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Full Length Research Paper

The FTIR spectroscopy investigation of the cellular components of cassava after sensitization with plant growth promoting rhizobacteria, *Bacillus subtilis* CaSUT007

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To evaluate the response of cassava stakes to plant growth promoting rhizobacteria, *Bacillus subtilis* CaSUT007, the changes in cellular compositions and phytohormone were investigated using the fourier transform infrared (FTIR) and high-performance liquid chromatography (HPLC) approach. The objective of this study was to test the hypothesis that CaSUT007 stimulates production of plant cellular components and phytohormone involved in metabolism and growth development mechanisms. Cassava stake treated with CaSUT007 or with sterile distilled water were germinated in sterile soil, after incubation for 28 days, CaSUT007 treated cassava stakes had more lateral root, longer roots, shoot length and greater biomass than the control which enhanced more than 1.3 fold of the cassava's phytohormone as indole-3-acetic acid content of non-treated control. We also focused on plant cellular composition and cassava stake tissues from the two treatments were harvested for FTIR analysis. FTIR analyses revealed that higher accumulated of lipid in response to the strain CaSUT007. The cassava stake treated with the beneficial bacteria *B. subtilis* strain CaSUT007 showed the higher content of the lipid content as (shown in the spectral regions of CH stretching and CH bending mode associated with cell membrane structure lipids) when compared with those of the cassava stake treated with distilled water. Our results initially demonstrated that CaSUT007 can enhance plant growth under greenhouse conditions by direct stimulation of plant lipid and phytohormone as indole-3-acetic acid production.

Key words: Plant growth promotion, *Bacillus subtilis* CaSUT007, cassava, cellular composition, FTIR spectroscopy.

INTRODUCTION

The beneficial bacteria, *Bacillus* spp. are widely used as commercial bacteria for control of plant pathogens and enhance plant growth promotion. In addition to directly affecting plant growth and development through plant growth regulator, *Bacillus* spp. can colonize roots and trigger plant biochemical and physiological systems to promote the growth enhancement. The plant growth

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promoting rhizobacterium (PGPRs) as *Bacillus amyloliquefaciens* strain KPS46, can enhance growth in several economic crops such as soybean, vegetable soybean, corn, rice, Chinese kale and cauliflower etc. (Prathuangwong and Kasem, 2004; Prathuangwong et al., 2005; Prathuangwong and Buensanteai, 2007), this process mediated in part by the excretion of phytohormones such as auxin and indole-3-acetic acid (IAA), lipopeptides and extracellular proteins (Buensanteai et al., 2008a). This could account for interacts with plants that the Bacilli group can synthesize phytohormones similar to the plant endogenous growth regulator and enhanced those levels in plant which involved in the initial processes of lateral and adventitious root formation and elongation (Buensanteai et al., 2008a, 2008b; Erturk et al., 2010). In regards to the latter mode of action, there is no information as to the proteome expression activated or the growth and development responses triggered in cassava upon treatment with PGPR as Bacilli group.

Cassava, (*Manihot esculenta* Crantz) of the family Euphorbiaceae, is a major field crop in Asia, especially Thailand, but there are have no studies on the use of PGPR to enhance growth in cassava stake. However, enhanced plant growth promotion studies using the strains of *Bacillus* spp. on model plants revealed that bacilli can trigger signalling pathways leading to the growth promotion phenotype (Idriss et al., 2002). Plant response to PGPR requires alterations in the physiology and biochemistry level that directly and indirectly result from the modification of genes and proteins expression (Wan et al., 2005; Buensanteai et al., 2009). Such PGPR-induced modifications may lead to the accumulation of certain metabolites and alterations in the level (increase or decrease) or the presence (appearance or disappearance) of some cellular proteins and hormones (Erturk et al., 2010; Wan et al., 2005). The growth and development of plants are the process by which a plant increases in the number, size of leaves, stems, root, and changing from one growth stage to another. The result of plant growth and development is production and the amount harvested of plant yield.

