African Journal of Biotechnology Vol. 11(84), pp. 15082-15090, 18 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2396
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Biochemical adaptation of phytopathogenic fungi, *Sclerotium rolfsii,* in response to temperature stress

Natthiya Buensanteai[1,2]*, Kanjana Thumanu[3], Khanistha Kooboran[1], Dusit Athinuwat[4] and Sutruedee Prathuangwong[5]

[1]School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000 Thailand.
[2]Unité Joint Research Eco and Soils (Functional Ecology and Biogeochemistry of Soils and Agroecosystems), UMR 1222 INRA-IRD-Montpellier SupAgro, Place Pierre Viala, 34060 Montpellier cedex 01, France.
[3]Synchrotron Light Research Institute (Public Organization), Muang, Nakhon Ratchasima, 30000 Thailand.
[4]Major of Organic Farming Management, Faculty of Science and Technology, Thammasat University, Pathumthani, 12121 Thailand.
[5]Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, 10900 Thailand.

Accepted 20 June, 2012

Temperature stress plays a critical influence on microbial survival and ecology. It has been reported to be associated with direct effects on microbial metabolisms, but there are very few studies in literature which have reported it in phytopathogenic fungi. In this study, we investigated the impact of two different temperature conditions, 28 and 32°C on the changes of *Sclerotium rolfsii* cellular components using Fourier transform infrared (FT-IR) spectroscopy and enzyme activities measurement. Our results demonstrate that growth, sclerotia germination and biomass of *S. rolfsii* were obviously increased at 32°C. The changes in defense enzymes activity as peroxidase (POX) generally decrease and changed at 32°C. Moreover, the culture of *S. rolfsii* grown at 32°C shows the higher content of the lipid content as shown in the spectral regions of CH stretching and bending bands, when compared with those of this fungi culture under temperature at 28°C, indicating that these indicators played a role in biochemical adaptation in *S. rolfsii*, probably due to enhanced activity of the fungal metabolism pathway and cell wall/membrane protection to temperature stress. Our findings illustrate that temperature stress caused increase biomass and lipid composition; whereas decrease in POX activities, which is a key enzyme helps *S. rolfsii* cope with survival at higher temperature.

Key words: *Sclerotium rolfsii*, enzyme activities, stress response, cellular composition, FT-IR spectroscopy.

INTRODUCTION

Phytopathogenic fungi, *Sclerotium rolfsii* is a soil borne fungus which causes diseases in several agricultural and horticultural crops (Paintin, 1928; Wydra, 1996). This pathogen always occurs and out-breaks in tropical climate areas, especially at high temperature and high moistures country as Thailand. *S. rolfsii* causes several types of symptoms such as damping-off, stem canker, crown blight, root, crown, bulb, tuber and fruit rots (Aries, 1997). Thus, it is essential to gain a better understanding of how this phytopathogenic fungus interact with the environment in order to control directly and indirectly, its activity, pathogenicity and virulence factors.

Environmental conditions, such as temperature, humidity, pH, ultra violet (UV), nutrient and salinity, have especially, critical effect establishment on fungal cellular composition and biochemical metabolism, which involve a role in virulence, pathogenicity, ecology and colonization (Michea-Hamzehpour et al., 1980; Bennett et al., 1992; Fargues et al., 1997; Feder and Hofmann, 1999; Jessup

*Corresponding author. E-mail: natthiya@sut.ac.th. Tel: 66 4 422 4204. Fax: 66 4 422 4281.

et al., 2004; Fels and Kaltz, 2006; Toyoda et al., 2009). Several studies have reported that the temperature-responsive cellular component including tannin, phenol compounds and lipid content are involved in the synthesis of exported polysaccharides and secondary metabolites (Leroi et al., 1994; Mejia et al., 1995; Li et al., 2003; Riehle et al., 2003; Garrett et al., 2006; Szeghalmi et al., 2006; Tharayil et al., 2011). In *Sclerotium rolfsii*, some research was already initially performed on the effects of difference in temperature (Chet et al., 1967; Georgiou et al., 1997, 2000; Kwon and Park, 2002), but no extensive experiment was done concerning cellular composition and enzyme activities response to temperature stress. Fourier transform infrared (FT-IR) spectroscopy has been introduced as a new tool for understanding the total cellular and biochemical components of organisms and microorganism cells (Orsini et al., 2000; Schmitt, 1998; Pandey and Pitman, 2003; Adt et al., 2006; Szeghalmi et al., 2006). The infrared spectrum of biological samples can provide detailed spectral information on cellular components such as polysaccharide, protein and lipid known as “fingerprint region” of the spectral domain (Irudayaraj et al., 2002). Therefore, the valuable information on the cellular and biochemical compositions of the organisms and microorganisms cells can be investigated by FT-IR (Beekes et al., 2007). There are several publications on the application of this FT-IR technique to detect change at the cellular level of cells under the different stress conditions (Kamnev, 2008). To analyze the impact of toxicity and stress response on yeast cell, Adt et al. (2006) and Szeghalmi et al. (2006) reported FT-IR as a credible procedure to detect changes in cellular and chemical composition of compounds that may respect the overall changes in metabolic processes of carbohydrates and lipids.

