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Biocontrol of postharvest green mold of oranges by *Hanseniaspora uvarum* Y3 in combination with phosphatidylcholine

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ABSTRACT

The effect of phosphatidylcholine (soybean extract) on the biocontrol efficacy of the antagonistic yeast *Hanseniaspora uvarum* Y3 against green mold of oranges was investigated. The results showed that *H. uvarum* Y3 significantly inhibited green mold of oranges at different concentrations compared with the control. Besides, phosphatidylcholine was observed to enhanced the biocontrol efficacy of *H. uvarum* Y3. Among the different concentrations, phosphatidylcholine at 1.5% w/v combined with *H. uvarum* Y3 exhibited the best control effect, the green mold incidence of oranges was only 4.63%, while the incidence was 33.33% when treated with *H. uvarum* Y3. After 24 h incubation, phosphatidylcholine at the different concentrations did not significantly influence the population of *H. uvarum* Y3 in NYDB. However, it was found that, phosphatidylcholine (1.5% w/v) significantly improved the population dynamics of *H. uvarum* Y3 in orange wounds at 20°C. The scanned electron microscope (SEM) results showed that *H. uvarum* Y3 combined with phosphatidylcholine (1.5% w/v) significantly inhibited the spore germination and mycelial growth of *Penicillium digitatum* in orange wounds. Moreover, *H. uvarum* Y3 in combination with 1.5% phosphatidylcholine treatment did not impair postharvest qualities of oranges. Conclusively, *H. uvarum* Y3 in combination with phosphatidylcholine offers a great potential as an alternative method against postharvest green mold of oranges.

Keywords: phosphatidylcholine; *Hanseniaspora uvarum* Y3; oranges; green mold; biocontrol

1. Introduction

Green mold, caused by *Penicillium digitatum*, is one of the most endemic postharvest diseases of oranges (Eckert and Ogawa, 1985). This pathogen is of main concern as it is responsible for 90% of oranges deterioration during the storage period, resulting in serious economic losses (Ariza et al., 2002). Control of postharvest diseases of oranges is mainly carried out by the application of fungicides (Eckert and Ogawa, 1985; Palou et al., 2001). However, a growing environmental contamination and human health problems caused by misapplication of synthetic fungicides on food crops have raised global concern (Norman, 1988). Besides, the frequent application of synthetic fungicides have led to the proliferation of fungicide-resistant strains and increasingly degenerated the effectiveness of these fungicides (Holmes and JW, 1999; Kinay et al., 2007; Salvatore et al., 2006; Spotts and Cervantes, 1986). *Geotrichum citri-aurantii*, for instance, has recently been found to be resistant to thiabendazole (TBZ) and imazalil (IMZ) (Montesinos-Herrero and Palou, 2010). Therefore, it is necessary to develop alternative methods to synthetic chemicals.

Microbial antagonists have shown potential as alternative measures to synthetic fungicides for disease control (Ippolito et al., 2000; Smilanick, 2004). Many microbial antagonists including *Bacillus subtilis* (Rodrigues et al., 2010), *Candida oleophila* (Droby et al., 2002), *Pichia guilliermondii* (Lahlali et al., 2011), *Cryptococcus laurentii* (Zhang et al., 2005) were reported to greatly inhibit *P. digitatum* and *P. italicum* of oranges. However, their biocontrol efficacy under semi-commercial conditions are often lower than synthetic fungicides (Droby et al., 1998; Spadaro and

Gullino, 2004). In order to replace synthetic fungicides, the efficacies of antagonists need to be enhanced (Janisiewicz and Korsten, 2002). Previous studies showed that, sodium bicarbonate improved the efficacy of antagonistic microorganisms *B. amyloliquefaciens* (Hong et al., 2014), *Pantoea agglomerans* (Usall et al., 2008) and *Trichoderma harzianum* (Alvindia, 2013) against postharvest green mold, blue mold and sour rot of oranges. Chitosan combined with *Rhodosporidium paludigenum* (Lu et al., 2014) and *C. saitoana* (El-Ghaouth et al., 2000) respectively inhibited postharvest green mold and blue mold of oranges and the efficacies were better than the antagonists treated alone.

Soybean lecithin is an excellent natural amphoteric surfactant with the characteristics of good surface activity, unique physiological activity, non-toxic, non-pollutant, non-stimulant and easily biodegradable. Phosphatidylcholine is regarded as an emulsifier, dispersion agent, viscous blending agent, lubricant, anti-splashing oil agent, leavening agent, antioxidant and nutritional agent (Rydhag and Wilton, 1981). Phosphatidylcholine is widely used in pharmaceutical, chemical, textile, food, pesticides, cosmetics and other industries. In the last decade, Japanese scholars found that, lecithin greatly controlled preharvest powdery mildew of cucumber, eggplant, strawberry and green pepper and developed the lecithin organic pesticide (Lecithinon). However, to the best of our knowledge, there is no research on the prevention of postharvest green mold of orange fruits by using the combination of yeast and phosphatidylcholine.