Fourier transform infrared (FTIR) spectroscopy is known to propose the high ability for understanding the total cellular and biochemical components of organism and micro-organism cells (Szeghalmi et al., 2007), because of all the bio-organic and cellular composition functional groups absorb specific infrared wavelength. Moreover, there are several publications on the application of this FTIR technique to detect changes in metabolic processes of carbohydrates and lipids under the different stress conditions (Kamnev, 2008; Szeghalmi et al., 2007). FTIR spectroscopy is also a rapid, versatile, and sensitive tool that has been used for elucidating the structure, physical properties and interactions of carbohydrates (Kacurakova and Wilson, 2001). The carbohydrates show high absorbance in the region 1200–950 cm^{-1} that is within the so-called fingerprint region, where the position and intensity of the bands is specific for every polysaccharide (Kacurakova and Wilson, 2001). FTIR spectroscopy may therefore be used to evaluate carbohydrate changes and profiles in plants exposed to biotic and abiotic stresses. For example, FTIR spectroscopy was used, in association with chemometrics and automatic variable selection, in metabolic fingerprinting of salt-stressed tomatoes (Johnson et al., 2003) and grapevine (Oliveira et al., 2009).

The aim of this study was to determine whether or not strain CaSUT007 can interact directly with plants to cause growth promotion. In our experiment, the cassava was chosen as an economic crop model. The changes in the cellular and some phytohormone composition of the cassava cell treated with the *B. subtilis* CaSUT007 were assessed by using the FTIR and HPLC. By selecting the FTIR procedures related to PGPR-enhance growth promotion, our report will displayed novel perspectives in the use of FTIR in the change of the cellular composition enhanced by beneficial bacteria as Bacilli group.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in cassava experiments included *B. subtilis* strain CaSUT007. Cells of the strain CaSUT007 stored in nutrient glucose broth with 10% glycerol at -80°C were revived by streaking onto nutrient glucose agar (NGA) and cultured at 28 ± 2°C for 48 h. The strain was transferred to 500 ml of nutrient glucose broth (NGB) containing 2% glucose and incubated for 48 h at 28 ± 2°C with constant shaking at 180 rpm. Cells were collected and washed twice by centrifugation at 13,000 rpm for 20 min (Beckman model JS13.1) and the bacterial cell pellet was washed three times in sterile saline (0.85% NaCl). The cells were re-suspended in sterile distilled water, cell concentrations were determined turbidimetrically and adjusted to optical density of 0.2 at 600 nm, corresponding to 1 × 10^8 CFU ml^{-1} (Buensanteai et al., 2008a, 2008b).

Plant materials and treatment

The stake of cassava were surface disinfested by treatment with 95% ethanol (v/v) for 2 min, followed by soaking in 20% commercial bleach (v/v) for 20 min. The stakes were then washed with sterile distilled water 5 times in order to remove the bleach. Before planting, 30 g of cassava stakes were mixed thoroughly with 5 ml of a liquid treatment for 15 min. The cell concentration in the whole culture and cell suspensions were adjusted to 1x10^8 cfu ml^{-1} on the basis of absorbance. The experiment conducted under greenhouse conditions, treated cassava stake were placed onto sterile soil and place into the plastic pot. There were four replicate pots per treatment with one cassava stake per pot. The pots were maintained in a greenhouse with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 200 umol m^2s^{-1}, and constant temperature of 24°C. At 28 days after germination, cassava stakes were harvested for measurement of growth parameters (root and shoot lengths; fresh and dry weights; and numbers of lateral roots).

The experiment was performed three times. Finally, for cellular components and phytohormone analysis, cassava stakes from

untreated (control) and CaSUT007 treated plants were used from 28 days old stakes.

Extraction and measurement of endogenous indole-3-acetic acid from cassava stakes by HPLC

The extractions of endogenous phytohormone, indole-3-acetic acid were carried out from cassava stake tissues using the slightly methods described by Majcherczyk et al. (1986) and Buensanteai et al. (2009). Plant tissues were homogenized with acetonitrile containing 2, 6-ditertiary butyl-p-cresol to extract indole-3-acetic acid. Partitioning with chloroform was used for hormone clean-up. Fractions corresponding to indole-3-acetic acid were separated using the HPLC. Methanol (500 µl) was added as diluents and only 6 µl were injected onto the analytical RP-HPLC. The conditions of the analysis were the mobile phase was methanol containing 0.05% acetic acid/water (20/80 v/v), linear gradient over 30 min to the final concentration 70/30 (v/v), the flow rate was 1 $mlmin^{-1}$, wavelength was 255 nm, indole-3-acetic acid content was calculated using indole-3-acetic acid corresponding on the standard curve.

