme activities, salicylic acid (SA) and H$_2$O$_2$ concentration assay

To investigate the plant defense-related compounds and the antioxidant enzyme activities change in response to the co-inoculation AM with SUT47, the four treatments were set as in the proteomic analysis and the plant growth condition was adjusted same as described above. The maize roots in each treatment were used to determine the activities of the antioxidant enzymes, including the total superoxide dismutase (SOD), ascorbate peroxidase (APX) (Enzyme Commission number (EC):1.11.1.11), and peroxidase (POD) (EC:1.11.1.17), as well as the content of salicylic acid (SA) and hydrogen peroxide (H$_2$O$_2$), at 7, 14, 21 and 30 dai.
Determination of APX and POD activities: The frozen roots of 500 mg were ground to the fine powder in liquid nitrogen by a pestle and mortar. The fine powder was moved into the 2 mL microcentrifuge tube. Then, 500 μL of 50 mM potassium phosphate buffer containing 2% (w/v) polyvinylpolypyrrolidone (MW. 360,000) with different pH and EDTA concentrations were added into each sample depending on the enzyme activity assay (pH 7 with 0.5 mM EDTA for APX; and pH 7 with 0.1 mM EDTA for POD) (Krantev et al. 2008; Munoz et al. 2015). The homogenate samples were centrifuged at 12,000×rpm for 30 min at 4°C, and the supernatant was transferred into a sterilized tube and used for subsequent determination of enzymatic activities. The activity of APX and POD was measured by monitoring the change in absorption at 290 and 470 nm, respectively.

Determination of SA content: The SA content in maize roots was measured by adding 1:1 volume of 0.1% aqueous solution of iron trichloride (FeCl₃) into a suspension of maize root extracted using a pestle and mortar in liquid nitrogen. The suspension was centrifuged at 4000 rpm for 10 min, and the supernatant was used to determine the SA content compared with the known amount of pure SA. The series of standard complex solutions (100–1000 μg/ml) were prepared using >99% purity of SA (Sigma-Aldrich) (St. Louis, Mo, USA), and the absorbance was measured at 540 nm by spectrophotometer (Thermo Scientific, USA) (Warrier et al. 2013).

Determination of SOD activity and content of H₂O₂: The SOD activity and H₂O₂ content were determined by total superoxide dismutase (T-SOD) and hydrogen peroxide (H₂O₂) assay kits according to the manufacturing protocol (Elabscience, Wuhan, China).

2.7. Statistical analysis

Statistical analyses were performed using the SPSS software (Version 14.0; SPSS, Chicago, IL) (Colman and Pulford 2011). All measurements on each experiments were subjected to the Kolmogorov–Smirnov test or Shapiro–Wilks test for conformity to a normal distribution before analyses. For the screening experiment, % root length colonized and spore number per plant were analyzed using one-way ANOVA. The mycorrhizal colonization parameters were analyzed using two-way ANOVA with ‘the sampling date’ and ‘SUT47 inoculation’ as fixed factor. The antioxidative enzyme activity, and the SA and H₂O₂ contents experiments were analyzed using three-way ANOVA with ‘AM inoculation,’ ‘SUT47 inoculation,’ and ‘the sampling date’ as fixed factor. The data of mycorrhizal colonization parameters and antioxidiant enzyme activities were square root-transformed or log-transformed to give a normal distribution. Significance level of p < .05 was used for all analyses. For ANOVA analyses, a post hoc Tukey test was performed when significant effects were found. The data presented are means and standard error of means (SEM) of three replicates (one root system per replicate).