The aim of this study was to investigate whether, and to what extent, temperature stress event are potentiated by increased temperature at 32°C in *S. rolfsii* that was chosen as a phytopathogenic fungal model. This experiment was to determine the effects of temperature on sclerotia germination, morphology and defense enzymes. Furthermore, FT-IR spectroscopy technique was applied to evaluate cellular composition changes in *S. rolfsii* exposed to temperature stress. By combining with multivariate statistical approach (such as principle component analysis, hierarchical cluster analysis), this technique seems to have the potential to be applied as a screening tool to identify, and characterize the changes of cells related to induced phytopathogenic fungi stress. Our report will display novel perspectives in the use of FT-IR in phytopathogenic fungi responses to temperature change.

MATERIALS AND METHODS

Sclerotium rolfsii and growth conditions

S. rolfsii was routinely maintained and grown on potato dextrose agar (PDA) (Difco) at 28°C. Then, *S. rolfsii* mycelia were collected 7 days after inoculation and re-suspended in sterile distilled water containing 0.05% (v/v) Tween20 at the concentration of 1×10^8 fragments mL^{-1} according to the slightly modified method of Jilkine et al. (2008). Then, aliquots of 50 μL mycelia suspension were placed to PDA and incubated at 28 and 32°C. Moreover, the aliquots of 1 mL mycelia suspension were added to 100 mL potato dextrose broth (PDB) in 250 mL conical flasks and cultured at the same both temperature conditions above on a rotary shaker, at 150 rpm. After incubation for 14 days under the difference temperature conditions, the fungal tissue were directly sampled for microscopy and/or collected by centrifugation and used for subsequent experiments, including cellular composition changes and metabolic enzyme activity measurements. *S. rolfsii* fresh and dry weight determination was performed on samples of fungal tissue harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) no. 5 filter. The fungal tissue were washed twice with distilled water and dried to a constant weight at 105°C for 5 days. Moreover, the number of sclerotia was also investigated from each sample on PDA. All statistical analysis was performed by one-way analysis of variance followed by SAS version 9.1 (SAS, 2008).

Microscopy for sclerotia germination

After sclerotia incubation for 16 h, sclerotia were sampled from each temperature treatment and observe using microscope to calculate germination rate. The experiment was conducted three times independently. For each experiment, each treatment was replicated three times by sampling from different cultures. At least 100 sclerotia were randomly selected in each treatment.

Enzymatic activity assays

The fungal total proteins extract were prepared by slight modification of a protocol described by Buensanteai et al. (2009). Briefly, tissue of *S. rolfsii* was harvested by filtration, washed in sterile distilled water, and then in cold 50 mM potassium phosphate buffer (pH 7.8), and finally resuspended in the same buffer. The samples were lyophilized for 48 h. The fungal cell samples were ground in a mortar and pestle containing extraction buffer (0.1 M Tris-HCl buffer pH 7, 0.1 M KCl, 1 mM phenylmethanesulfonyl fluoride (Sigma), 10 mL/L Triton X-100, 30 g/L polyvinylpyrrolidone (Sigma). The homogenate was centrifuged at 12000 rpm at 4°C for 10 min and the supernatant was kept on ice until the enzyme activity assayed. Total protein concentration in the extracts was measured with a modified assay described by Bradford (1976), in which 1 mL of Bradford reagent was added to 0.1 mL of extract, and absorbance of the mixture was read at 595 nm after a reaction time of 2 min. Sample protein content was determined from a standard curve generated with bovine serum albumin. Peroxidase (POX) activity was determined by a spectrophotometric method described by Hammerschmidt et al. (1982) and Buensanteai et al. (2006). The reaction mixture consisted of 10 μl leaf extract and a substrate solution containing 125 μL guaiacol and 153 μL hydrogen peroxide in 50 mL of 10 mM sodium phosphate buffer, pH 6.0. The reaction mixture was incubated in a water bath at 30°C and absorbance readings at 460 nm were taken every 30 s for 15 min. The level of specific peroxidase activity in a sample was determined by measuring the difference in optical density, and expressed in U $(mg\ protein)^{-1}$.