Cassava cellular composition measurement using FTIR

Dried cassava stakes were ground in a crystal mortar and pestle. FTIR sample preparation and measurements were performed according to Kamnev et al. (2008). In brief, 1 mg of the resulting dry biomass in a micro sampling cup lightly presses the surface of the powdered sample with a flat glass spatula and mounting the sampling cup into the sample holder of the FTIR spectrometer (Tensor 27). The Infrared spectra were collected using the Attenuated Total Reflectance (ATR)-FTIR Spectroscopy with single reflection ATR sampling module with and coupled with MCT detector cooled with liquid nitrogen over the measurement range from 4000-600 cm^{-1}. The measurements were performed with a spectral resolution of 4 cm^{-1} with 64 scans co-added (Bruker Optics Ltd, Ettlingen, Germany).

Spectra from each group were analyzed using Principal Component Analysis (PCA). Individual spectra from each group were analyzed using PCA to distinguish different chemical components of the samples using the Unscrambler 9.7 software. (CAMO, Norway). The spectra were processed using 2nd derivative and vector normalized by the Savitzky-Golay method (3^{rd} polynomial, 9 smoothing points) and then normalized using Extended Multiplicative Signal Correction in the spectral regions from 1750-850 cm^{-1}.

Unsupervised hierarchical cluster analysis (UHCA)

UHCA was performed on second derivative spectra using Ward's algorithm which utilizes a matrix defining inter-spectral distances to identify the most similar IR spectra. Spectral distance was calculated as D-values. Ward's Algorithm tries to find homogeneous groups as possible. This means that only two groups are merged which show the smallest growth in heterogeneity factor *H*. Instead of determining the spectral distance, the Ward's Algorithm determines the growth of heterogeneity *H*. *n(i)* was the number of spectra merged in the *i* cluster . *H(r,i)* was calculated according to the following equation:

n(p) was the number of spectra which are merged in the *p* cluster
n(i) was the number of spectra which are merged in the *i* cluster, and
n(q) was the number of spectra which are merged in the q cluster.

The spectral distance between the new *r* cluster and the *i* cluster was calculated as follows;

$$D(r,i) = \frac{[n(p) + n(i)].D(p,i) + [n(i) + n(q)].D(q,i) - n(i).D(q,i)}{n+n(i)}$$

RESULTS

Effect of CaSUT007 on growth parameter of cassava stakes

The strain CaSUT007 was effective in promoting the growth of cassava stakes under greenhouse conditions, the length, weight and lateral root of the stakes were measured after 14 days of planting. These strain, when applied to cassava stake, increased shoot lengths, by more than 35% (Figure 1a), and increased dry weights by more than 90% (Figure 1b) compared to the distilled water control. Cassava stakes treatment with the strain CaSUT007 also increased the stake germination and number of lateral root more than 30% and more than 90%, respectively when compared to the control treatment (Figures 1c and 1d). Similar results were obtained when the experiment was repeated. In order to investigate the effects of CaSUT007 at the early stage of plant growth and development, the cassava stakes of untreated and CaSUT007-treated stakes were excised, the cellular composition and phytohormone accumulation levels were determined, and cellular components and biochemical phytohormone analysis were conducted.

Indole-3-acetic acid increased in the cassava stakes after sensitization with CaSUT007

The indole-3-acetic acid was measured to determine whether this phytohormone accumulates in enhance growth promotion mechanism. Cassava stakes grown in greenhouse condition amended with the CaSUT007 had significantly ($P < 0.05$) higher endogenous indole-3-acetic acid levels than those plants grown in the control treatment in all plant growth parameter. As the CaSUT007 treated, the indole-3-acetic acid of the cassava stakes increased (Figure 2), the indole-3-acetic acid was approximately more than 1.3 fold compared to the distilled water control.

Changes in cassava stake cellular components in response to CaSUT007 treatment using FTIR analysis

In this current study, the FTIR spectroscopy was performed in order to explore the cellular and biochemical changes of cassava plant after sensitization with the beneficial bacteria *B. subtilis* strain CaSUT007. The FTIR spectra of cassava plant reflect the cellular components of the cell wall and membrane such as polysaccharides, proteins secondary structure and lipid content. The conformational change of protein amide I noted between 1700-1600 cm^{-1} can give information of protein secondary