Hanseniaspora uvarum has been previously reported as a potential biological

control agent for the control of postharvest gray mold decay of grapes (Liu et al., 2010a; Liu et al., 2010b; Qin et al., 2015) and preharvest disease of strawberry (Cai et al., 2015). However, there is little information concerning the effect of *H. uvarum* on postharvest green mold of oranges and its effect on quality parameters of fruits. The objective of this study was to evaluate the synergetic effect of *H. uvarum* Y3 and phosphatidylcholine on postharvest control of green mold of oranges and (1) to determine its efficacy in biocontrol of green mold of oranges, (2) to determine the population of *H. uvarum* Y3 *in virto* and *in vivo*, (3) to determine the spore germination and mycelial growth of *P. digitatum*, (4) to evaluate the effects of *H. uvarum* Y3 and phosphatidylcholine on the natural decay and storage quality of oranges, and (5) to investigate the inhibition of the growth of *P. digitatum* hyphase on orange woulds by *H. uvarum* Y3 treated with water and phosphatidylcholine, using Scanned electron microscope, in order to explore the possible mechanisms involved.

2. Materials and methods

2.1. Fruits

Oranges (*Citrus sinensis* (L.) Osbeck) cultivar 'Gongchuan' were harvested from the orchard in Zhenjiang, China, and selected randomly based on uniformity of size, ripeness and absence of apparent injuries or infections before any commercial postharvest treatments were applied. Fruits were soaked by 0.1% sodium hypochlorite (Sangon Biotech (Shanghai) Co., Ltd) for 1-2 min, rinsed with tap water, and allowed to air dry at room temperature.

2.2. Phosphatidylcholine

Phosphatidylcholine (soybean lecithin powder), was obtained from Guangzhou Haisha Biological Technology Co. Ltd., China.

2.3. Pathogen

P. digitatum was isolated from decayed oranges and inoculated on potato dextrose agar medium (PDA: 200 mL of extract from boiled potatoes, 20 g of dextrose, 20 g of agar and 800 mL of distilled water). PDA plates were incubated at 25°C for 7 days, then transferred to 4°C refrigerated condition before use. Spore suspensions were prepared by removing the spores from the sporulating edges of PDA culture with inoculating loop and suspended in sterile distilled water. Spore concentrations were adjusted as required to 1×10^5 spores/mL in sterile distilled water by a hemocytometer (XB-K-250, Jianling Medical Device Co., Danyang, China).

2.4. Antagonist

The yeast antagonist *H. uvarum* Y3 was isolated by Wanhai Li, from the surfaces of unsprayed grapes in vineyard, Zhenjiang, China and maintained at 4°C on nutrient yeast dextrose agar (NYDA; 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose, 20 g of agar and 1000 mL of distilled water). The yeast strain was preserved in the China General Microbiological Culture Collection Center, and numbered as CGMCC2.5414. *H. uvarum* Y3 was cultured in 250 mL Erlenmeyer flasks containing

50 mL of nutrient yeast dextrose broth (NYDB; 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose and 1000 mL of distilled water). Flasks were incubated in a rotary shaker at 180 rpm for 20 h at 28°C. Following incubation, cells were centrifuged (TGL-16M Centrifuge, Xiangyi Company, Changsha, China) at 8000 × g for 6 min and washed twice with sterile distilled water in order to remove the growth medium. Yeast cell pellets were re-suspended in sterile distilled water and phosphatidylcholine solution, adjusted to the required concentration by a hemocytometer (XB-K-250, Jianling Medical Device Company, Danyang, China).

2.5. Effect of H. uvarum Y3 *at different concentrations on green mold incidence and lesion diameter of oranges*

Treatments used in this experiment were sterile distilled water (CK), and *H. uvarum* Y3 at different concentrations (1×10^6 cells/mL; 1×10^7 cells/mL; 1×10^8 cells/mL; 1×10^9 cells/mL). Three uniform wounds (5 mm diameter and 3 mm deep) were made at the equator of each orange fruit using a sterile borer (using a spirit lamp to sterilize, 5 mm diameter). An aliquot (30 μL) of cell suspensions of each treatment was pipetted into each wound site, respectively. Two hours later, 30 μL of *P. digitatum* suspension (1×10^5 spores/mL) was inoculated into each wound. Treated fruits were stored in incubator at 20°C and 95% RH, and the fruits were randomized block. Seven days later, decay incidence and lesion diameter of the *H. uvarum* Y3 and the control treatments on the fruits were measured with a slide caliper. There were three replicates of twelve fruits for each treatment, and the experiment was repeated twice.

2.6. Effect of phosphatidylcholine on biocontrol efficacy of H. uvarum Y3 (1×10^8 cells/mL) *to inhibit green mold of oranges*

The treatments applied on the fruits were sterile distilled water (CK), phosphatidylcholine (P) at the different concentrations (0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, and 5% w/v), *H. uvarum* Y3 (Y) (1×10^8 cells/mL), *H. uvarum* Y3 combined with phosphatidylcholine (P) at the different concentrations (0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, and 5% w/v), and make the final concentration of *H. uvarum* Y3 was 1×10^8 cells/mL (yeast cells were resuspended in phosphatidylcholine suspension at different concentrations). Three uniform wounds (5 mm diameter and 3 mm deep) were made at the equator of each orange fruit using a sterile borer. An aliquot (30 μL) of cell suspensions of each treatment was pipetted into each wound site, respectively. Two hours later, 30 μL of *P. digitatum* suspension (1×10^5 spores/mL) was inoculated into each wound. Treated fruits were stored in incubator at 20°C and 95% RH, and the fruits were randomized block. After 7 days, decay incidence and lesion diameter of the treated fruits were measured with a slide caliper. There were three replicates of twelve fruits for each treatment, and the experiment was repeated twice.