Cellular composition measurement using FT-IR

Lyophilized cells of *S. rolfsii* were ground in a crystal mortar and

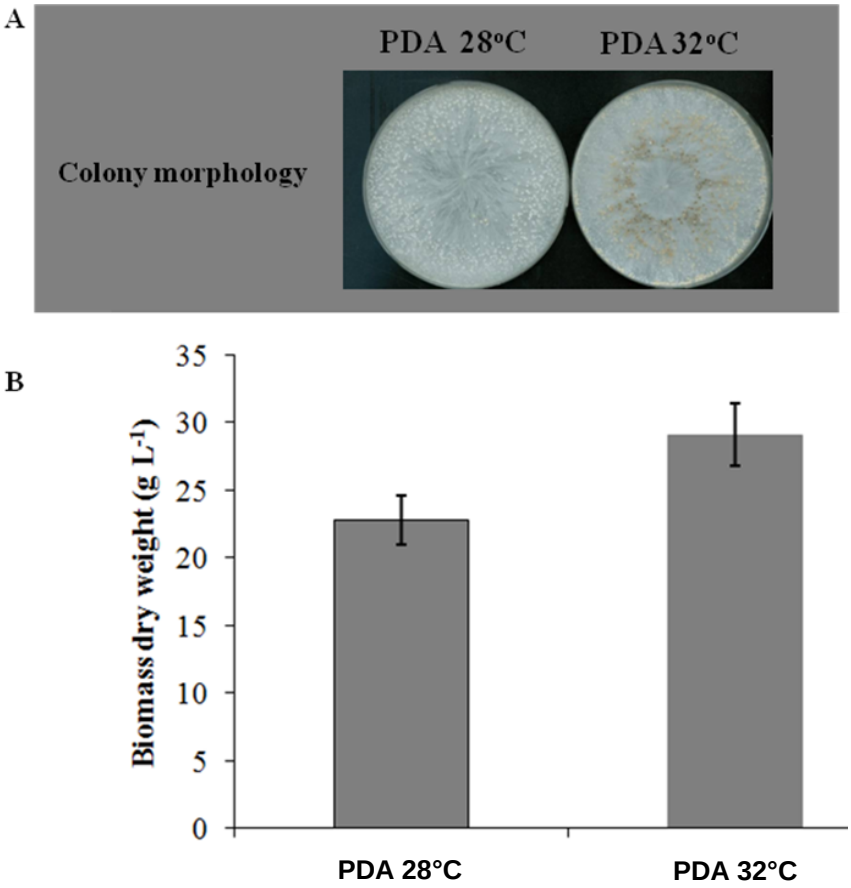

Figure 1. The effects of temperature stress on *Sclerotium rolfsii* colony morphology (A) and fungal biomass (B) after 14 days of culture in PDA with different temperature. The data represent mean ± standard deviation from three different experiments.

pestle. FT-IR sample preparation and measurements were performed according to Kamnev et al. (2008). In brief, 1 mg of the resulting dry biomass was placed in a micro sampling cup, the surface of the powdered sample was lightly pressed with a flat glass spatula and the sampling cup mounted into the sample holder of the FT-IR spectrometer (Tensor 27). The IR spectra were collected using the Attenuated total reflectance (ATR)-FTIR spectroscopy with single reflection ATR sampling module and coupled with MCT detector, cooled with liquid nitrogen over the measurement range from 4000 to 600 cm^{-1}. The measurements were performed with a spectral resolution of 4 cm^{-1} with 64 scans co-added. (Bruker Optics Limited, 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 (3rd polynomial, 9 smoothing points) and then normalized using Extended multiplicative signal correction in the spectral regions from 1750 to 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 distances were calculated as D-values.

RESULTS

Effect of temperature on *S. rolfsii* growth

When exposing the *S. rolfsii* in the exponential-early stationary growth phase (14 days after the start of growth) to either on PDA at 28 and 32°C, the growth patterns of *S. rolfsii* were significantly different (Figure 1A). Moreover, the biomass of *S. rolfsii* grown in PDB at 32°C was 0.4 times higher, compared to this fungus grown in the same media at 28°C (Figure 1B).

