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Farnesol produced by the biocontrol agent *Candida ernobii* can be used in controlling the postharvest pathogen *Penicillium expansum*

Pu Liu[1,2], Yuan-yuan Shi[2], Lifeng Chen[3] and Chao-an Long[1]*

[1]National Centre of Citrus Breeding, Key Laboratory of Horticultural Plant Biology of Ministry of Education, Huazhong Agricultural University, Wuhan 430070, P. R. China.
[2]Key Lab Pomology, Anhui Agricultural University, Hefei 230036, P. R. China.
[3]Agricultural Bureau of Changle County, Changle 262400, P. R. China.

This study was conducted to investigate the effect of *Candida ernobii* strain Pg2-5 against postharvest disease of *Penicillium expansum* and to evaluate the possible mechanisms involved. Disc diffusion assay showed that Pg2-5 exhibited an inhibitory effect against *P. expansum* growth. An antifungal compound was isolated from the cell-free supernatant culture of Pg2-5 and identified as farnesol. The minimal inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) of farnesol for *P. expansum* were 15 and 100 µM, respectively. The addition of farnesol promoted reactive oxygen species (ROS) burst, and increased ultrastructure disintegration of mitochondria. These results indicate that antifungal compound production is one of the modes of action of *C. ernobii* Pg2-5 against postharvest pathogen of *P. expansum*. ROS may be an inducer, and mitochondria are potential target of the cytotoxicity process of farnesol.

Key words: Postharvest, *Candida ernobii*, biocontrol, farnesol.

INTRODUCTION

Postharvest losses of fresh fruits and vegetables are very high, ranging from 10 to 40% depending on species, harvest methods, length of storage and technologies used in the packing houses (Xu et al., 2013). *Penicillium* spp. as ubiquitous soil inhabitants of spores, play an important role in industry, agriculture and medicine. *Penicillium expansum*, one of the most frequently isolated fungal pathogens of plants, can attacks fruits such as apple, pear and citrus, causing blue mold rot (Morales et al., 2008). Traditionally, the main use of synthetic fungicides to control postharvest diseases of fruits but there are several concerns, including side effects of synthetic fungicide and development of fungicide-resistant strains of pathogens (Ren et al., 2012). It is

always necessary to explore new agents or alternative approaches of controlling fungal pathogens.

Biocontrol using antagonists has been proved as one of promising alternative way to synthetic fungicides (Perello et al., 2008; Menel et al., 2013). The beneficial of yeast are generally considered safe and have long been accepted by consumers. Some of them have been investigated as potential antifungal biocontrol agents and several have been launched on the market (Nguefack et al., 2009). The complex interactions between host, pathogen, antagonist and other microorganisms lead to the study of the mode of action for the antagonists investigated is still incomplete (Spadaro and Gullino, 2004; Droby et al., 2009; Liu et al., 2013a). However, a

*Corresponding author. E-mail: postharvest@mail.hzau.edu.cn. Tel:+86-27-87280499. Fax:+86-27-87282010.

good understanding of the mode of action is essential for application, official approval and consumer acceptance. In the present, several possible mode of action have been suggested to be effective against rots on fruits include production of antibiotics (Zhou et al., 2008; Chan et al., 2009) and extracellular hydrolytic enzymes such as chitinase (Li et al. 2008), competition for nutrients and space (Liu et al., 2013b); parasitism and induced resistance in the host tissue (Hershkovitz et al., 2012). However, little information is available regarding the antifungal compound of antagonistic yeast.

Previously studies, *in vitro* and *in vivo*, have shown that *Candida ernobii* Pg2-5 was an effective antagonist against postharvest pathogen of citrus caused by *Diplodia natalensis* (Chen et al., 2007). The objectives of the present work were to evaluate the potential of *Candida ernobii* strain Pg2-5 in controlling postharvest pathogen *P. expansum* and to identify the potential antifungal substances.

MATERIALS AND METHODS

Microorganisms and growth conditions

P. expansum L was obtained from typical blue mold rot symptoms and cultured on potato-dextrose agar (PDA: extract of boiled potatoes, 200 mL; dextrose, 20 g; agar, 20 g and distilled water, 800 mL) and stored at 4°C. Spore suspensions were prepared by removing the spores from a seven day old culture, and suspended them in 5 mL of sterile distilled water (containing 0.05% Tween 80). Suspensions were filtered through four layers of cheesecloth to remove fungal mycelium and the spore concentrations were adjusted with a hemocytometer.

