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Infrared spectroscopy: Methods for investigating cellular components of phytopathogenic fungi response to temperature stress

Mathukorn Sompong[1], Kanjana Thumanu[2], Inchana Prakhongka[1], Bhop Burapatpong[1], Dusit Athinuwat[3], Sutruedee Prathuangwong[4] and Natthiya Buensanteai[1]*

[1]School of Crop Production Technology, Institute of Agriculture Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000 Thailand.
[2]Synchrotron Light Research Institute (public organization), Ministry of Science and Technology of the Royal Thai Government, Muang, 30000 Nakhonratchasima, Thailand.
[3]Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, 10900 Thailand.
[4]Major of Organic Farming Management, Faculty of Science and Technology, Thammasat University, 12121, Pathumthani, Thailand.

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Fourier-transform infrared (FTIR) spectroscopy is a sensitive method for the investigation of fungi biochemical composition changes in cells. FTIR spectroscopy can be used to study fungal cell biology by fingerprinting varieties of carbohydrates, proteins, and lipids at about 6 µm spatial resolution. FTIR can distinguish fungal changes during stressed conditions, exhibit dramatic biochemical changes without obvious morphological effects. The objective of this study was to test the hypothesis that the temperature stress, as one of the most important environmental conditions, plays a critical influence on fungal cellular components involved in pathogenic mechanisms. In the current study, we investigated the impact of temperature on the changes of cellular components of phytopathogenic fungi, *Sclerotium rolfsii* and *Colletotrichum gloeosporioides* using FTIR spectroscopy with respect to the lipid, protein and polysaccharide composition. Our results demonstrate that fungal growth of *S. rolfsii* and *C. gloeosporioides* obviously increased at temperature of 32°C. The culture of *S. rolfsii* and *C. gloeosporioides* grown at 32°C shows higher content of the lipid as shown in the spectral regions of CH stretching and bending bands when compared with those of the fungi culture under temperature at 28°C, indicating that all these indicators played a role in temperature stress condition in *S. rolfsii* and *C. gloeosporioides*, probably due to enhanced activity of the fungal metabolism pathway and cell wall protection. Our findings illustrate that temperature stress caused increase biomass and the lipid composition that might be these key biochemical composition help phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* cope for survival at higher temperature. FTIR spectroscopy can be used as a new and sensitive tool to examine the biochemical changes within the fungal cell. This technique allows us to reveal structural chemical makeup and features within the different fungal growing conditions.

Key words: *Sclerotium rolfsii*; *Colletotrichum gloeosporioides*, temperature response, cellular composition, FTIR spectroscopy.

INTRODUCTION

Fourier transform infrared (FTIR) spectroscopy has been introduced as a new tool for understanding the total cellular and biochemical components of organisms and microorganisms 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, 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 FTIR (Beekes et al., 2007). There are several publications on the application of this FTIR 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). Szeghalmi et al. (2006) reported FTIR 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.

Phytopathogenic fungi, *Sclerotium rolfsii* and *C. gloeosporioides* are fungus disease in several agricultural and horticultural crops (Paintin, 1928; Wydra, 1996). This disease always occurs and out-breaks in tropical climate area, especially at high temperature and high moistures country as Thailand. The pathogens of this diseases cause several types of symptoms such as damping-off of seedlings, stem canker, crown blight, root, crown, bulb, tuber, anthracnose and fruit rots (Agrios, 1997). Thus, it is essential to gain a better understanding of how this phytopathogenic fungus interact with the environment in order to direct and indirect its activity, pathogenicity and virulence factors.

Environmental conditions, such as temperature, humidity, pH, UV, nutrient and salinity, have especially critical effect establishes 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 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* and *C. gloeosporioides*, some report was already initially performed on the effects of different temperature (Chet et al., 1967; Georgiou et al., 1997, 2000; Kwon and Park, 2002) but no extensive experiment was done concerning about cellular composition response to temperature stress and which methods was sensitive for investigating cellular components of phytopathogenic fungi response to temperature stress.

The aim of this study was to investigate FTIR spectroscopy technique to evaluate cellular composition changes in pathogenic fungi 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-pathogenic fungi stress.

MATERIALS AND METHODS

Fungal strains and growth conditions

The phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* were used in this study. The strains were routinely maintained on potato dextrose agar: PDA (Difco). The isolate was routinely grown on PDA plates for seven days at 28°C. Then spores were collected and suspended in sterile distilled water containing 0.05% (v/v) Tween20 at the concentration of 1×10^8 spores mL^{-1} according to the slightly modified method of Jilkine et al. (2008). Then aliquots of 50 uL spore suspension were placed to PDA media and incubated at 28 and 32°C. Moreover, the aliquots of 1 mL spore suspension were added to 100 mL potato dextrose broth (PDB) media in 250-mL conical flasks and cultured at the same temperature condition 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 measurement.