2.7 Effect of phosphatidylcholine at various concentrations on population dynamics of H. uvarum Y3 *in NYDB*

H. uvarum Y3 was cultured in 250 mL Erlenmeyer flasks containing 50 mL of

nutrient yeast dextrose broth (NYDB) at 180 rpm for 20 h at 28°C. Afterwards, 1 mL of *H. uvarum* Y3 was added into 50 mL NYDB alone or amended with phosphatidylcholine at the different concentrations (0.1%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, and 5% w/v). All flasks were incubated in rotary shaker at 180 rpm and 28°C for 24 h. The total cells of *H. uvarum* Y3 in the various liquid media were determined by optical microscope and expressed as Log_{10} cfu/mL. Serial 10-fold dilutions of various liquid media were made and 0.1 mL of each dilution was spread on NYDA. NYDA plates were incubated at 28°C and 95% RH. The live cells of *H. uvarum* Y3 in the various media were determined by counting single yeast colony on NYDA after 36-48 h incubation and expressed as Log_{10} cfu/wound. There were three replicates per treatment and the experiment was conducted twice.

2.8. Effect of phosphatidylcholine in combination with H. uvarum Y3 *on green mold of oranges after inoculation with P. digitatum*

The treatments applied on the fruits were sterile distilled water (CK), phosphatidylcholine (1.5% w/v), *H. uvarum* Y3 alone（1×10^8 cells/mL）or combined with phosphatidylcholine (1.5% w/v). The experiment was conducted according to the method of Abraham et al. (2010) with some slight modifications. Three uniform wounds (5 mm diameter and 3 mm deep) were made at the equator of each orange fruit using a sterile borer. An aliquot of 30 μL of *P. digitatum* suspension (1×10^5 spores/mL) was inoculated into each wound, respectively. Half an hour later, 30 μl of cell suspensions of each treatment was pipetted into each wound site. Treated fruits

were incubated at 20°C and 95% RH, and the fruits were randomized block. After 7 days, decay incidence and lesion diameter of the treated fruits were measured with a slide caliper. There were three replicates of twelve fruits for each treatment, and the experiment was repeated twice.

2.9 Effect of phosphatidylcholine on population dynamics of H. uvarum Y3 *in wounds of oranges*

The populations of *H. uvarum* Y3 were monitored in wounds of oranges. The oranges were wounded (5 mm diameter and approximately 3 mm deep) using a sterile borer. Each wound was treated with 30 μL suspension of *H. uvarum* Y3 (1×10^8 cells/mL) alone or in combination with phosphatidylcholine (1.5% w/v). *H. uvarum* Y3 was recovered from the wounds after incubation at 20°C for 0 (1 h after treatment), 1, 2, 3, 4, 5, 6 and 7 days, respectively. The wounded tissues were removed with a sterile knife and macerated in 30 mL of sterile 0.85% sodium chloride solution and quartz sand in a mortar. Serial 10-fold dilutions were made and 0.1 mL of each dilution was spread on NYDA. NYDA plates were stored in incubator at 28°C and 95% RH. The population of *H. uvarum* Y3 in wounds were determined by counting single yeast colony on NYDA after 36-48 h incubation and expressed as Log_{10} cfu/wound. There were three fruits replicates per treatment and the experiment was conducted twice.

2.10. Effects of phosphatidylcholine and H. uvarum Y3 *on spore germination and*

mycelial development of P. digitatum in vitro

The effects of phosphatidylcholine (1.5% w/v) and *H. uvarum* Y3 on spore germination and mycelial growth *of P. digitatum* were measured in potato-dextrose broth (PDB). Spores of *P. digitatum* and *H. uvarum* Y3 suspensions were prepared as described above. Erlenmeyer flasks of PDB 50 mL were prepared and 1mL of sterile distilled water was added as a control, in the second flask, 1 mL of *H. uvarum* Y3 was added and adjusted to a concentration of 1×10^8 cells/mL, and in the third flask, 1 mL of phosphatidylcholine combined with *H. avarum* was added and adjusted to a concentration of phosphatidylcholine at 1.5% w/v and *H. uvarum* Y3 concentration of 1×10^8 cells/mL. Aliquots of 1mL *P. digitatum* spore suspension (adjusted to the final concentration of 1×10^5 spores/mL) were added to each PDB flask, respectively. All flasks were incubated in rotary shaker at 75 rpm and 25°C for 18 h. Approximate 100 spores were observed by microscope and micrometer to determine germination rate and hyphae length per treatment within each replicate. Each treatment was replicated three times and the experiment was conducted twice.

2.11. Scanned electron microscope (SEM) analysis

Three treatments were tested in this experiment: (1) Control (sterile distilled water), (2) *H. uvarum* Y3, and (3) phosphatidylcholine (1.5% w/v) combined with *H. uvarum* Y3. Three uniform wounds of diameter 5 mm and depth 3 mm were made at the equator of each orange fruit using a sterile borer. An aliquot of 30 µL cell suspensions of each treatment was pipetted into each wound site, respectively. Two

hours later, 30 μL of *P. digitatum* suspension (1×10^5 spores/mL) was inoculated into each wound. Treated fruits were stored in incubator at 20°C and 95% RH. After 6 days, the effects of treatments were observed in wounds of oranges by SEM (XL30-ESEM).