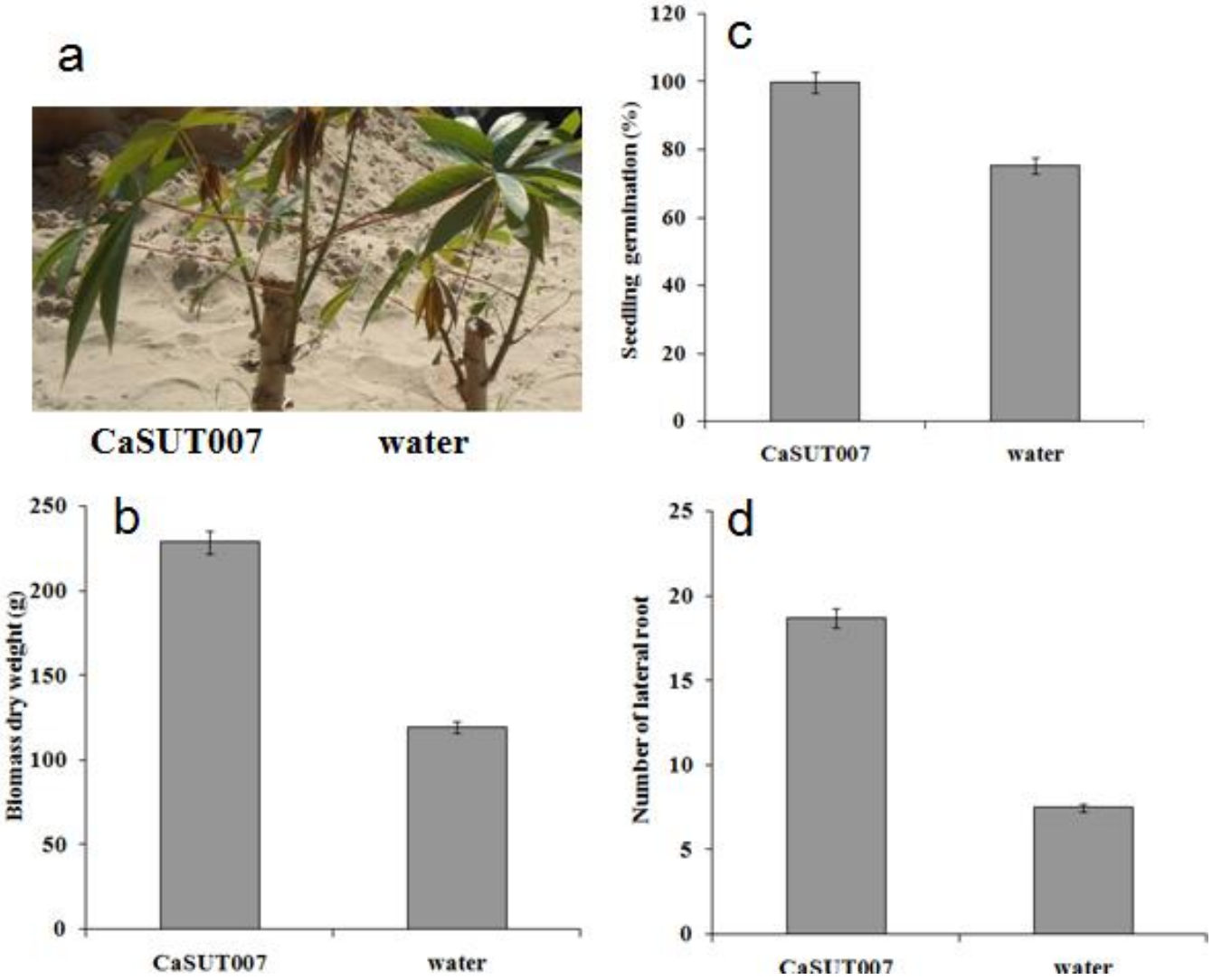

Figure 1. Effects of PGPR *Bacillus subtilis* CaSUT007 on the growth and development of cassava stake under greenhouse conditions, as measured at 14 days after inoculation. (a) cassava stake under greenhouse conditions in sterile soil, (b) percent of stake germination, (c) dry weight, (d) number of lateral root. The data are the average of four replications (three plants per replication) for each treatment. Error bars represent the standard deviation. For each growth parameter, different letters indicate significant differenced ($P \leq 0.05$) among treatments.

structure such as alpha-helix (centered at 1653 cm^{-1}), beta-sheet (centered at 1635 cm^{-1}), beta-turn (centered at 1685 cm^{-1}). The conversion of the original spectra to their second derivatives was used in order to find the exact peak locations and reveal spectral shifting and intensity variations among spectra. Indeed, the second derivative transformation of FTIR spectra made the differences in two spectral regions more distinctive when cassava stake plants treated with CaSUT007 were used. Our results indicated that the average FTIR spectra of cassava plants (Figure 3) treated with distilled water and the beneficial bacteria *B. subtilis* strain CaSUT007 were difference in biochemical components upon plant growth promoting bacteria sensitizaton. The cassava stake treated with the beneficial bacteria *B. subtilis* strain CaSUT007 shows the higher content of the lipid content as shown in the spectral regions of CH stretching (3000-2800 cm^{-1}) and CH bending mode (1467 cm and 1373 cm^{-1}) associated with cell membrane structure lipids compared with those of the cassava stake treated with distilled water. The spectra showed in Fig. 4 indicated that there are variations in polysacharide component. Clearly, the higher content of polysaccharide in the spectral region of C-O-C stretching (1150-900 cm^{-1}) represent under distilled water negative control when compared with cassava stake treated with the beneficial bacteria *B. subtilis* strain CaSUT007.