Cellular composition measurement using FTIR

Lyophilized cells of *S. rolfsii* and *C. gloeosporioides* were ground in a crystal mortar and pestle. FTIR sample preparation and measurements were performed according to the study of 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).

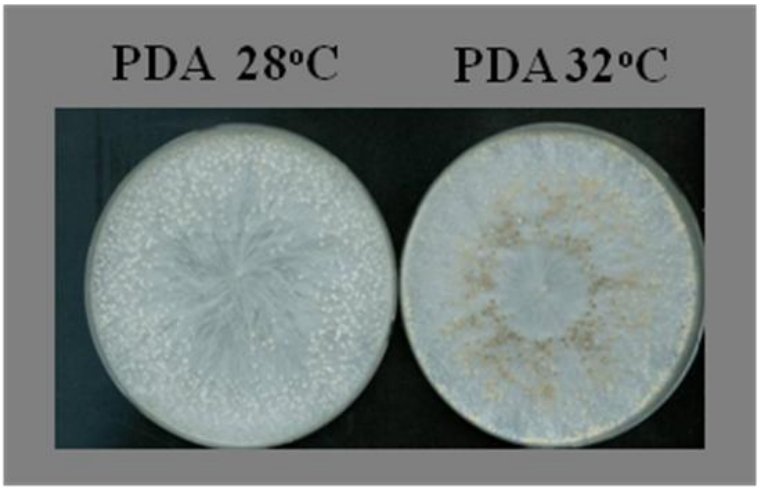
Spectra from each group were analyzed using Principal Component Analysis (PCA). Individual spectra from each group were analyzed using PCA to distinguish different chemical components of the samples using the Unscrambler 9.7 software (CAMO, Norway). The spectra were processed using 2nd derivative and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

RESULTS

Effect of temperature on the growth of phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides*

When exposing the cells of tested fungal strains in the

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



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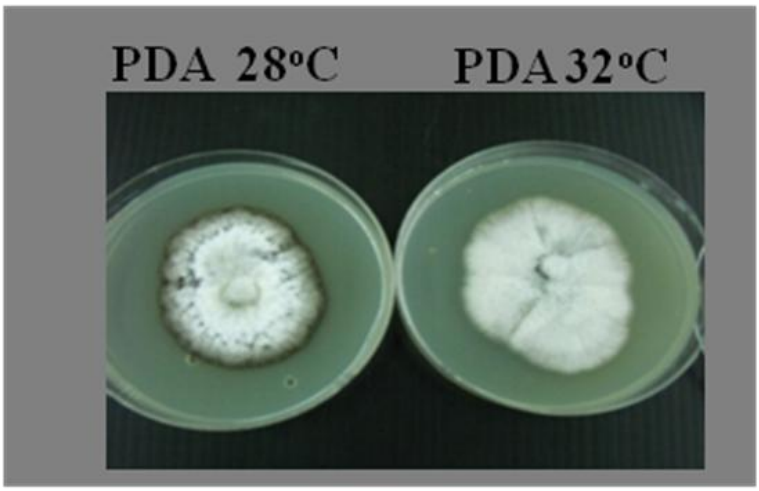

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Figure 1. The effects of temperature stress on the phytopathogenic fungi, *Sclerotium rolfsii* colony morphology (A) fungal biomass (B) 14 day of culture in PDA media with different temperature. The data represent mean ± standard deviation from three different experiments. Columns with different letters are significantly different from each other by the least significant difference test ($p < 0.05$).

exponential-early stationary growth phase (14 days after the start of growth) to either PDA at temperature 28 or 32°C, the growth patterns of the phytopathogenic fungi *S. rolfsii* (Figure 1A) and *C. gloeosporioides* (Figure 1B) treated with these temperature conditions were signify-cantly different.