2.12. Effects of H. uvarum Y3 *and phosphatidylcholine on natural decay and storage quality of oranges*

To evaluate the effects of *H uvarum* and phosphatidylcholine on natural decay and storage quality of postharvest oranges, we sprayed the disinfected oranges with (1) the control treatment (sterile distilled water), (2) *H. uvarum* Y3 (1×10^8 cells/mL) and (3) phosphatidylcholine (1.5% w/v) combined with *H. uvarum* Y3 (1×10^8 cells/mL), and then air dried. The treated fruits were sealed in polyethylene-lined plastic baskets to retain high humidity. Fruits were stored in incubator (20°C, 95% RH) for 30 days, and the fruits were randomized block. Natural decay (the rate of the decay fruits in all treated fruits) and quality parameters were measured after storage. There were three replicates of 48 fruits for each treatment. The storage quality parameters of the treated fruits were determined as described below:

Weight loss: The weight of orange was measured by an MP2000-2 balance (±0.001 g) (Shanghai Balance Instruments, China). Weight loss of treated fruits was determined by the formula (A-B)/A where A is defined as weight before treatment and B is the weight after treatment.

Fruit firmness: Firmness values of each individual orange were measured at the

points of the equatorial region by using the TA-XT Plus Texture Analyser (Microstable Instruments, UK) with a 3 mm diameter flat probe (Zhang et al., 2008). The probe descended towards the sample at 1.0 mm/s, and the distance that the probe travelled through the sample is 6 mm. The maximum force (N) was defined as firmness.

Total soluble solids: Total soluble solids (TSS) were determined by measuring the refractive index of the fruits juice with a hand refractometer and the results expressed as percentages (g per 100 g fruit weight) (Manganaris et al., 2007).

Titratable acidity: Titratable acidity was determined according to the method of Wright and Kader (1997) with some slight modifications. Titratable acidity was measured by titrating 0.1 N NaOH to pH 8.1; 4 g of juice diluted with 20 mL of distilled water. All replicates were evaluated. Titratable acidity was calculated as percent citric acid.

Ascorbic acid: Ascorbic acid was evaluated using UV spectrophotometer method. Firstly, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mL of 100 μg/mL vitamin C standard solution were added into test tubes and adjusted with distilled water to 10.0 mL. The solution was homogenized. The absorbance of vitamin C in the solution was determined at the wavelength of 243 nm. Taking the content of vitamin C (g) as the horizontal coordinates, the absorbance (A243) was used as y-coordinates to make the standard curve. Thirty grams of pulp from 5 oranges was placed in a mortar and 20 mL of 1% HCl was added to it. The homogenate was then transferred to a 50 mL volumetric flask and adjusted to 50 mL with distilled water. The solution was centrifuged at

10000 × g for 10 min and 0.4 mL supernatant was added to a 10 mL volumetric flask with 0.8 mL 10% HCl, adjusted with distilled water to the required mark and vortexed to homogenize the solution. In the other sample, distilled water as blank, the absorbance was determined at the wavelength of 243 nm, 0.4 mL extraction solution, 4 mL distilled water and 1.6 mL1 mol/L NaOH solution were added to the 10 mL volumetric flask, mixed, 15 min later, 1.6 mL 10% HCl was added, mixed, distilled water was added to the required mark. Distilled water as the blank, the absorbance of solution was measured at 243 nm. According to the absorbance differences between analyte liquid and alkali treatment liquid. The results of vitamin C content in the sample were determined automatically on the Vc standard curve of the UV spectrophotometer.

2.13. Statistical analysis

The data were analyzed by the analysis of variance (ANOVA) in the statistical program SPSS/PC version II.x, (SPSS Inc. Chicago, Illinois, USA) and the Duncan's Multiple Range Test and Tukey's Test were used for means separation. The statistical significance was applied at the level $P < 0.05$. In order to determine the type of effect (additive, synergistic or antagonistic) of phosphatidylcholine and *H. uvarum* Y3, the methodology and statistical analysis by De Curtis et al. (2012) was cited.

3. Results

3.1. Effect of H. uvarum Y3 *at different concentrations on green mold incidence and*

lesion diameter of oranges

Efficacy of *H. uvarum* Y3 at different concentrations against green mold incidence and lesion diameter of oranges was examined. The results of the decay incidence of orange wounds treated with *H. uvarum* Y3 at different concentrations reduced by 75.3%, 60.5%, 35.8% and 27.2%. The decay incidence of all treatments were significantly lower than the control treatment (Fig. 1A). From Fig. 1A, the lowest decay incidence was observed at concentrations of 1×10^8 cells/mL and 1×10^9 cells/mL. For lesion diameter, the same trend was observed. *H. uvarum* Y3 at different concentrations significantly inhibited lesion diameter of oranges compared with the control. When *H. uvarum* Y3 concentration was 1×10^8 cells/mL or 1×10^9 cells/mL, the lesion diameter recorded the least value (Fig. 1B).

3.2. Efficacy of H. uvarum Y3 *in combination with phosphatidylcholine in inhibiting postharvest green mold of oranges*

The effect of phosphatidylcholine on the antagonistic activity of *H. uvarum* Y3 against *P. digitatum* on oranges was examined. After 7 days, phosphatidylcholine at all tested different concentrations did not reduce the green mold incidence but inhibited lesion diameter of *P. digitatum* in postharvest orange wounds. *H. uvarum* Y3 alone and *H. uvarum* Y3 combined with different concentrations of phosphatidylcholine all significantly reduced the green mold incidence and lesion diameter of *P. digitatum* compared with the control (Fig. 2A and B). Different concentrations of phosphatidylcholine provided synergistic effect of *H. uvarum* Y3 on

postharvest *P. digitatum* in orange wounds (SF>1). Especially, the combined treatment of phosphatidylcholine (1.5% w/v) with *H. uvarum* Y3 was the most effective treatment and reduced the green mold incidence and lesion diameter of fruits up to 93.5% and 5.6 mm, respectively.