Moreover, the multivariate statistical analysis techniques

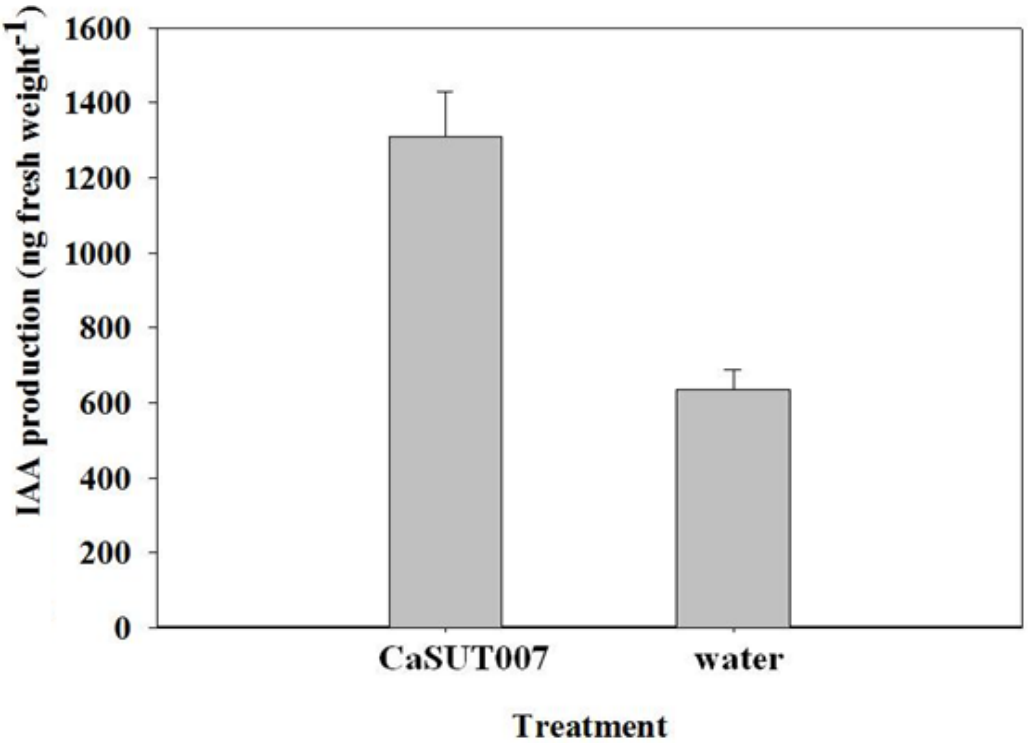

Figure 2. Effect of PGPR *Bacillus subtilis* CaSUT007 on indole-3-acetic acid content in the stake of cassava. Cassava stakes were treated with or without *Bacillus subtilis* CaSUT007, and the indole-3-acetic acid content was measured 28 days after inoculation by HPLC. The data are the average of four replications (three plants per replication) for each treatment. Error bars represent the standard deviation. For each growth parameter, different letters indicate significant differenced ($P \leq 0.05$) among treatments.