3. Results

3.1. Brevibacillus sp. SUT47 promotes mycorrhizal colonization

After 90 days of co-inoculation between each AM fungus and each bacterial isolate on maize root, the percentage root length colonization and number of spores were determined (Figure 1). Brevibacillus sp. strain SUT47 and Pseudomonas sp. strain SUT19 significantly promoted root length colonization of A. tuberculata in approximately 15-20% more than that of plant inoculated with A. tuberculata alone. However, none of the bacterial strains significantly promoted root length colonization of C. etunicatum (Figure 1(a)). Moreover, the mycorrhizal roots co-inoculated with SUT47 were found to have a significantly higher number of spores than that of mycorrhizal roots without bacterial co-inoculation. The spores number of A. tuberculata and C. etunicatum increased around 3.5 and 2.5 folds, respectively when compared with plant inoculated with AM alone (Figure 1(b)). A significant increase of spore number for both AM fungal genera was also found in mycorrhizal root co-inoculated with SUT19. However, the spore number of A. tuberculata and C. etunicatum was still significantly lower than that of mycorrhizal roots co-inoculated with SUT47 around 1.3- and 1.2-fold, respectively, whereas mycorrhizal roots co-inoculated with SUT1 did not affect sporulation of both mycorrhizal genera in this plant host. Therefore, this preliminary screening showed that Brevibacillus sp. SUT47 promotes the A. tuberculata for colonization and sporulation.

To confirm the ability of SUT47 to promote maize root length colonization by A. tuberculata, another set of experiment was conducted to estimate the levels of mycorrhizal colonization by determination of different mycorrhizal colonization parameters at 7, 14, 21, and 30 dai (Figure 2). The result obtained from the two-way ANOVA (Sampling date × SUT47 inoculation) (Supplementary Table S2) clearly showed that the increases of all mycorrhization parameters were mainly influenced by the duration of plant-AM symbiosis interaction at different in each sampling date, while the SUT47 inoculation clearly had a significant impact on the arbuscule and vesicle development (A%,V%, a%, and v%). However, it was found that co-inoculation with SUT47 stimulated the increase in frequency of mycorrhizal colonization (F%) level higher than that of control treatment (without SUT47 co-inoculation) at 7, 21, and 30 dai. The F% increased around 1.4 folds from the control treatment at 30 dai (Figure 2(a)). In the case of the intensity of mycorrhizal colonization in the whole root system (M%) and in a root fragment (m%), these two parameters showed a similar response where the intensity of mycorrhizal roots with SUT47 was significantly enhanced around 1.6 folds at 30 dai (Figure 2(b,c)). In addition, the arbuscule abundances in the whole root system (A%) and in a root fragment (a%) of mycorrhizal roots inoculated with SUT47 were greatly increased. However, no significant difference of A% and a% was observed in mycorrhizal roots with and without SUT47 at 7, 14, and 21 dai. The levels of A% and a% were significantly stimulated around 18 and 14 folds, respectively in mycorrhizal roots with SUT47 at 30 dai (Figure 2(d,e)). In terms of vesicular abundance in the whole root system (V%) and in a root fragment (v%), the levels of these two parameters started to increase after 14 dai. Significant differences in the vesicle abundance (V% and v% levels) of samples were detected at 21 and 30 dai. The mycorrhizal roots with SUT47 showed around 1.9 folds increase in either V% or v% levels when compared to non-SUT47 mycorrhizal roots at 30 dai (Figure 2(f,g)). The increase of arbuscule and vesicle abundances in the mycorrhizal roots co-inoculated with SUT47 at 21 and 30 dai was also correlated to the presence of more arbuscules and vesicles in mycorrhizal roots with SUT47 when observed under compound microscope.
Brevibacillus with A. tuberculata from all treatments are presented in Figure 3. As compared to the 2DE analyses. The 2DE images of root proteins extracted examining the changes of maize root proteins expression in association with inoculation of A. tuberculata and SUT47.