The antagonist *C. ernobii* Pg2-5 was isolated from the surface of citrus fruit by Chen et al. (2007). The yeast cells were obtained from a culture grown in 250 mL Erlenmeyer flasks with 50 mL yeast peptone dextrose (YPD) on a rotary shaker at 200 rpm for 48 h at 28°C. Cells were harvested by centrifugation at 10,000 × g for 10 min, and resuspended in sterile distilled water. The cells were adjusted to the desired concentration (10^7 cells/mL) with a hemo-cytometer.

Trans-trans farnesol (Sigma-Aldrich, USA) was added into the indicated concentrations from a 100 mM stock solution prepared fresh in methanol before each experiment. The original flask containing farnesol was de-gassed with nitrogen gas each time after being opened in order to avoid degradation by oxygen. All other chemicals and solvents were of analytical grade.

Candida ernobii antimicrobial detection

The agar diffusion assay was used to assess the antifungal active of *C. ernobii* strain Pg2-5 as describes by Helal et al. (2007). The plate containing 20 mL PDA was seeded with 1 mL *P. expansum* L spores suspension ($1.0 × 10^5$ spores/mL). 5 mm diameter strain Pg2-5 colonies agars from 48 h-old cultures were placed in the centre of the plates. Clear zones of inhibition were recorded after 48 h incubation.

Meanwhile, Co-culture assays also achieved as described by Semighini et al. (2006). Briefly, strain Pg2-5 and *P. expansum* L were grown separately or in co-culture in YPD at 28°C for 12 h (initial inoculum size: $1.0 × 10^3$ cells/mL). The cultures were washed and plated onto YPD at 28°C for five days.

Activity compound extraction

The culture of strain Pg2-5, incubated in 80 mL YPD on a rotary shaker at 200 rpm for 48 h at 28°C, was centrifuged at 12,000 × g for 10 min. The supernatant was filtrated by a polyethersulfone membrane (0.45 µm). The cell-free supernatant culture was extracted by 1/2 volume ethyl acetate for twice, which was collected and removed by rotary evaporation. The residue was resuspended in 250 µL methanol.

Activity compound detection

The extract was filtrated by a polyethersulfone membrane (0.45 µm), and then analyzed by Agilent 6890N gas chromatography with GB-35 in electron ionization (EI) modes as described by Hornby et al. (2001). Briefly, GC used a 1 µL sample, injector and detector temperatures of 250 and 280°C. The GC column used an initial temperature of 100°C, held at temperature for 3 min, and then 20°C/min until 280°C, with a 3 min hold at 290°C.

Antimicrobial detection

The agar diffusion assay was carried out as describes above. Plates were incubated at 28°C after sealing with Parafilm[TM] (Pechiney Plastic Packaging, Chicago, IL). Clear zones of inhibition were recorded after 48 h incubation. Meanwhile, minimal inhibitory concentration (MIC) for germ tubes was determined in 250 mL flasks with 50 mL PDB using a modified version of the antimicrobial bioassay method of Hwang et al. (2001). Briefly, $1.0 × 10^5$ spores/mL of strain *P. expansum* L were incubated on a rotary shaker at 200 rpm for 12 h at 28°C, the cultures were added with the extract and farnesol to different concentrations, and further incubated for additional 48 h. The experiment was repeated twice.

Minimum fungicidal concentrations (MFC) of farnesol

The germ tubes cultures were prepared as described above. The effects of farnesol on the rapid growth of *P. expansum* L were determined by MFC, Briefly, the cultures were exposed to different concentration of farnesol (25 µM to 100 µM) and incubated at 28°C for additional 2 h. Cells were washed, diluted to $1.0 × 10^4$ cells/mL and plated onto PDA and incubated at 28°C for 3 day. The colony-forming units (CFU) were counted per plates.

Transmission electron microscopy (TEM) and reactive oxygen species (ROS) analysis

TEM was performed after the germ tube cultures were treated with 100 µM farnesol (or methanol control) for additional 2 h, according to the following standard procedure as described by Helal et al. (2007). Intracellular ROS production was examined with the oxidant-sensitive probe dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, California, USA) according to Leiter et al. (2005). Briefly, the germ tubes cultures were treated with 40 µM H_2DCFDA for 30 min, and then exposed to 100 µM farnesol (or methanol control). The contents of ROS was immediately measured with Spectrofluorophotometer and excitation/emission at 480/530 nm.