3.3 Effect of phosphatidylcholine at various concentrations on population dynamics of H. uvarum Y3 *in NYDB*

As shown in Fig. 3, after 24 h incubation, NYDB amended with phosphatidylcholine at the different concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, and 5% w/v) did not significantly affected the total and live cells of *H. uvarum* Y3 compared with NYDB alone.

3.4. Effect of phosphatidylcholine (1.5% w/v) in combination with H. uvarum Y3 *on green mold of oranges after inoculation with P. digitatum*

When orange wounds were treated with *H. uvarum* Y3 alone or combined with phosphatidylcholine (1.5% w/v) after inoculation with the pathogen at 20°C, both treatments significantly reduced green mold incidence and lesion diameter of postharvest oranges compared with the control (Fig. 4. A and B and Fig. 5.).

3.5. Effects of phosphatidylcholine on population growth of H. uvarum Y3 *in orange wounds*

The population of *H. uvarum* Y3 in orange wounds treated with water or

phosphatidylcholine both increased to more than 30-fold in the first day at 20℃. *H. uvarum* Y3 treated with phosphatidylcholine (1.5% w/v) multiplied rapidly in orange wounds within 3 days at 20℃, and reached the maximum at the 3rd day, which was approximately 4.2-fold as *H. uvarum* Y3 treated with water at the same day (2.45×10^8 cells/wound) (Fig. 6). After the 3rd day, *H. uvarum* Y3 decreased then stabilized at a high level ($3.75\text{-}7.67 \times 10^8$ cells/wound). Similarly, *H. uvarum* Y3 treated with water multiplied rapidly in orange wounds within 4 days, and reached the maximum at the 4th day (6.69×10^8 cells/wound), after which *H. uvarum* Y3 decreased then stabilized at a high level ($2.83\text{-}5.50 \times 10^8$ cells/wound). The population of *H. uvarum* Y3 in orange wounds treated with phosphatidylcholine was higher than that treated with water at 2, 3 and 7d.

3.6. Effects of phosphatidylcholine and H. uvarum Y3 *on spore germination and mycelial growth of P. digitatum in vitro*

The results showed that *H. uvarum* Y3 alone or combined with phosphatidylcholine (1.5% w/v) significantly decreased spore germination and hyphae length *of P. digitatum in vitro* from 86.7% and 321.6 μm to 24.3% and 87.6 μm, 11.7% and 26.4 μm compared with the control, respectively (Fig. 7A and B).

3.7. Scanned electron microscope (SEM) of the germination and growth of P. digitatum in orange wounds

Scanned electron micrographs showed that there were dense long mycelia in

orange wounds for control treatment (Fig. 8A). Spore germination and mycelial growth of *P. digitatum* in orange wounds were inhibited with *H. uvarum* Y3 treatment alone (Fig. 8B). There were just a few short mycelia together with some yeast cells in orange wounds treated with *H. uvarum* Y3 alone (Fig. 8B). However, when *H. uvarum* Y3 was treated with phosphatidylcholine (1.5% w/v), more yeast cells and almost no mycelia were found in orange wounds (Fig. 8C).

3.8. Effects of H. uvarum Y3 *combined with phosphatidylcholine on storage quality of oranges*

As shown in Table 1, *H. uvarum* Y3 treatment alone and *H. uvarum* Y3 combined with phosphatidylcholine (1.5% w/v) all significantly reduced natural decay of postharvest oranges compared with the control, the natural decay of oranges treated with *H. uvarum* Y3 combined with phosphatidylcholine (1.5% w/v) was 20.83%, significantly lower than treated with *H. uvarum* Y3 (31.94%) and the control (44.44%). Phosphatidylcholine (1.5% w/v) enhanced the efficacy of *H. uvarum* Y3 in controlling natural decay of postharvest oranges. There were no significant influences with respect to storage quality parameters such as firmness, soluble solids, titratable acidity, ascorbic acid except weight loss to oranges in three treatments. Weight loss of oranges treated with *H. uvarum* Y3 in combination with phosphatidylcholine (1.5% w/v) was significantly less than control treatment or *H. uvarum* Y3 treatment alone.

4. Discussion

H. uvarum has been previously reported as a potential biological control agent for the control of postharvest gray mold decay of grapes (Liu et al., 2010a; Liu et al., 2010b; Qin et al., 2015) and preharvest disease of strawberry (Cai et al., 2015). However, there is little information concerning the effect of *H. uvarum* on postharvest green mold of oranges and its effect on quality parameters of fruits. Combination of biocontrol agents with additives have proved to be effective methods to inhibit postharvest pathogens and reduce chemical residues on fruits in other studies (Arrebola et al., 2010). The objective of the study was evaluating the feasibility of combined application of microbial antagonist *H. uvarum* Y3 and soybean extract phosphatidylcholine to control postharvest green mold of oranges.

The results showed that *H. uvarum* Y3 has a potential as a biocontrol agent for the control of postharvest green mold of oranges caused by *P. digitatum*. *H. uvarum* Y3 at different concentrations significantly inhibited green mold of oranges compared with the control. The higher the concentration, the better and the efficacy was. This finding is in agreement with Lahlali et al. (2004), Lahlali et al. (2005) , Droby et al. (1989) and Nunes et al. (2001) who indicated that the biological efficacy of antagonistic yeasts dependent on the biocontrol agent's concentration and the pathogen inoculum concentration. Different concentrations of phosphatidylcholine significantly improved the efficacy of *H. uvarum* Y3 against postharvest green mold incidence compared with control at 20°C.