3.2. Alteration of protein profile in response to the co-inoculation of AM with SUT47

To identify the maize root proteins altered by A. tuberculata co-inoculated with SUT47, the proteins extracted from the 30 dai roots of non-inoculated maize (control), maize inoculated with Brevibacillus sp. SUT47 (SUT47), maize inoculated with A. tuberculata (AM), and maize co-inoculated A. tuberculata with Brevibacillus sp. SUT47 (AM+SUT47) were subjected to the 2DE analyses. The 2DE images of root proteins extracted from all treatments are presented in Figure 3. As compared to the protein spots presented in non-inoculated maize (control) (Figure 3(a)), total of 23 up-regulated protein spots and 9 down-regulated protein spots were significantly altered in maize roots inoculated with AM (Figure 3(c)), while 8 up-regulated protein spots and 10 down-regulated protein spots were found in maize roots inoculated with AM+SUT47 (Figure 3(d)) when compared with non-inoculated maize (Supplementary Table S3). Then, 15 spots were selected for further protein identification as the representative protein expression profile based on two criteria, (i) protein must be expressed differently in mycorrhizal roots (AM or AM+SUT47) when compared with non-inoculated roots (control), and (ii) protein must be expressed differently in root inoculated with AM+SUT47 when compared to root inoculated with AM alone. The identified proteins are shown in Table 1. Based on the protein ontology and biological process, the proteins were classified into 4 groups: (i) plant defense response; (ii) ROS-scavenging enzyme; (iii) energy metabolism; and (iv) others. High fold change ratio in protein expression was mainly found in plants treated with AM+SUT47. Protein in the group of plant defense response, the pathogenesis-related protein 1 (PR1) and DIMBOA-UDP-glucosyltransferase (BX9) were highly up-regulated in plant treated with AM+SUT47. Interestingly, the BX9 protein was up-regulated higher in either plants treated with SUT47 or AM+SUT47 than that of plants inoculated with AM alone. On the other hand, the putative uncharacterized protein which has the role in defense response to fungus via hydro lyase activity was down-regulated in plants inoculated with AM+SUT47, but highly up-regulated in plant inoculated with AM alone. Protein in the group of ROS-scavenging enzyme, the superoxide dismutase (SOD_Mn) and ascorbate peroxidase (APX) were highly up-regulated in AM root and higher in AM+SUT47, while these enzymes including peroxidase (POD1), glutathione S-transferase I (GST-I), and APX1-cytosolic ascorbate peroxidase (APx1) were mainly down-regulated in most inoculated plants. Protein in the group of energy metabolism, there were three proteins that had high level of fold change ratio, including the lactoylglutathione lyase (GlxI), the malate dehydrogenase (MDH) and triose-phosphate isomerase (TPI or TIM), while other proteins were altered in the low level. The GlxI protein was up-regulated in all AM or SUT47 inoculated roots, while the MDH and TPI (or TIM) proteins were highly up-regulated in plants inoculated with AM+SUT47. However, the enzyme glutamine synthetase (GLN4) was down-regulated in all inoculated plants. Lastly, other proteins that showed an alteration when compared with non-inoculated plants were the chaperonin (CPN20), actin, and uncharacterized protein containing transferase activity. The CPN20 was up-regulated in plants inoculated with the single inoculum of AM or SUT47, but down-regulated in plants inoculated with AM+SUT47. Nevertheless, the putative actin was down-regulated in all inoculated plants (Table 1). The protein–protein interaction network construct by STRING system was explained in Supplementary Fig. S2.

3.3. Changes of antioxidant enzymes activities and amount of plant defense-related compounds in maize roots associated with AM and SUT47 inoculation