Microscopy for sclerotia germination

The effect of two different temperature stress inductors, 28 and 32°C, on sclerotia viability was investigated. When sclerotia from different temperature condition were

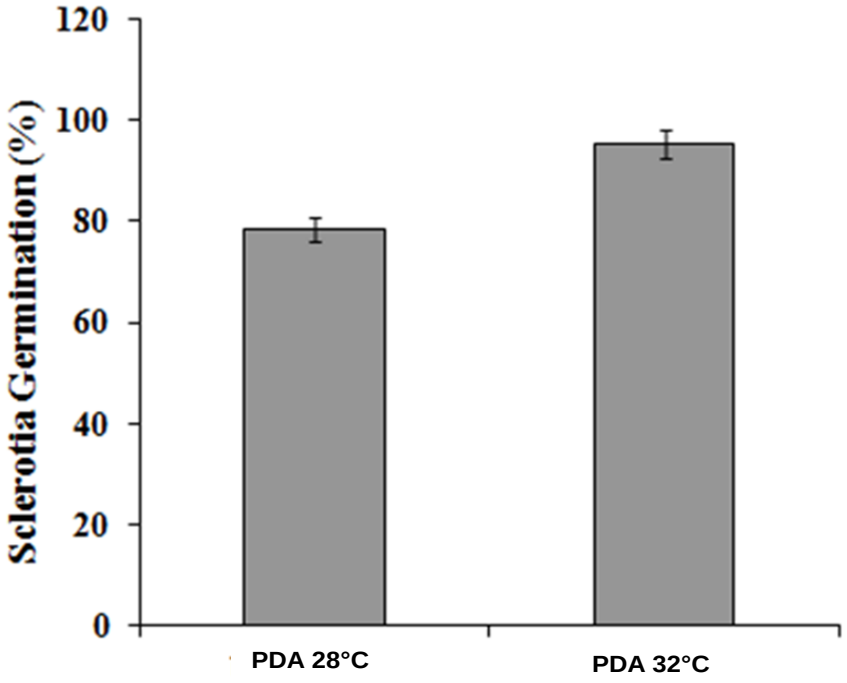

Figure 2. The effects of temperature stress on sclerotia germination after 16 h of culture in PDB with different temperature. The data represent mean ± standard deviation from three different experiments.

exposed for 16 h under temperature at 32°C, there was a marked increase in germination. This induction was 0.2-fold higher in the presence of the *S. rolfsii* sclerotia germination at 28°C (Figure 2).

Enzymatic activity assays

To confirm the relationship between stress situations and effect of temperature stress, we measured activity of defense enzymes as an alternative index of fungal cellular stress response. The results in this study indicated that the peroxidase (POX) activity levels in *S. rolfsii* decreased after inoculation at 32°C, by approximately 0.4-fold when compared to the fungal growth under temperature at 28°C (Figure 3).

FT-IR analysis of *S. rolfsii* cellular composition

In this study, the FT-IR spectroscopy was performed in order to explore the cellular and biochemical changes of phytopathogenic fungi *S. rolfsii* cells after incubation with the difference temperature, 28 and 32°C. The FT-IR spectra of *S. rolfsii* 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 to 1600 cm^{-1} can give information of protein secondary structure such as alpha-helix (centered at 1653 cm^{-1}), beta-sheet (centered at 1635 cm^{-1}) and 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 FT-IR spectra made the difference in two spectral regions more distinctive when different temperatures were used. Our results indicate that the average FT-IR spectra of *S. rolfsii* (Figure 4), treated with each different temperature as 28 and 32°C, were different in biochemical components upon environmental stress. The culture of *S. rolfsii* grown at 32°C shows the higher content of the lipid content as shown in the spectral regions of CH stretching (3000 to 2800 cm^{-1}) and CH bending mode (1467 cm and 1373 cm^{-1}) associated with cell membrane structure lipids, compared with those of the fungi culture under temperature at 28°C. The spectra showed in Figure 5 indicated that there is variation in the amide I secondary structure component. Clearly, the beta sheet secondary structure was shift from 1618 cm^{-1} under temperature of 28°C to beta sheet at 1646 cm^{-1} under stress condition at 32°C. Besides, *S. rolfsii* culture at 32°C significantly shows the band of the beta (1- > 6) glucans centered at993 cm^{-1}. In consistency at this temperature, the results represent the higher content of polysaccharide in the spectral region of C-O-C stretching (1150 to 900 cm^{-1}) from sugars.