RESULTS

Inhibitory effect of *Candida ernobii* on *Penicillium expansum* growth

The inhibitory potential of *C. ernobii* Pg2-5 against *P.*

Table 1. Antifungal activity of Antagonist or the extract.

Parameter	Zone of inhibition (mm)
C. ernobii	14.3 ± 1.40
The extract	13.5 ± 1.26
Farnesol	16.2 ± 3.58

Antifungal activity was accounted by the agar diffusion method. Means are averaged values of three trials and given as ± SE.
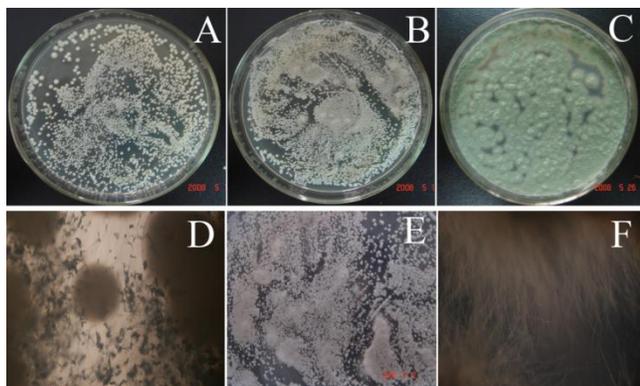
Table 2. GC analysis of the extract and farnesol.

Parameter	t_R (min)	peak H	peak W	areas
Farnesol	9.773	2460.0	0.0315	5323.7
	9.945	2583.2	0.0340	5931.3
The extract	9.756	163.1	0.0639	803.3
	9.920	162.3	0.0658	825.1

The samples were analyzed by GC with GB-35 in electron ionization (EI) modes. GC used a 1µl sample, injector, and detector temperatures of 250 and 280°C, and a temperature program of 100°C for 3 min and then 20°C/min until 280°C. Finally, GC retained in 290°C for 3 min. Peak areas are averaged values of three trials.

Figure 1. Pg2-5 inhibits growth and development of *P. expansum* in YPD. Strains of Pg2-5(A), *P .expansum* was mixed with Pg2-5 (B, D and E), and only *P. expansum* (C and F) in YPD medium, and incubated at 28°C for 5d. Each treatment was replicated three times and the experiment was repeated twice.

expansum L assessed in PDA medium as given in Table 1, *C. ernobii* Pg2-5 showed inhibitory effects on mycelial growth of *P. expansum* L. The zone of inhibitory reached to 14.3 mm after 48 h by using agar diffusion assay. Co-culture assays showed that *C. ernobii* Pg2-5 can remarkably restrained spore germination and growth of *P. expansum* L (Figure 1).

The antifungal compound was identified for farnesol

It was noticed that the *P. expansum* L colonies around antagonistic agars became transparent, and on examination of this portion of the culture it is found that practically all the hyphae were dissolved, indicating that the antifungal substance has continued to diffuse into the agar in sufficient concentration to induce dissolution of the fungi. The active compound of *C. ernobii* Pg2-5 was availably extracted by ethyl acetate, and then analyzed by GC. The extract presented special retention time (t_R) of 9.756 and 9.920 min was identified as farnesol in comparison with standard farnesol that had the same t_R (Table 2). Heating the extract for 20 min at 100 °C has no obviously impact on the antifungal power, but keeping or

using exposed on the air have impact on antifungal power. These characters of the extract were identical to that of standard farnesol. Moreover, the efficacy of the extract and farnesol against *P. expansum* L was evaluated in by disc diffusion assay. The zone of inhibitory was 13.5 mm for the extract (50 µL) and 16.2 mm for farnesol (20 mM, 50 µL) (Table 1). In PDB, the MIC value was 15 µM for farnesol and 125 µL for the extract. Accounting GC peak areas (Table 2) indicated the concentration of farnesol in the extract was 14.47 mM. Comparatively, the concentration of farnesol in *C. ernobii* Pg2-5 cell-free supernatant was 45.22 µM.

Farnesol induced *Penicillium expansum* germ tubes death

The germ tubes cultures were exposed to 25, 50 and 100 µM farnesol for additional 2 h. Compared with methanol-treated control, the activities of cells were determined by the number of colonies per plate in PDA (Figure 2). At 25 µM farnesol, 52.87% (control, 100%) hyphae kept alive (Figure 3) and grew into colonies. However, in higher concentrations, cells were almost completely dead. The MFC values of farnesol for *P. expansum* L was 100 µM.