The results of 2DE analysis were again confirmed by measuring the contents of plant defense-related compounds, such as H2O2 and SA, as well as determining some antioxidant enzyme activities (Figure 4). Based on these results, the Three-way ANOVA (Supplementary Table S4) indicated that the alteration of antioxidative enzyme activities and the contents of H2O2 and SA in maize roots were significantly influenced by the factor of sampling date and the AM inoculation. Although, inoculation with SUT47 had an impact on most parameters, the alteration of APX activity in maize roots was not influenced by SUT47. Starting with the H2O2 content, the obvious differences of H2O2 content were found in 7 dai maize roots. Maize roots inoculated with
SUT47 and inoculated with AM were found to produce higher H2O2 content than that of control (non-inoculated maize roots). Interestingly, the H2O2 content in root inoculated with AM+SUT47 was significantly lower than that of other treatments at 7 dai. Then, the H2O2 content in all treatments was fluctuated during 14, 21, and 30 dai, but no apparent difference among the treatments was observed during these d. However, all treatments of inoculated maize roots were found to produce H2O2 lower than that of control at 30 dai (Figure 4(a)). For the SA production at 7 and 14 dai, the non-inoculated roots contained similar SA content with mycorrhizal roots (AM), and these levels were significantly higher than those of roots inoculated with AM+SUT47 and with SUT47 alone. At 21 dai, SA contents in all inoculated maize roots were significantly reduced when compared with non-inoculated roots. Then, the mycorrhizal roots (AM)
tended to accumulate SA in a high level at 30 dai, while the SA content in AM+SUT47 roots was significantly lower than other treatments (Figure 4(b)). For the total SOD activity, all inoculated maize roots were found to produce higher level content than that of control. Interestingly, the SOD activity in AM+SUT47 root was significantly lower than that of root inoculated with AM alone at 7 dai. Then, the fluctuation of SOD activity in all treatments was found during 14, 21, and 30 dai. However, at 30 dai, all treatments of inoculated roots had a lower level of SOD activity than that of control (Figure 4(c)). In the case of APX, an obvious increase of activity was found after 21 dai. The APX activity detected in control roots was lower than that of mycorrhizal roots (AM), while roots inoculated with AM+SUT47 and with SUT47 alone showed the highest APX activity at 30 dai (Figure 4(d)). A clear difference of POD activity among the treatments

Figure 3. The representative 2DE images of maize roots. (a) non-inoculated (control), (b) maize roots inoculated with SUT47 (SUT47), (c) maize roots inoculated with A. tuberculata (AM), (d) maize root co-inoculated A. tuberculata with SUT47 (AM+SUT47), and (e) the expanded regions of selectively differentially expressed protein spots. Spot number refers to spot identified in Supplementary Table S3.
Table 1. Identified proteins of maize root extracts differentially expressed after the inoculation with AM and SUT47.

| Spot No. | Top hit protein               | Gene symbol | Protein Ontology/Biological Process                                      | Organisms | No. of peptides matched | Mascot score | Mass (kDa) | Isoelectric point (PI) | Fold change ratio* |
|----------|-------------------------------|-------------|-------------------------------------------------------------------------|-----------|-------------------------|--------------|------------|------------------------|-------------------|
| 0        | Putative uncharacterized protein | Sb07g006390 | Hydro-lyase activity / defense response to fungus Sorghum bicolor |           | 6                       | 223          | 24.5       | 9.27                   | 44.67             |
| 1        | Pathogenesis-related protein 1 | PR1         | Cellular component                                                    | Z. mays   | 1                       | 55           | 15.3       | 4.38                   | 5.67              |
| 2        | Superoxide dismutase          | SOD_Mn      | Superoxide dismutase activity                                          | Z. mays   | 10                      | 466          | 25.6       | 6.71                   | 24.33             |
| 5        | Ascorbate peroxidase          | APX         | Peroxidase activity                                                   | Z. mays   | 15                      | 524          | 27.5       | 5.67                   | 28.00             |
| 6        | Triosephosphate isomerase     | TPI         | Isomerase activity                                                    | Z. mays   | 5                       | 223          | 26.9       | 5.12                   | 5.67              |
| 7        | Lactoylglutathione lyase      | GlXI        | Lactoylglutathione lyase activity                                      | Z. mays   | 17                      | 684          | 35.3       | 6.62                   | 18.00             |
| 12       | Malate dehydrogenase          | MDH         | Malate dehydrogenase activity                                         | Z. mays   | 4                       | 268          | 35.7       | 7.63                   | 5.67              |
| 30       | Glutamine synthetase          | GLN4        | Catalytic activity                                                    | Z. mays   | 8                       | 342          | 39.6       | 5.24                   | 0.14              |
| 19       | Chaperonin                    | CPN20       | ATP binding                                                            | Z. mays   | 7                       | 364          | 25.8       | 7.72                   | 1.42              |
| 21       | Putative actin family protein isoform 1 | Actin | ATP binding                                                            | Z. mays   | 13                      | 477          | 42.0       | 5.24                   | 0.59              |
| 18       | Uncharacterized protein -     |             | Transferase activity                                                  | Z. mays   | 5                       | 284          | 19.5       | 5.14                   | 4.26              |

Fold change ratio: AM/SUT47

Note: - vanished protein.