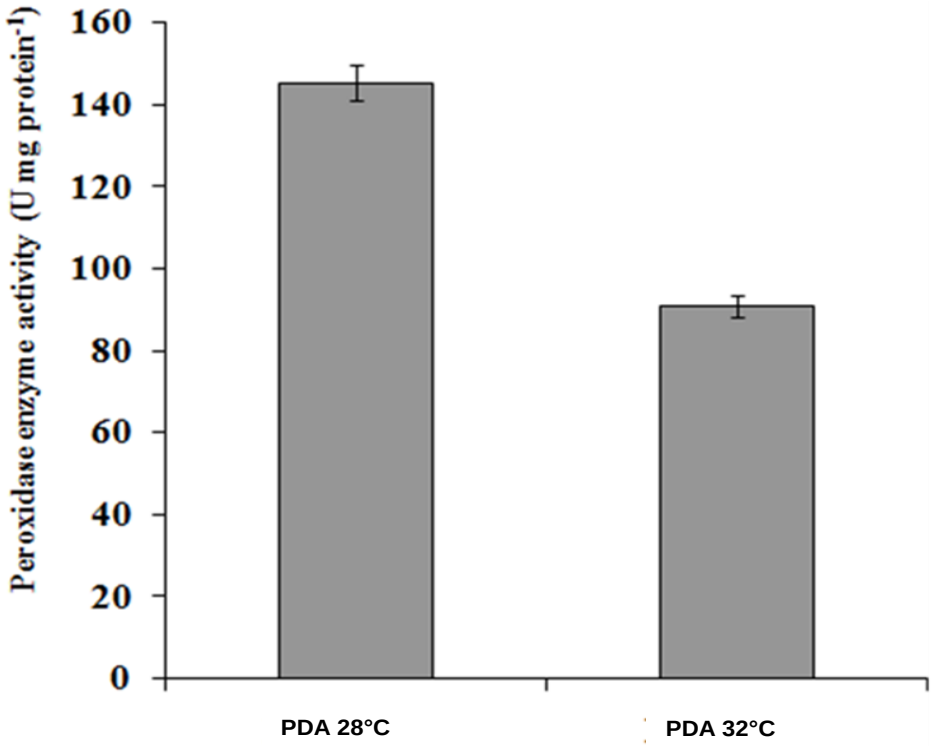

Figure 3. Activities of peroxidase in *Sclerotium rolfsii* treated with difference temperature. The data represent mean ± standard deviation from three different experiments.

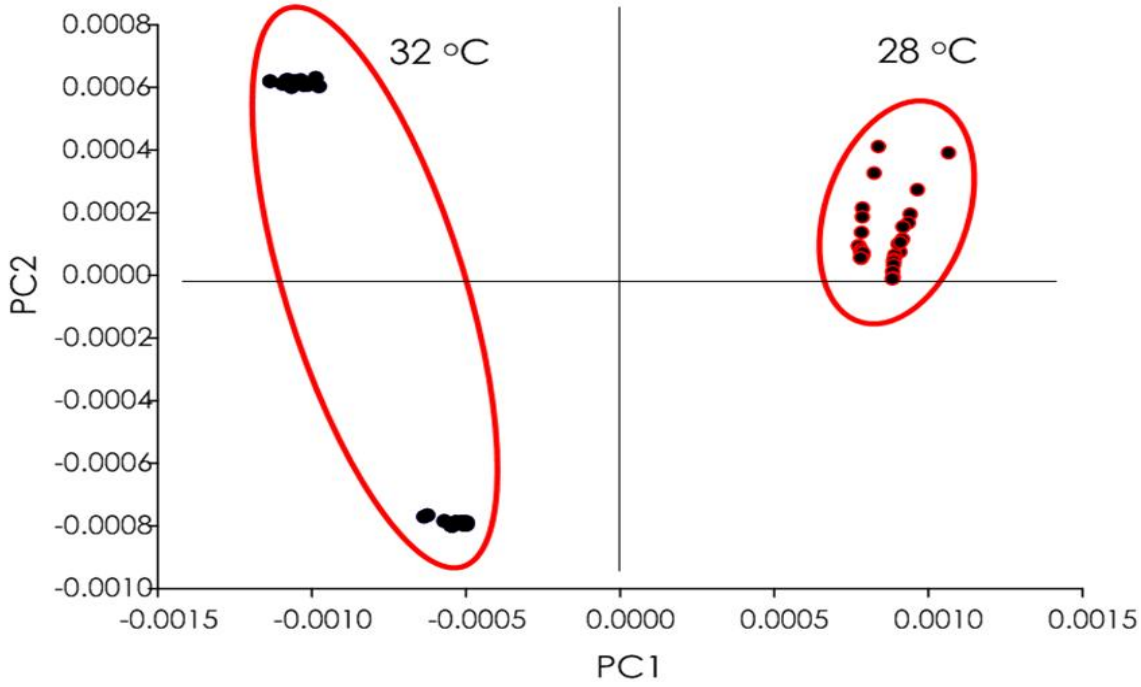

Figure 4. PCA analysis of *Sclerotium rolfsii* at different temperature (A) score plot and (B) loading plot of independent spectra from different condition. The chemical compositions of two groups were classified with PC1 versus PC2 score plot. PC1 and PC2 explained 69 and 27% of the total variance, respectively. Spectra were derived using second derivative processing with the entire biochemical cell fingerprint region (3000 to 2800 and 1750 to 850 cm^{-1}).