The ability of antagonists to survive and proliferate in fruits wounds when applied in orchards is a critical factor (Li et al., 2014; Macarisin et al., 2010). The

efficacy of antagonistic yeasts in inhibiting fruits decay caused by pathogen decreased because of the lower viability in wounds or on fruit surfaces (Liu et al., 2011). The population dynamics of *H. uvarum* Y3 *in vitro* suggested that, after 24 h incubation, phosphatidylcholine at the different concentrations did not significantly influence the total and live cells of *H. uvarum* Y3. While the population of *H. uvarum* Y3 *in vivo* showed that *H. uvarum* Y3 increased rapidly in orange wounds in both control and phosphatidylcholine at 20°C . The nutritional environment at the wounds might have favored *H. uvarum* Y3, which rapidly colonized the fruit tissues and allowed the yeast to easily have access to nutrients and space. The population of *H. uvarum* Y3 were greatly increased in wounds treated with phosphatidylcholine (1.5% w/v) at 20°C. Competition for nutrients and space has been cited as a main mechanism of antagonistic microorganisms (Droby et al., 2002; Sharma et al., 2009). These results suggest the possible mechanism by which *H. uvarum* Y3 might achieve biological control is through effective competition with the pathogen for space and nutrients. Phosphatidylcholine enhanced the biocontrol efficacy of *H. uvarum* Y3 to postharvest green mold of oranges by facilitating the growth of *H. uvarum* Y3 in orange wounds through the supply of nutrients.

H. uvarum Y3 alone or combined with phosphatidylcholine (1.5% w/v) significantly inhibited spore germination and mycelial development of *P. digitatum in vitro*. It was observed that, 1.5% phosphatidylcholine significantly improved the efficacy of *H. uvarum* Y3 in controlling germination and mycelial development of *P. digitatum* compared with *H. uvarum* Y3 treatment alone.

By scanning electron microscope (SEM) of the growth of *P. digitatum* and *H. uvarum* Y3 in orange wounds, we found spore germination and mycelial growth of *P. digitatum* in orange wounds were inhibited when treated with *H. uvarum* Y3 alone (Fig. 8B) or *H. uvarum* Y3 combined with 1.5% phosphatidylcholine (Fig. 8C). The results also showed that, the more the yeast cells grew in orange wounds, the less the spores of *P. digitatum* germinated. This study further showed that, antagonistic *H. uvarum* Y3 could rapidly colonized fruit wounds first and gained advantage to nutrients and space over the pathogen. Under these conditions, spore germination and mycelial development were greatly restricted and inhibited. These results suggest that the inhibitory effect on spore germination and mycelium growth may be one of the major mechanisms of *H. uvarum* Y3 and phosphatidylcholine inhibiting postharvest fungal disease. However, the precise interaction among *H. uvarum* Y3, phosphatidylcholine and spores of postharvest fungal pathogens remains unknown and needs to be studied further.

With regards to natural decay, the results showed that phosphatidylcholine (1.5% w/v) significantly enhanced the efficacy of *H. uvarum* Y3 against natural decay incidence of postharvest oranges. Results of the postharvest storage parameters showed that, there were no significant influence on storage quality parameters such as firmness, total soluble solids, titratable acidity and ascorbic acid to orange among three treatments. Furthermore, postharvest treatment with *H. uvarum* Y3 combined with 1.5% phosphatidylcholine significantly reduced weight loss in comparison with the control treatment. These results agree with the findings of Qin et al. (2015) who

used *H. uvarum* Y3 together with salicylic acid or sodium bicarbonate to reduce weight loss in grapes.

In conclusion, phosphatidylcholine at 1.5% w/v significantly enhanced the biocontrol activity of *H. uvarum* Y3 against postharvest green mold of oranges and influenced *H. uvarum* Y3 to increase rapidly in fruit wounds. Application of *H. uvarum* Y3 alone or combined with phosphatidylcholine significantly inhibited spore germination and mycelial development in orange wounds and had no influence on storage quality parameters. Postharvest treatment of *H. uvarum* Y3 combined with 1.5% phosphatidylcholine significantly reduced weight loss in comparison with the control treatment. Thus, *H. uvarum* Y3 in combination with phosphatidylcholine (1.5% w/v) may be a potential biocontrol method against postharvest green mold of oranges.

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References

Abraha, O., Mark, D., John, p., 2010. Isolation and in vivo screening of yeast and *Bacillus* antagonists for the control of *Penicillium digitatum* of citrus fruit. Biol. Control 53**,** 32-38.

Alvindia, D.G., 2013. Sodium bicarbonate enhances efficacy of *Trichoderma harzianum* DGA01 in controlling crown rot of banana. J. Gen. Plant Pathol. 79**,** 136-144.

Ariza, M.R., Larsen, T.O., Duus, J., Barrero, A.F., 2002. *Penicillium digitatum* metabolites on synthetic media and citrus fruits. J. Agric. Food Chem. 50**,** 6361-6365.

Arrebola, E., Jacobs, R., Korsten, L., 2010. Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* against postharvest fungal pathogens. J. Appl. Microbiol. 108**,** 386-395.

Cai, Z., Yang, R., Xiao, H., Qin, X., Si, L., 2015. Effect of preharvest application of *Hanseniaspora uvarum* on postharvest diseases in strawberries. Postharvest Biol. Technol. 100**,** 52-58.