*The fold change ratio of one protein was obtained from the protein abundance ratios between the un-inoculated and inoculated treatments.
was found in sampling date at 14, 21, and 30 dai. The POD activity of the non-inoculated roots tended to decrease after 14 dai. At 21 dai, the POD activity highly increased in AM roots, while the highest POD activity was detected in AM+SUT47 roots at 30 dai. Maize roots inoculated with SUT47 alone showed the lowest level of POD activity during 7–21 dai, while the activity tended to increase at 30 dai similar to the AM+SUT47 roots (Figure 4(e)).

4. Discussion

Here, different PGPR were investigated of their ability to promote maize root colonization by two AM fungal genera. Not all PGPR strains promote maize root colonization in our study. *Brevibacillus* sp. SUT47 and *Pseudomonas* sp. SUT19, but not *Bacillus* sp. SUT1, were found to enhance root colonization only by *A. tuberculata*. However, SUT19 and SUT47 could increase the spore number of both *A. tuberculata* and *C. etunicatum*. Therefore, PGPR strain is one of the factors that affect mycorrhization and sporulation. However, other factors may also be involved in this interaction. The AM fungal specie should also be considered since the root colonization of *C. etunicatum* did not increase when co-inoculated with our tested bacteria. However, it might depend on the plant host preference of this AM fungal specie. As reported by Pivato et al. (2009), ability of mycorrhiza development in roots was influenced by host plant, specie of bacteria, and specie of AM itself. Sylvia et al. (2003) proposed that the colonization and spore production ability also depend on the genetic control of host plant and the AM symbiont.
Moreover, Siqueira et al. (1984) and Bartolome-Esteban and Schenck (1994) reported the influence of pH on the adaptation, spore germination, root colonization, and hyphal growth of AM, where each fungus varied in pH tolerance. Thus, the ability of the fungus species to tolerate different pH also plays a predominant role on plant root infection and sporulation by AM. Hence, the compatibility of plant host, AM, pH, and specie of PGPR should be considered. In addition, the levels of plant nutrient may influence on the symbiosis interaction. In our experiment, the full strength Hoagland’s solution was used to fertilize the plant, although the effect of PGPR on promoting mycorrhization was clearly found when tested under low nutrient conditions (Valdenegro et al. 2001). In this case, we have tested the effect of nutrient levels on the ability of SUT47 to promote sporulation of A. tuberculata in maize roots. The results showed that the plant nutrient levels (full- and low-strength Hoagland’s solution with normal and low P contents) did not significantly affect the number of spore produced in mycorrhizal roots with SUT47 (preliminary data). It is possible that A. tuberculata could tolerate in several levels of soluble phosphorus. Please note that the presence of soluble fertilizer might affect the growth of AM as well. A. tuberculata may have ability to tolerate moderately in high soluble phosphorus than that of C. etunicatum.

The ability of Brevibacillus sp. SUT47 to promote maize root colonization by A. tuberculata was confirmed by estimation the levels of intraradical colonization. The high F% value in mycorrhizal roots indicates the high frequency of roots that are infected by AM. It is logical to assume that the high frequency of root length colonization occurs from the high number of spore germination and high mycelium development. Thus, it is possible that SUT47 may also promote the spore germination or hyphal growth of A. tuberculata and lead to an increase of the frequency of mycorrhizal root colonization. It has been proved that B. brevis improves in vitro spore germination and mycelial growth of F. mosseae even under the presence of heavy metal (Vivas et al. 2005). However, further experiments are needed to prove the direct role of SUT47 on these aspects. Interestingly, although SUT47 could clearly promote the colonization of AM in maize roots especially at 30 dai (Figure 2), the presence of arbuscules and vesicles was observed under microscope since 7 dai at a low value of abundance (Supplementary Fig. S1). These results indicated that A. tuberculata was fully established in maize roots since 7 dai and continuously develop their colonization along the period of time.