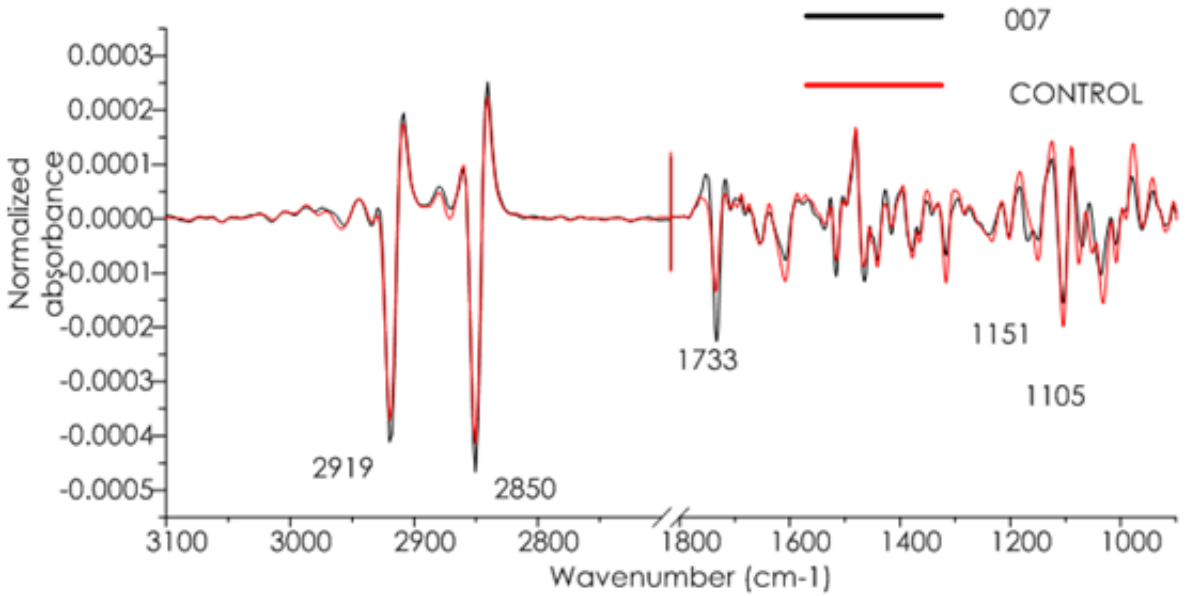

Figure 3. Average second derivative FTIR spectra of treated with the beneficial bacteria *B. subtilis* strain CaSUT007 and distilled water in the region of (a) 1800-850 cm^{-1} and (b) 3000-2800 cm^{-1}. Spectra were measured with 64 scans co added for each individual spectra. Spectra were pre-processed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range.

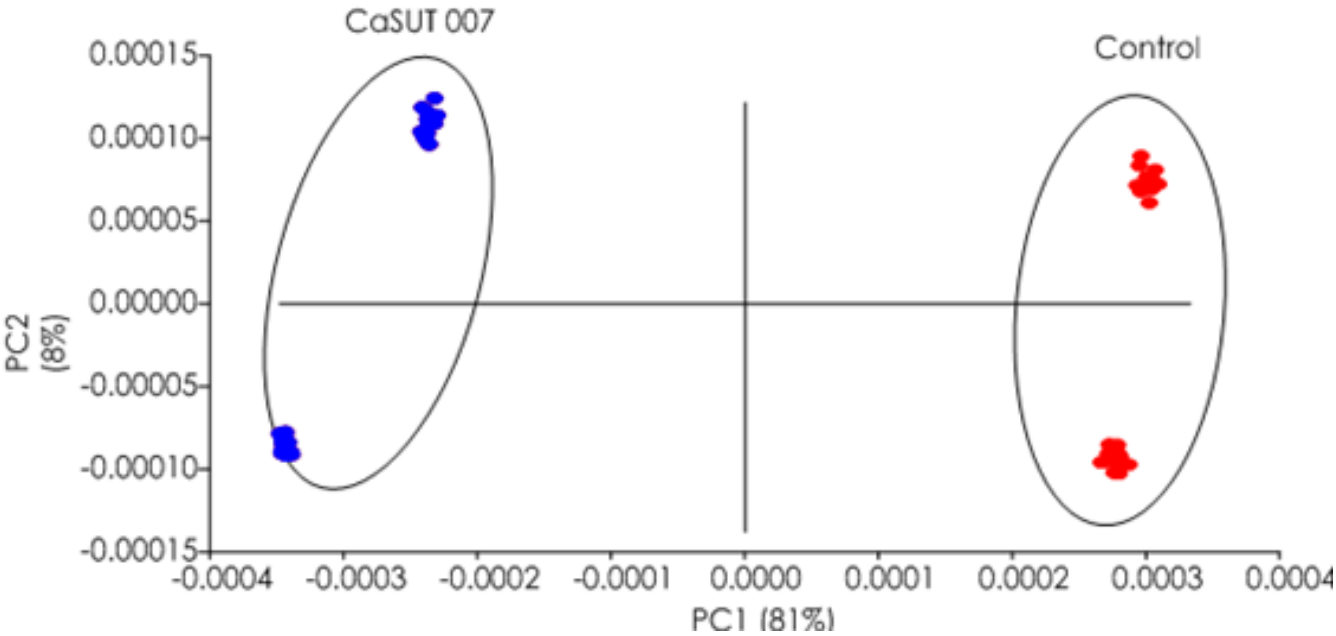

Figure 4. PCA analysis of cassava stake treated with the beneficial bacteria *B. subtilis* strain CaSUT007 and distilled water (a) score plot and (b) loading plot of independent spectra from different conditions. The chemical compositions of two groups were classified with PC1 versus PC2 score plot. PC1 and PC2 explained 81% and 8% of the total variance respectively. Spectra were derived using second derivative processing with the entire biochemical cell fingerprint region.