Farnesol induced cytoplasmic degeneration and reactive oxygen species (ROS) eruption in *Penicillium expansum* hyphal cells

In order to examine the effects of farnesol on the morphology of *P. expansum* L at the ultrastructural level, hyphae were cultivated on 100 µM farnesol for 2 h and examined by TEM. Control hyphae cells showed typical cellular structures (Figure 4A and B). Cytoplasmic organelles of cells such as mitochondria, nucleus could be clearly distinguished. Cell wall and plasma membrane were also observed as intact structure. In hyphae, mitochondria, the most visible submicroscopic organelles, as engine for hyphal growth, indicated that the hyphae were in a very rapidly growing process. Vacuoles were
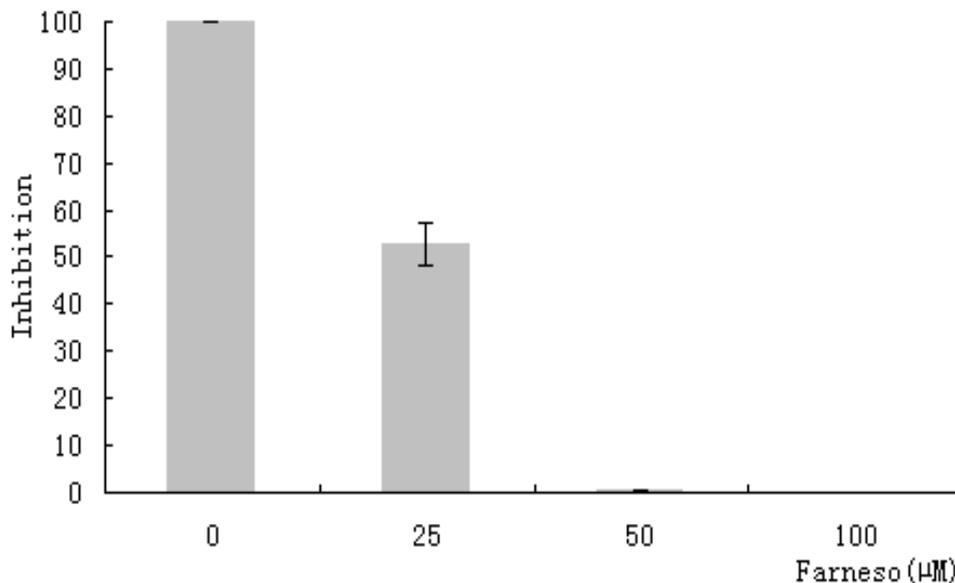
Figure 2. Farnesol inhibits the growth of *P. expansum.* The results were acquired by comparing farnesol-treated cultures formed the number of colonies per plate with control. Each data point represents the average of three replicates and Bars represent standard deviations of the means.
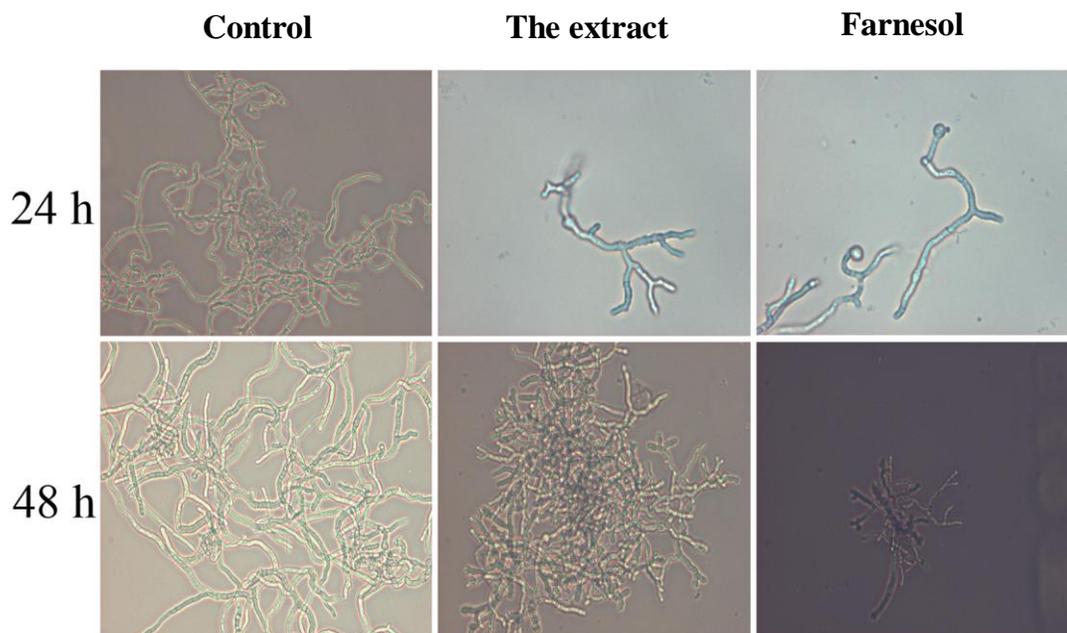