The proteomic analyses were performed with maize roots at 30 dai. The results demonstrated that some proteins involved in plant defense response, ROS-scavenging enzymes, and energy metabolism were mainly changed in mycorrhizal maize roots in consequence of the symbiosis (Table 1). However, these proteins might have been a secondary effect of changes in plant minerals or carbon nutrition during the symbiosis. The proteomics analysis presented in Bona et al. (2016) also suggested that proteins involved in energetic metabolism were altered along the maize seed development either with or without AM inoculation. Therefore, we focused on the groups of plant defense response and ROS-scavenging enzymes since alteration of proteins in group of energy metabolism may receive the main effect from changing in plant nutrition. From the results of protein classification, protein in the group of plant defense response, PR1, which is the protein produced in plants in the event of a pathogen attack and induced as part of systemic acquired resistance (SAR) in many plant species (Biliou et al. 2000), was up-regulated in mycorrhizal roots and highly up-regulated when co-inoculated with SUT47 (AM+SUT47). Since the cell wall components of AM are similar to fungal pathogen, PR1 protein is induced to control the AM infection. However, AM must deal with the plant immune system for successful colonization of the host plant. Jung et al. (2012) proposed that once AM has established, the plant must regulate the level of fungal spread in the root to prevent excessive colonization and carbon drainage to maintain the level of mutualism. Moreover, BX9, which is an enzyme that transforms DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoazin-3-one), an alkaloid toxic to bacteria, fungi, and insect into toxic or non-toxic forms (Song et al. 2011), was also up-regulated in a high level in maize roots treated with SUT47 and AM+SUT47. It is possible that SUT47 might contain some elicitors that induce this enzyme when inoculated onto maize roots. On the other hand, the putative uncharacterized protein which has the role in defense response to fungus via hydro-lyase activity was down-regulated in roots treated with AM+SUT47, but highly up-regulated in roots treated with AM alone. These results suggested that proteins involving in the plant defense response were differently respond to AM and SUT47 inoculation in maize roots at 30 dai. Protein in the group of ROS-scavenging enzymes, the superoxide dismutase (SOD_Mn) and ascorbate peroxidase (APX) were highly up-regulated in mycorrhizal roots and higher in the roots co-inoculated AM+SUT47, while other proteins were mainly down-regulated. ROS are usually generated in normal metabolic processes, such as a result of electron transport chain in mitochondria. In case of the plant-AM symbiosis, plant must sustain a balance between ROS generation and ROS-scavenging pathways to allow AM colonization (Lehr et al. 2007; Wu, Zou et al. 2014). Thus, up-regulation of some ROS-scavenging enzymes in mycorrhizal roots may be required to cope with the accumulation of ROS in plant cells. However, Bona et al. (2016) presented that the proteins linked to ROS response were up-regulated in mycorrhizal maize roots during seed development and maturation stages, which were also conferred as a response to plant physiological and environmental changes. More discussions about plant defense response and ROS-scavenging enzyme activities are described below.