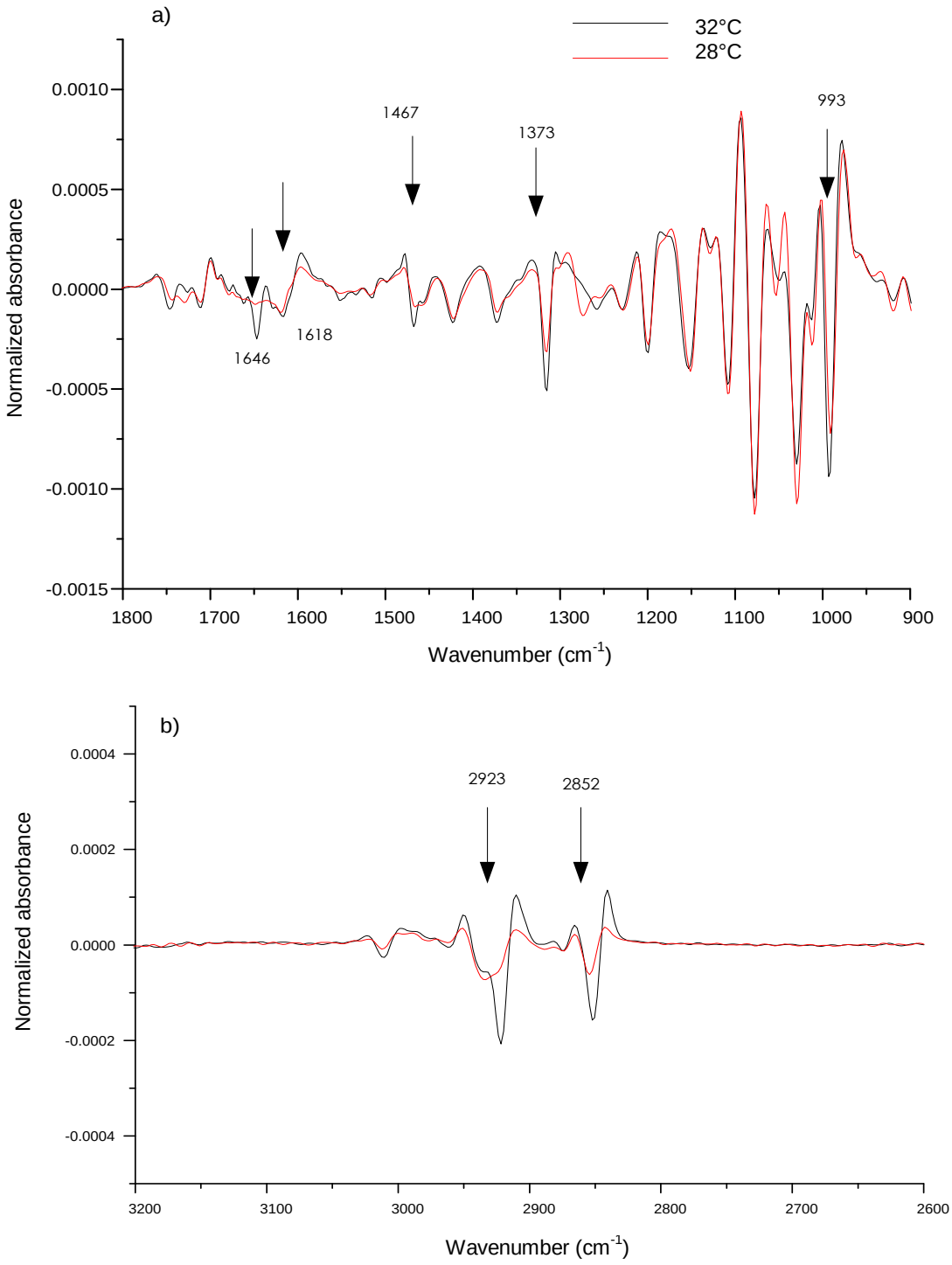

Figure 5. Average second derivative FT-IR spectra of *Sclerotium rolfsii* at different temperature in the region of (a) 1800 to 850 cm^{-1} and (b) 3000 to 2800 cm^{-1}. Spectra were measured with 64 scans co added for each individual spectra. Spectra were preprocessed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range of 3000 to 2800 and 1750 to 850 cm^{-1}.