based on PCA was used to statistical analyze the significant spectral data of cassava stake (Figure 4 and 5). Our results shown as clearly separate with distinct sample clusters were observed among the cassava stake in two spectral regions. Discrete grouping of samples originating from the use of the beneficial bacteria *B. subtilis* strain CaSUT007 and distilled water in these two spectral regions were readily evident within the PC1 and PC2 appeared the highest variance, accounting for 81and 8% of the variability, respectively.

Our findings clearly support a specific effect of the beneficial bacteria *B. subtilis* strain CaSUT007 on the lipid contents and polysaccharide that all involve in the cellular composition of cassava stake, while leaving also affected to another cellular composition and some phytohormone as IAA in cassava stake cell.

DISCUSSION

Enhanced plant growth promotion effects using PGPR strains in different economic crops were clearly investigated and studied (Idriss et al., 2002, 2007; Buensanteai et al., 2008, 2009). The beneficial PGPR inoculants are able to enhanced plant growth and germination rate, improve stake emergence and vigor, responses to biotic/abiotic stress factors and protect plants from phytopathogens infection (Buensanteai et al., 2008, 2009; Gravel et al., 2007).

This current study confirms the earlier experiments which revealed that under the greenhouse conditions, cassava stake treatment with PGPR as Bacilli group strain CaSUT007 improved seed germination, stake vigor and stake emergence over the control. Similar improvement of seed germination parameters by rhizobacteria has been reported in other field crop such as soybean (Buensanteai et al., 2009). The improvement in seed germination by PGPR was also found in work with Buensanteai et al. (2008, 2009), where it was found that some PGPR induced increases in seed emergence, in some cases achieving increases up to 100% greater than controls. These findings may be due to the increased synthesis of hormones like IAA and the high lipid band could assume that is related with the increase of IAA which have the carbonyl bond around 1743 cm^{-1}, which would have triggered the activity of specific enzymes that promoted early germination, such as amylase, which have brought an increase in availability of starch assimilation. Beside, significant increase in stake vigor would have occurred by better synthesis of auxins (Idriss et al., 2002). These results are also similar with the findings of Buensanteai et al. (2008a) who assessed the inoculation effect of PGPR *B. amyloliquefaciens* strain *KPS46* on growth of vegetable soybean. They observed that inoculated plants resulted in better germination, early development and flowering and also increase in dry weight of both the root system and the upper plant parts (Buensanteai et al., 2008a; 2008b).

Similarly, promotion in growth parameters and yields of various crop plants in response to inoculation with PGPR