Differences in plant defense-related compounds and ROS-scavenging enzyme activities were produced dominantly at 7 and 30 dai. Several reports have shown the different levels of ROS production and induction of antioxidative enzyme activities upon the plant-mycorrhizal symbiotic stages. Most studies have shown that the H2O2 level was increased in the late phase of symbiosis. Hause et al. (2002) found an increase of ROS and jasmonate production at the later stage of symbiosis with barley roots, and Salzer et al. (1999) also observed the accumulation of H2O2 in the cell containing arbuscules at late stages of mycorrhizal development. Nevertheless, the induction of MrRBOHE3 which is involved in ROS generation occurred at early hyphal root colonization of M. truncatula by AM (Kiiro et al. 2012). In our study, the H2O2 content in mycorrhizal maize roots increased at 7 dai, while the level decreased lower than non-inoculated roots at 30 dai. It was notified that mycorrhizal roots with SUT47 (AM+SUT47) produced lowest level of H2O2 at 7 dai (Figure 4...
For SA content, the level of SA content has been proposed as the first induction in the presymbiotic state and a second induction at later stages of root colonization probably to control the extension of colonization by AM (Blilou et al. 1999; Gallou et al. 2012). In our experiment, the SA contents of inoculated maize roots were lower than that of non-inoculated maize at 7 dai, while the SA content in mycorrhizal roots increased again at 30 dai at the level higher than non-inoculated one (Figure 4(b)). It is possible that the induction of SA level also depended on the AM and plant species. Fernández et al. (2014) found that only F. mosseae induced the SA-related pathway in tomato and soybean. However, no effect was observed in these two plants when infected with R. irregularis. Interestingly, the levels of SA in maize roots inoculated with AM+SUT47 was again lower than that of other treatments in all sampling dates similar to H2O2 content (Figure 4(b)). Since the negative effect of SA on mycorrhizal colonization has been described (García-Garrido and Ocampo 2002; Medina et al. 2003), further investigations are required to prove how SUT47 is involved with AM in the lowering of SA as well as H2O2 levels in maize roots.

In terms of ROS-scavenging enzyme activities, the total SOD activity was observed in this experiment since several isoforms of SOD were present in plant. The results showed that induction of SOD activity in all inoculated maize roots was higher than that of non-inoculated roots at 7 dai, and the SOD activity in mycorrhizal roots was reduced to the level similar to non-inoculated roots at 30 dai (Figure 4(c)). The induction of SOD in the presence of AM has been reported to depend on the compatibility, the stage of mycorrhizal development, and plant growth condition. Lambais et al. (2003) indicated that more infective AM induced high SOD activity in bean roots at late stage of symbiosis under low P condition, while the activity was induced at an early stage under high P condition. In the cases of POD and APX activities, higher levels of these enzyme activities were found in mycorrhizal maize roots at 30 dai, where the levels were higher than that of non-inoculated roots (Figure 4(d, e)). It has been shown that AM initiated the plant defense response in plant roots. AM infection was reported to increase activity of chitinase (Spanu et al. 1989). However, the chitinase activity and other plant defense responses decreased after reaching a maximum in mycorrhizal alfalfa roots (Volpin et al. 1994). Spanu and Bonfante-Fasolo (1988) also reported the presence of cell-wall-bound peroxidase (POD) activity in Allium porrum L. roots during AM development in planta. The POD activity was higher in the infected plant than in non-infected during the initial stage of AM penetration. However, the activity decreased when AM was established and highly colonized in roots. Moreover, the transient induction of CAT and ascorbate peroxidase (APX) activities were reported in tobacco roots coinciding with the stage of appressoria formation in the root surface (Blilou et al. 2000). Thus, high activity of POD and APX induction in mycorrhizal maize roots at 30 dai suggested that the maize roots were associated with higher intensity of AM colonization in maize roots than that of 7 dai. The induction of POD and APX activities or other plant defense responses may occur from the possible presence of new colonized roots that could alter the enzyme activity.

Our results present the possible evidence that SUT47 can promote colonization of AM and spore production in maize roots. SUT47 co-inoculated with AM could also enhance the frequency of mycorrhizal colonization. The protein expression at 30 dai and the alteration of plant defense response at different periods of time demonstrate how plant responds to AM infection in the presence of SUT47, which is related to the levels of the intraradical colonization. These alterations of protein expression and development of AM in plant roots could be changed if the incompatibility between plant-AM-PGPR occurs. Thus, these results may be a clue to further explore the direct mechanisms of PGPR that interact on plant and AM to increase the colonization and sporation efficiency.

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