The dendrogram represented in the Figure 6 shows the identification of two groups of spectra based on similarities and differences between spectra from different temperature condition, using spectral information

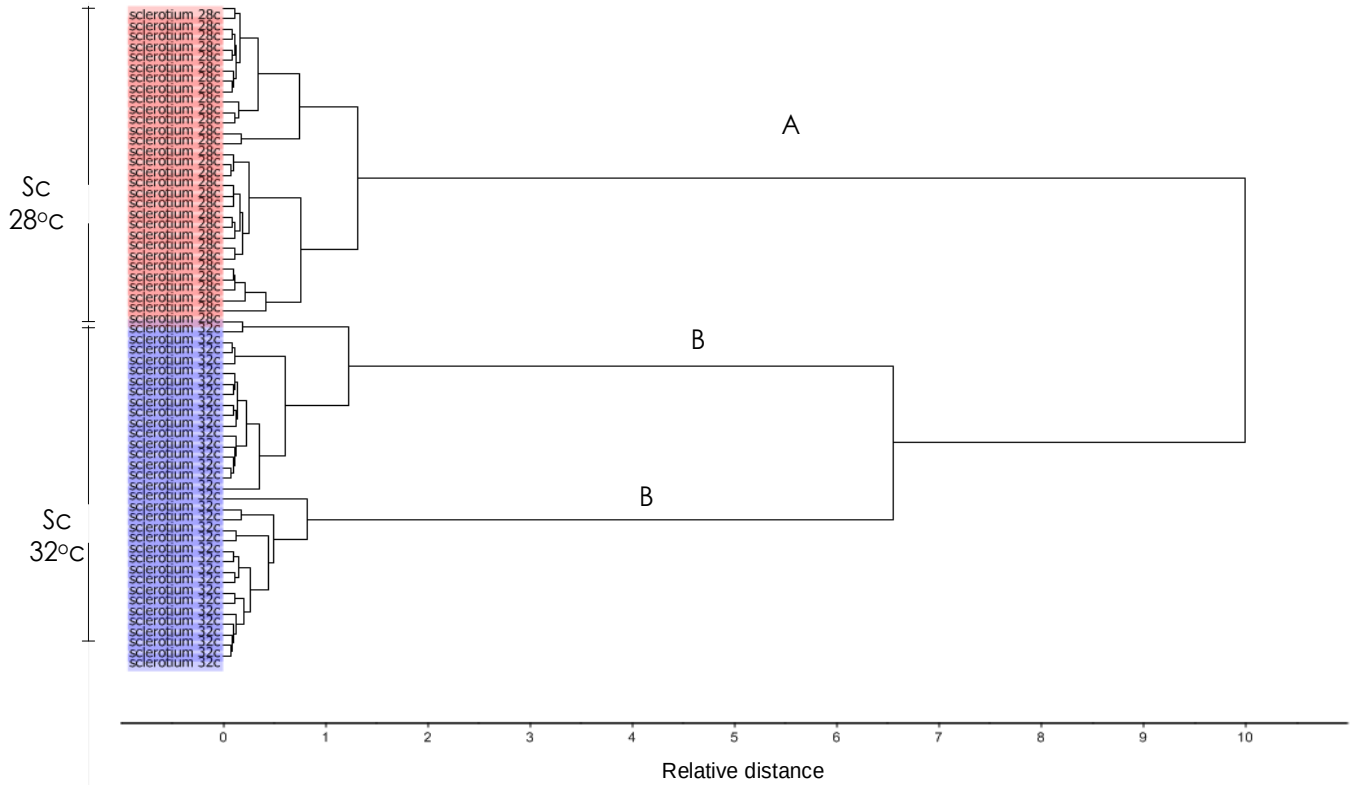

Figure 6. Dendrogram obtained by cluster analysis of *Sclerotium rolfsii* at different temperature. Cluster analysis was employed by Ward's algorithm using second derivative and then vector normalized over the spectra region from 3000 to 2800 cm^{-1} and 1750 to 850 cm^{-1}. Note that the larger the separation between clusters indicated by connecting lines, the lower the similarity of the spectra.

in the ranges of 3000 to 2800 and 1800 to 850 cm^{-1}. The upper branch (A) and lower branch (B) were clearly separated which corresponds to the spectra at 28 and 32°C, respectively. This is confirming the evidence of the distinct FT-IR spectral profiles revealed by PCA. Moreover, the multivariate statistical analysis techniques based on PCA was used to statistically analyze the significant spectral data of *S. rolfsii* (Figure 4). Our results are shown as a clearly separate, with distinct sample clusters observed among *S. rolfsii* cells in two spectral regions. Discrete grouping of samples originating from the use of different temperature of 28 and 32°C in these two spectral regions were readily evident within the PC1 and PC2 in which appeared the highest variance, accounting for 69 and 27% of the variability, respectively. Our findings clearly support a specific effect of the temperature stress on the lipid content, protein and polysaccharide which are all involved in the cell membrane and the cell wall of *S. rolfsii*, while leaving also the effect of other cellular composition and some defense enzymes as POX in the *S. rolfsii*.