Figure 3. Effect of the extract (0.1% v/v) and farnesol (25μM) to mycelium growth of *P. expansum.*

small, empty or loosely distributed. In contrast, farnesol-treated cells exhibited extensive cytoplasmic damages (Figure 4C and D). The shrunk cytoplasm which kept slippery and integrates was detached from the cell wall (Figure 4C). Mitochondria were less well defined and often displayed discontinuous or missing inner mem-branes and cristae (Figure 4C), and large globular mitochondria that were partially or completely invaded by a vacuole (white arrow) served as another prominent feature. The rapid growth of hyphae was evidently inhibited. These results indicate that disruption of cytoplasmic structures and internal cellular disintegration
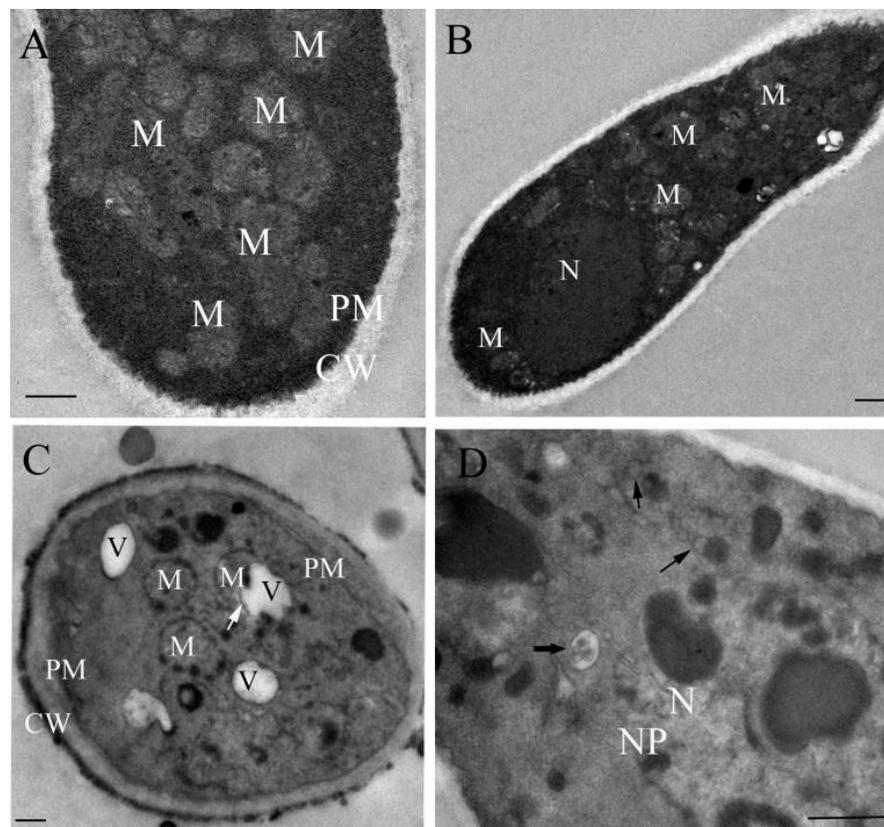
Figure 4. TEM images of the cellular ultrastucture of *P. expansum*. Hyphae were treated with 100μM farnesol for 2 h at 28°C. Specimens were fixed immediately with 2.5% glutaraldehyde for 24 h. A and B: control; C and D: treatment; CW, cell wall; M, mitochondrion; N, nucleus; NP, nuclear envelope; PM, plasma membrane; V, vacuole; Scale bars: 0.5 μm.

played a major role in the cytotoxicity of farneol.

ROS generation of *P. expansum* hyphal cells was visualized by H$_2$DCFDA, which was a cell-permeable indicator for ROS. The results showed that the contents of ROS was progressively increased to nearly twentyfold within 30 min after treatment with 100 μM farnesol compared with control (Figure 5).