FTIR analysis of phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* cellular composition

In this current study, the FTIR spectroscopy was performed in order to explore the cellular and biochemical changes of phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* cells after incubation with the difference temperature. The IR spectra of phytopathogenic fungi 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 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 different

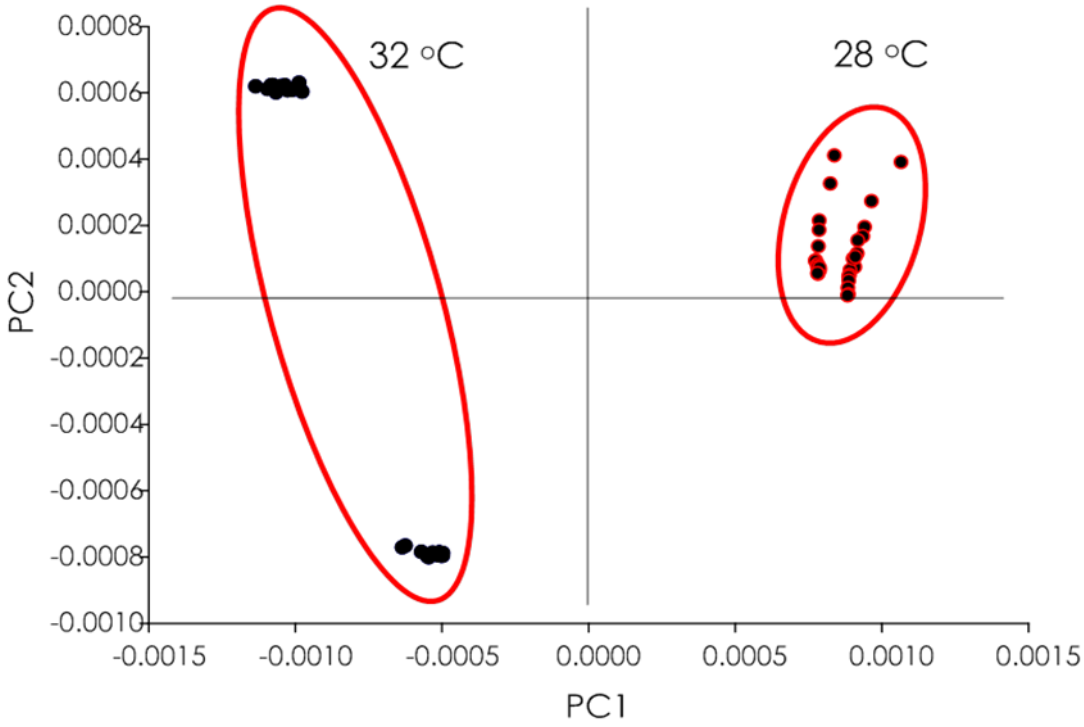

Figure 2. PCA analysis of phytopathogenic fungi, *Sclerotium rolfsii* at different temperature (a) score plot and (b) loading plot of independent spectra from different condition . The chemical compositions of two groups were classified with PC1 versus PC2 score plot. PC1 and PC2 explained 69% and 27% of the total variance respectively. Spectra were derived using second derivative processing with the entire biochemical cell fingerprint region (3000-2800 and 1750-850 cm^{-1}).

temperatures were used. Our results indicated that the average FTIR spectra of phytopathogenic fungi *S. rolfsii* (Figure 1) and *C. gloeosporioides* (Figure 2) treated with each different temperature at 28 and 32°C were different in biochemical components upon environmental stress. The culture of phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* grown at 32°C 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 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 Figures 1 and 2 indicated that there are variations in the amide I secondary structure component phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides*. Clearly, the beta sheet secondary structure was shift from 1618 cm^{-1} under temperature 28°C to beta sheet at 1646 cm^{-1} under stress condition at 32°C for *S. rolfsii*. In contrast, the high alpha helix secondary structure (centered at 1656 cm^{-1}) was shown under temperature at 28°C, but the beta sheet secondary structure (centered at 1629 cm^{-1}) was significantly dominant at 32°C. Besides, significantly the fungal culture of phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* at 32°C showed the band of the beta (1->6) glucans centered at 993 cm^{-1}, in constant in this temperature the results represent the higher content of polysaccharide in the spectral region of C-O-C stretching (1150-900 cm^{-1}) from sugars .

Moreover, the multivariate statistical analysis techniques based on PCA was used to statistical analyze the significant spectral data of phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* cells (Figures 3 and 4). Our results clearly separate with distinct sample clusters were observed among the phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* cells in two spectral regions. Discrete grouping of samples originating from the use of different temperature at 28 and 32°C for *S. rolfsii* were readily evident within the PC1 and PC2 appeared the highest variance, accounting for 69 and 27% of the variability respectively. While, spectra groups for *C. gloeosporioides* were clearly separate along *PC1 and PC2* with the highest variance of 75 and 17 % respectively.