De Curtis, F., De Cicco, V., Lima, G., 2012. Efficacy of biocontrol yeasts combined with calcium silicate or sulphur for controlling durum wheat powdery mildew and increasing grain yield components. Field Crops Res. 134**,** 36-46.

Droby, S., Chalutz, E., Wilson, C., Wisniewski, M., 1989. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Can. J. Microbiol. 35**,** 794-800.

Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M., Shachnai, A., 1998. Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. Biol. Control 12**,** 97-101.

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E., Porat, R., 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*. Phytopathology 92**,** 393-399.

Eckert, J.W., Ogawa, J.M., 1985. The chemical control of postharvest diseases: subtropical and tropical fruits. Ann. Rev. Phytopathol. 23**,** 421-454.

El-Ghaouth, A., Smilanick, J.L., Wilson, C.L., 2000. Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. Postharvest Biol. Technol. 19**,** 103-110.

Holmes, G.J., JW, E., 1999. Sensitivity of *Penicillium digitatum* and *P. italicum* to Postharvest Citrus Fungicides in California. Phytopathology 89**,** 716-721.

Hong, P., Hao, W., Luo, J., Chen, S., Hu, M., Zhong, G., 2014. Combination of hot water, *Bacillus amyloliquefaciens* HF-01 and sodium bicarbonate treatments to control postharvest decay of mandarin fruit.Postharvest Biol. Technol. 88**,** 96-102.

Ippolito, A., Ghaouth, A.E., Wilson, C.L., Wisniewski, M., 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biol. Technol. 19**,** 265–272.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Ann. Rev. Phytopathology 40**,** 411-441.

Kinay, P., Mansour, M.F., Gabler, F.M., Margosan, D.A., Smilanick, J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum*

collected in California. Crop Prot. 26**,** 647–656.

Lahlali, R., Hamadi, Y., Jijakli, M.H., 2011. Efficacy assessment of *Pichia guilliermondii* strain Z1, a new biocontrol agent, against citrus blue mould in Morocco under the influence of temperature and relative humidity. Biol. Control 56**,** 217-224.

Lahlali, R., Serrhini, M., Jijakli, M., 2004. Efficacy assessment of *Candida oleophila* (strain O) and *Pichia anomala* (strain K) against major postharvest diseases of citrus fruits in Morocco. Commun. Agric. Appl. Biol. Sci. 69**,** 601-609.

Lahlali, R., Serrhini, M., Jijakli, M., 2005. Development of a biological control method against postharvest diseases of citrus fruits. Commun. Agric. Appl. Biol. Sci. 47-58.

Li, C., Zhang, H., Yang, Q., Komla, M.G., Zhang, X., Zhu, S., 2014. Ascorbic acid enhances oxidative stress tolerance and biological control efficacy of *Pichia caribbica* against postharvest blue mold decay of apples. J. Agric. Food Chem. 62**,** 7612-7621.

Liu, H., Guo, J., Cheng, Y., Liu, P., Long, C., Deng, B., 2010a. Inhibitory activity of tea polyphenol and *Hanseniaspora uvarum* against *Botrytis cinerea* infections. Letters in Appl. Microbiol. 51**,** 258-263.

Liu, H.M., Guo, J.H., Cheng, Y.J., Luo, L., Liu, P., Wang, B.Q., Deng, B.X., Chao'an, A., 2010b. Control of gray mold of grape by *Hanseniaspora uvarum* and its effects on postharvest quality parameters. Annals Microbiol. 60**,** 31-35.

Liu, J., Wisniewski, M., Droby, S., Vero, S., Tian, S., Hershkovitz, V., 2011. Glycine

betaine improves oxidative stress tolerance and biocontrol efficacy of the antagonistic yeast *Cystofilobasidium infirmominiatum*. Int. J. Food Microbiol. 146**,** 76-83.

Lu, L., Liu, Y., Yang, J., Azat, R., Yu, T., Zheng, X., 2014. Quaternary chitosan oligomers enhance resistance and biocontrol efficacy of *Rhodosporidium paludigenum* to green mold in satsuma orange. Carbohydr. Polym. 113**,** 174-181.

Macarisin, D., Droby, S., Bauchan, G., Wisniewski, M., 2010. Superoxide anion and hydrogen peroxide in the yeast antagonist–fruit interaction: a new role for reactive oxygen species in postharvest biocontrol. Postharvest Biol. Technol. 58**,** 194-202.

Manganaris, G., Vasilakakis, M., Diamantidis, G., Mignani, I., 2007. The effect of postharvest calcium application on tissue calcium concentration, quality attributes, incidence of flesh browning and cell wall physicochemical aspects of peach fruits. Food Chem. 100**,** 1385-1392.

Montesinos-Herrero, C., Palou, L., 2010. Combination of physical and low-toxicity chemical postharvest treatments for integrated disease management of citrus fruit: A Review. Stewart Postharvest Rev. 6**,** 1-11(11).

Norman, C., 1988. EPA sets new policy on pesticide cancer risks. Science 242, 366-367.

Nunes, C., Usall, J., Teixidó, N., Viñas, I., 2001. Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2. Int. J. Food

Microbiol. 70**,** 53-61.

Palou, L., Smilanick, J.L., Usall, J., Viñas, I., 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate, and sodium bicarbonate. Plant Dis. 85**,** 371-376.