DISCUSSION

C. ernobii strain Pg2-5 isolated from the surface of citrus has been proved to availably inhibit postharvest stem-end rot disease of *citrus* that caused by *D. natalensis* (Chen et al., 2007). In this study, the strain Pg2-5 also has the potency in controlling postharvest pathogen *P. expansum* L. The identity of the compounds produced by strain Pg2-5 was confirmed to be farnesol. Farnesol is a 15-carbon isoprenoid alcohol widely distributed in nature as an odoriferous component that may be generated within cells by enzymatic dephosphorylation of farnesyl pyro-phosphate, an intermediate of the metabolic pathway

yielding sterols and other isoprenoid compounds from mevalonate (Lan et al., 2013). It has been reported that farnesol inhibits the growth of various important plant pathogenic and zoopathogenic fungi such as *A. nidulans* (Semighini et al., 2006), *C. albicans* (Shirtliff et al., 2009) and *B. cinerea* (Cotoras et al., 2013). In this study, farnesol strongly inhibited *P. expansum* hyphal growth and promoted cell death. ROS burst and mitochondria disruption may be the target for the cytotoxicity of farnesol.

In microbes, cell-cell communication is referred to as quorum sensing (QS) (Bassler, 2002). The signals of QS systems are small molecules called auto-inducers. Many microbes possess the capability of producing antimicrobials, such as phenylcaetic acid and sodium phenyacetate (Hwang et al., 2001). Maybe, these antifungal compounds secreted from microorganism just as one type of QS molecules protect themselves from others microorganisms in a limited circumstance. In the previous study, antibiotics purified from biocontrol agents of bacteria have been shown to be effective in controlling postharvest pathogens (Alemu and Alemu, 2013), such
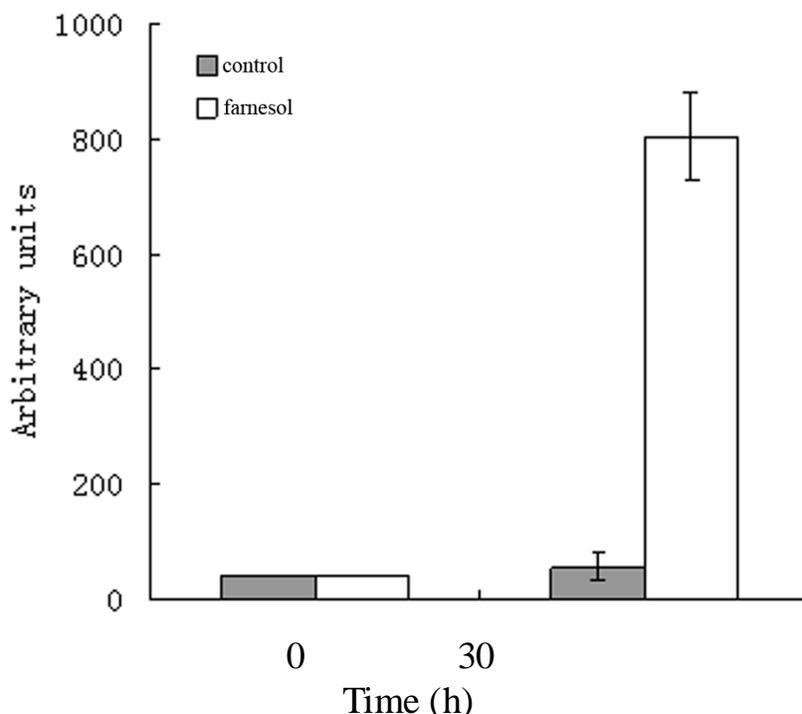
Figure 5. Intracellular ROS production in *P. expansum*. Each data point represents the average of three replicates and Bars represent standard deviations of the means.

as pyrrolnitrin produced by *Pseudomonas cepacia* was applied in controls gray and green mold on apples and pears (Rinez et al., 2013). Now, some antifungal compounds have been directly utilized for the biocontrol of fungal pathogens (Nguyen et al., 2009; Zhang et al., 2012; Fan et al., 2013).

To conclude, a good understanding of the mechanism of action of antagonist yeasts is important for selection more effective biocontrol methods. Based on the present studies, Pg2-5 that showed antagonistic activities against postharvest pathogen *P. expansum* were capable of producing antifungal substances. Further investigation will focus on other biological activities (hydrolytic enzymes, competition for nutrients and space, parasitism, and induced resistance in the host tissue).

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