DISCUSSION

In the current study we describe the fourier-transform infrared (FTIR) spectroscopy as sensitive method for the investigation of fungi biochemical composition changes in cells of phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* response to temperature stress. FTIR spectroscopy can be used to study fungal cell biology by

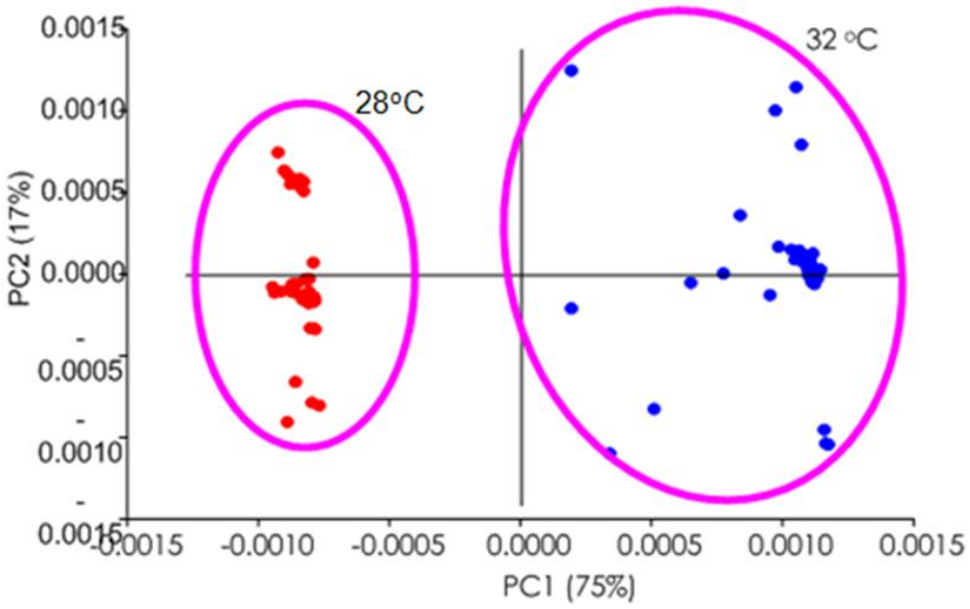

Figure 3. PCA analysis of phytopathogenic fungi, *C. gloeosporioides* at different temperature (a) score plot and (b) loading plot of independent spectra from different condition . The chemical compositions of two groups were classified with PC1 versus PC2 score plot. PC1 and PC2 explained 75% and 17% of the total variance respectively. Spectra were derived using second derivative processing with the entire biochemical cell fingerprint region (3000-2800 and 1750-850 cm^{-1}).

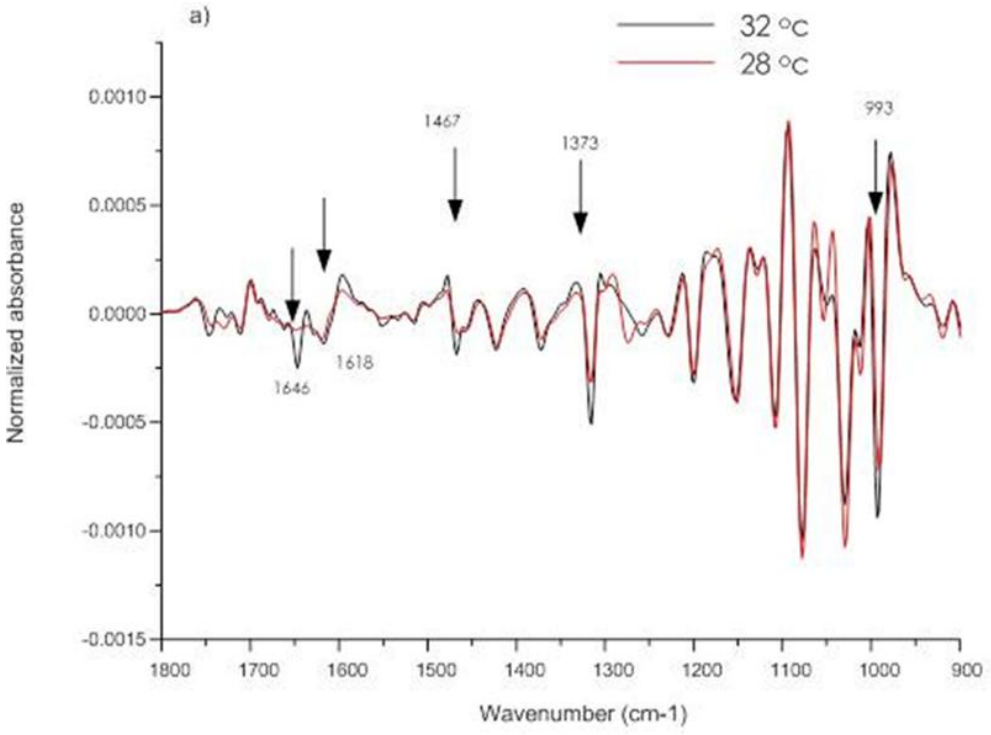

Figure 4. Average second derivative FTIR spectra of phytopathogenic fungi, *Sclerotium rolfsii* at different temperature in the region of (a) 1800-850 cm^{-1} and (b) 3000-2800 cm^{-1}. Spectra were measured with 64 scans co added for each individual spectra. Spectra were preprocessed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range of 3000-2800 and 1750-850 cm^{-1}.