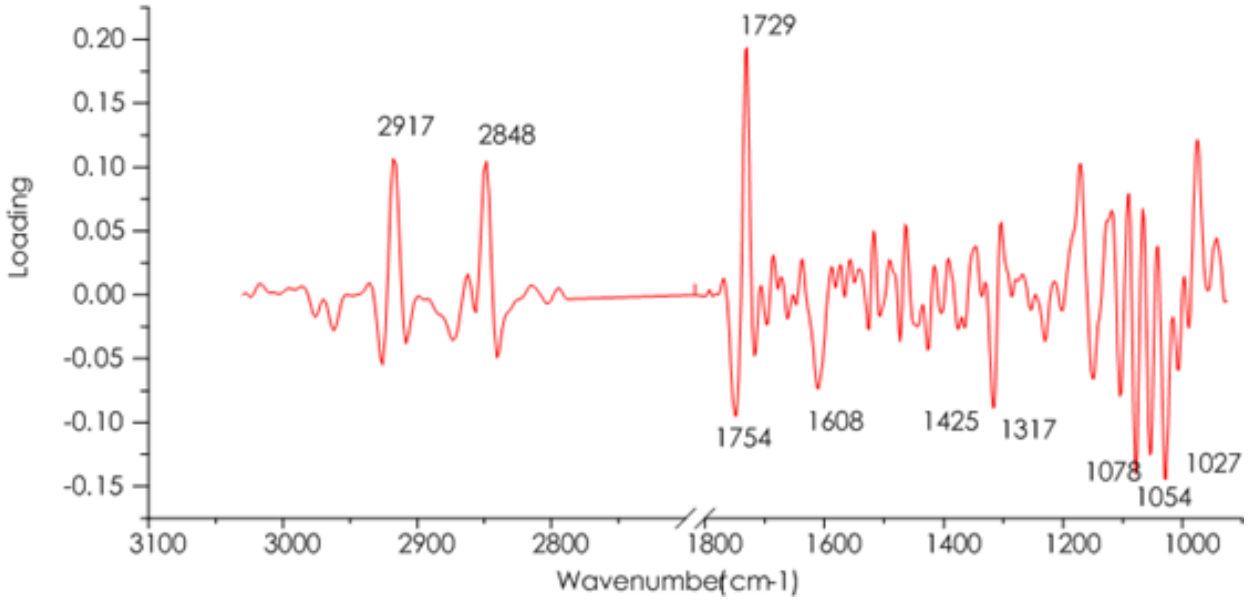

Figure 5. The second derivative FTIR spectra of treated with the beneficial bacteria *B. subtilis* strain CaSUT007. Spectra were measured with 64 scans co added for each individual spectra. Spectra were preprocessed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range.

were reported by other workers (Idriss et al., 2002; Gravel et al., 2007). Inoculation of maize seeds with *Azospirillum* strains compared with *Pseudomonas* strains under experiment conditions resulted in a more visible increase in shoot development, especially during the establishment of the plant.

In current study we are also describe the changes in the cellular components of cassava stakes after sensitization with plant growth promoting rhizobacteria, *B. subtilis* CaSUT007 using FTIR spectroscopy and phytohormone analysis**.** We found that CaSUT007 can influence the growth and development of cassava stakes. The results are consistent with the hypothesis that strain of PGPR promotes the growth of plant stakes by secretion of several types of compounds interact with directly and indirectly with plant growth enhancement mechanism (Buensanteai et al., 2008). Enhanced growth and development in plants inoculated with PGPR have been published (Vessey, 2003; and Banchino et al., 2008). And results of cassava stake in present study are consistent with previous documents. The effects of PGPR inoculation on plant growth and development varied depending on the microbial genus and species, *B. subtilis*, *B. amyloliquefaciens*, *B. polymyxa, B. cereus, B. megaterium, P. fluorescens*, *Bradyrhizobium* sp., *S. meliloti*. The increasing in maize stake biomass when *B. amyloliquefaciens* FZB42 treated the seeds has been reported previously (Idriss et al., 2007). And various effects of PGPRs on root morphology have been reported (Vessey, 2003; Banchino et al., 2008; Buensanteai et al., 2008). Enhanced formation of lateral roots leads to increased root surface area and nutrient uptake potential (Zhang et al., 2007).

However, the present results confirm that cassava stake is response to PGPR (Idriss et al., 2002). The harmful effects of beneficial microbe are mainly due to the resultant regulation of phytohormone. The indole-3-acetic acid was the predominant component in the indole extract and its role in plant growth stimulation by PGPR has been well established (Majcherczyk et al., 1986; Araujo et al., 2005; Idriss et al., 2007), it was most likely the compound responsible for the activity of that extract. The effects of the indole extract on root development apparent in the gnotobiotic experiments are consistent with the effects of exogenous IAA. The peak concentrations of IAA detected in the indole extract of KPS46 were the higher than that reported for any other strain of PGPR (Araujo et al., 2005; Buensanteai et al., 2008). For example, *B. amyloliquefaciens* FZB42 also exhibited increased IAA production with greater tryptophan availability (Lambrecht et al., 2000; Idriss et al., 2007). This is the importance study to examine the interaction of cassava with a PGPR at the cellular level. In our analysis of cassava cellular components produced in response to CaSUT007 treatment, the number of lipid was higher accumulation than untreated treatment. The cellular components we investigated represent only proportion of candidate up-regulated cellular components and we also did not consider cellular components that were down-regulated by CaSUT007 treatment. Nevertheless, our results support the conclusion that CaSUT007 sensitizes and enhances growth of cassava plants. Besides IAA induced in inoculated and non-inoculated plants by CaSUT007 treatment was related to

plant growth regulator stimulation. This most likely reflects the strong positive direct effect that CaSUT007 has on cassava growth and development. In conclusion the results of this experiment suggest that simultaneous finding of beneficial bacteria for growth promotion under pot experiment is a valuable tool to select collect effective PGPR strain for our microbial biofertilizer in the near future.

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