DISCUSSION

In the present study, we described the changes in the cellular components of phytopathogenic fungi, *S. rolfsii* response to temperature stress, using FT-IR spectroscopy and enzyme activity assay. The results demonstrated that growth, sclerotia germination and biomass of *S. rolfsii* were obviously increased at a higher temperature of 32°C. There were changes in defense enzymes activity as POX was generally decreased, whereas lipid composition increased with higher temperature stresses, indicating that all five indicators played a role in temperature stress condition in *S. rolfsii* cells, probably due to enhanced activity of the fungal metabolism pathway, and protect cell wall/ membrane at the higher temperature. The changes of POX as signaling response to several abiotic and biotic stress conditions, and its altering have been closely correlated with some defense enzyme changes in induced thermotolerance in *Aspergillus niger* (Abrashev et al., 2005; Aguirre et al., 2005). These enzymes has also been shown to play a role as a stress protective composition under, several environment conditions, including oxidative stress, low temperature, high temperature, salt stress and osmotic stress (Hammerschmidt et al., 1982; Leroi et al., 1994; Fargues et al., 1997; Georgiou, 1997; Abrashev et al., 2005; Di Pasqua et al., 2006; Jilkine et al., 2008). Moreover, these enzymes may serve as a first line of defense against oxidative stress by preventing the

accumulation of reactive oxygen species (Hammerschmidt et al., 1982; Abrashev et al., 2005).

The accumulation of lipid may play an important role as protective response to temperature stress. Lipid has been shown to protect membranes and proteins from the stress *in vitro* and its accumulation have been closely correlated with changes in induced thermotolerance in yeasts and fungi (Fargues et al., 1997; Georgiou, 1997; Di Pasqua et al., 2006). When the temperature stress is applied to the growing cells, high levels of lipid accumulations have been detected in mycelia fungi (Abrashev et al., 2005; Di Pasqua et al., 2006, 2010). Lipid has been shown to play a role as a stress protective composition under several environment conditions, including oxidative stress, low temperature, high temperature, salt stress and osmotic stress (Hammerschmidt et al., 1982; Leroi et al., 1994; Fargues et al., 1997; Georgiou, 1997; Di Pasqua et al., 2006; Jilkine et al., 2008).

The spectra of *S. rolfsii* cells were comparable to the spectra reported for other microorganisms. Strong absorptions were detected in all two spectral regions that characterize the major cellular components. In order to rationalize this multivariate data set, by investigating putative changes in *S. rolfsii*, we proceeded with the PCA of the second derivative transformed spectra. PCA has been shown to be well suited for analysis of the FT-IR spectra, both for identification purposes and for analysis of the biochemical information in the spectra (Pandey and Pitman, 2003; Szeghalmi et al., 2006; Jilkine et al., 2008). Actually, PCA shows whether there are clusters in the data and describes similarities or differences from multivariate data sets (Jilkine et al., 2008). We demonstrated that when *S. rolfsii* are exposed to stress condition, both the lipid content, fatty acids of the cell membrane, protein and the polysaccharides of the cell wall are significantly affected. The mechanisms responsible for the observed experiment in this study are better understood about fungal temperature response. The investigated results are agreeable with cellular changes and evolution occurring in the different temperature stress conditions. The responses of *S. rolfsii* at the different temperatures may be the clue at the underlying physiological and biochemical mechanisms. At 32°C, the fungal biomass of *S. rolfsii* was increased higher than at 28°C. This is consistent with a constitutive temperature stress protection mechanism, such as altered membrane structure (Sasaki et al., 2006) and cell wall components.

Our results clearly showed that *S. rolfsii* from variable environments grow reasonably well at both 28 and 32°C, which is consistent with the evolution of a generalist strategy. Our findings indicate that temperature stress caused increase biomass, sclerotia germination and cellular components, as lipid contents and decrease in the defense enzyme activity, which might be the key compositions, and enzymes, help phytopathogenic fungi, *S. rolfsii* adapt and cope with temperature change.

ACKNOWLEDGEMENTS

The authors wish to express our special thanks to Suranaree University of Technology for funding support. Also, this research is partial support by funds from the France Embassy, European Community Mobility Programme Erasmus Mundus, Parnerships Action2 (EMA2) and European Academic Mobility Network with Asia. Moreover, we would like to express appreciation to Siam Photon Laboratory, thanks for providing the FT-IR instruments.

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