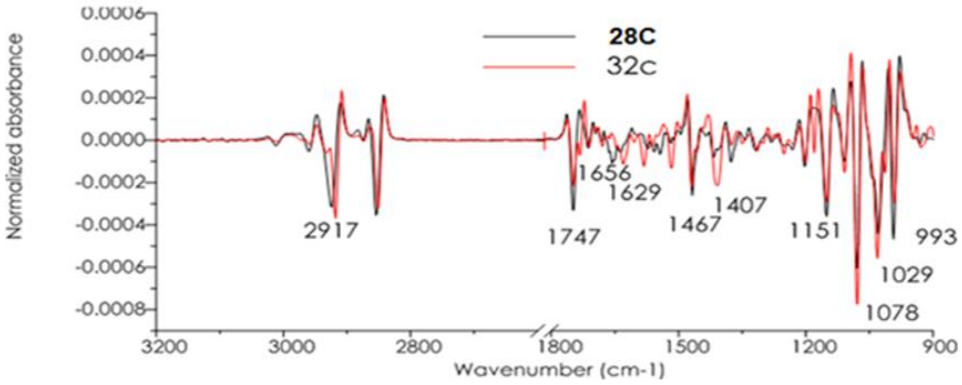

Figure 5. Average second derivative FTIR spectra of phytopathogenic fungi, *C. gloeosporioides* at different temperature in the region of (a) 1800-850 cm^{-1} and (b) 3000-2800 cm^{-1}. Spectra were measured with 64 scans co added for each individual spectra. Spectra were preprocessed by taken second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

fingerprinting varieties of carbohydrates, proteins, and lipids. FTIR that can distinguish fungal changes during stressed conditions exhibit dramatic biochemical changes without obvious morphological effects. The results demonstrated that spore germination of *S. rolfsii* and *C. gloeosporioides* obviously increased at higher temperature 32°C. The culture of *S. rolfsii* and *C. gloeosporioides* grown at 32°C shows the higher content of the lipid as shown in the spectral regions of CH stretching and bending bands when compared with those of the fungi culture under temperature 28°C, indicating that all these indicators played a role in temperature stress condition in *S. rolfsii* and *C. gloeosporioides*, probably due to enhanced activity of the fungal metabolism pathway and cell wall/membrane protection. The accumulation of lipid may play a 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 fungi cells, high levels of lipid accumulations have been detected in mycelia fungi (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 phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* 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, investigate putative changes in phytopathogenic fungi *S. rolfsii* and *C. gloeosporioides* cells, we proceeded with the PCA of the second derivative transformed spectra. PCA has been shown to be well suited for analysis of the FTIR 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). Taken as a whole, PCA revealed unique features of the FTIR spectra among phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* cells. We demonstrated that when phytopathogenic fungi, *S. rolfsii* cells and *C. gloeosporioides* are exposed to the stress condition, both the lipid content, fatty acids of the cell membrane, proteins and the polysaccharides of the cell wall are significantly affected.

The mechanisms responsible for the observed experiment in this study have more better understanding about fungal temperature response. The investigated results are agreeable with cellular change and evolution occurring in the different temperature stress conditions. The responses phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* from the different temperatures may clue at the underlying physiological, biochemical and pathogenesis mechanisms. At 32°C, the fungal biomass increase at the same rate as phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* from 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 phytopathogenic fungi, *S. rolfsii* and *C. gloeosporioides* from variable environments grow reasonably well at both 28 and 32 ^{o}C. According to, these changes in temperature may activate stress response pathways to cope with severe changes in environ-

mental temperature or may inform fungal pathogens of successful plant host infection and initiate virulence programs.

In summary, the study shows that FTIR spectroscopy can be used as a new tool to examine the biochemical changes within the fungal cell. This technique allows us to reveal structural chemical makeup and features within the different fungal growing conditions. However, further research is needed to better understand and characterize the chemical and structural changes reflecting direct effect from several temperatures as climate change systems. In the future, we plan to investigate the fungal cell from difference temperature conditions in order to compare spectral difference via synchrotron FTIR microspectroscopy that could be explained more clearly during fungal growth, development and pathogenesis in the plants.

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