Qin, X., Xiao, H., Xue, C., Yu, Z., Yang, R., Cai, Z., Si, L., 2015. Biocontrol of gray mold in grapes with the yeast *Hanseniaspora uvarum* alone and in combination with salicylic acid or sodium bicarbonate. Postharvest Biol. Technol. 100**,** 160-167.

Rodrigues, L.B., Santos, L.R.d., Tagliari, V.Z., Rizzo, N.N., Trenhago, G., Oliveira, A.P.d., Goetz, F., Nascimento, V.P.d., 2010. Quantification of biofilm production on polystyrene by *Listeria, Escherichia coli* and *Staphylococcus aureus* isolated from a poultry slaughterhouse. Brazilian J. Microbiol. 41**,** 1082-1085.

Rydhag, L., Wilton, I., 1981. The function of phospholipids of soybean lecithin in emulsions. J. Am. Oil Chem. Soc. 58**,** 830-837.

Salvatore, D.A., Mario, S., Amedeo, P., Alberto, A., Paolo, C., Quirico, M., 2006. Residue levels and effectiveness of pyrimethanil vs imazalil when using heated postharvest dip treatments for control of *Penicillium* decay on citrus fruit. J. Agric. Food Chem. 54**,** 4721-4726.

Sharma, R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A Review. Biol. Control 50**,** 205-221.

Smilanick, J.L., 2004. Postharvest Diseases of Fruits and Vegetables: Development and Control: R. Barkai-Golan, Elsevier, Amsterdam, 2001, pp. 418, ISBN 0-444-50584-9. Postharvest Biol. Technol. 31**,** 213.

Spadaro, D., Gullino, M.L., 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. Int. J. Food Microbiol. 91**,** 185-194.

Spotts, R.A., Cervantes, L.A., 1986. Populations, pathogenicity, and benomyl resistance of *Botrytis spp., Penicillium spp*., and *Mucor piriformis* in packinghouses. Plant Dis. 70**,** 106-108.

Usall, J., Smilanick, J., Palou, L., Denis-Arrue, N., Teixidó, N., Torres, R., Viñas, I., 2008. Preventive and curative activity of combined treatments of sodium carbonates and *Pantoea agglomerans* CPA-2 to control postharvest green mold of citrus fruit. Postharvest Biol. Technol. 50**,** 1-7.

Wright, K.P., Kader, A.A., 1997. Effect of controlled-atmosphere storage on the quality and carotenoid content of sliced persimmons and peaches. Postharvest Biol. Technol. 10**,** 89-97.

Zhang, H.Y., Zheng, X.D., Xi, Y.F., 2005. Biological control of postharvest blue mold of oranges by *Cryptococcus laurentii* (Kufferath) Skinner. Biol. Control 50**,** 331-342.

Zhang, H.Y., Wang, L., Dong, Y., Jiang, S., Zhang, H.H., Zheng, X.D., 2008. Control of postharvest pear diseases using *Rhodotorula glutinis* and its effects on postharvest quality parameters. Int. J. Food Microbiol. 126**,** 167-171.

Table 1 Effects of *H. uvarum* Y3 combined with phosphatidylcholine on natural decay and storage quality of oranges at 20 ℃ for 30 days. The values are means ± standard deviations of three independent experiments, different letters represent significant difference ($P < 0.05$). CK: oranges treated with sterile distilled water as the control, Y: oranges treated with *H. uvarum* Y3, Y+1.5%P: oranges treated with *H. uvarum* Y3 combined with phosphatidylcholine (1.5%, w/v).

Treatment	Natural decay (%)	Weight loss （%）	Firmness（N）	TSS（%）	Titratable acidity（%）	Vc（mg/100g）
CK	44.444±8.420a	10.705±0.301a	11.745±0.795a	11.733±0.977a	0.496±0.010a	18.183±0.040a
Y	31.944±3.182b	10.043±0.409a	11.860±0.664a	11.4±0.921a	0.505±0.005a	18.827±0.192a
Y+1.5%P	20.833±2.083c	8.963±0.325b	11.344±0.437a	11.567±0.175a	0.490±0.018a	19.044±0.732a

Figure Captions:

Fig. 1 Effect of *H. uvarum* Y3 at different concentrations on green mold incidence (A) and lesion diameter (B) in orange wounds at **20°C** for 7 days. Bars represent standard errors. Within plots, means with the different letters are significantly different according to Duncan's Multiple Range Test at $P<0.05$.

Fig. 2 Effect of *H. uvarum* Y3 and phosphatidylcholine on green mold incidence (A) and lesion diameter (B) in orange wounds at 20°C for 7 days. Bars represent standard errors. Data in columns with the different letters are significantly different according to Duncan's Multiple Range Test at $P<0.05$.

Fig. 3 Effect of phosphatidylcholine at various concentrations on population dynamics of *H. uvarum* Y3 in NYDB. Bars represent standard errors. Data in columns with the different letters are significantly different according to Duncan's Multiple Range Test at $P<0.05$.

Fig. 4 Effect of phosphatidylcholine (1.5% w/v) in combination with *H. uvarum* Y3 on green mold of oranges after inoculation with *P. digitatum.* Bars represent standard errors. Data in columns with the different letters are significantly different according to Duncan's Multiple Range Test at $P<0.05$. CK: oranges treated with sterile distilled water as the control, 1.5%P: oranges treated with phosphatidylcholine (1.5%, w/v), Y: oranges treated with *H. uvarum* Y3, Y+1.5%P: oranges treated with *H. uvarum* Y3

combined with phosphatidylcholine (1.5%, w/v).

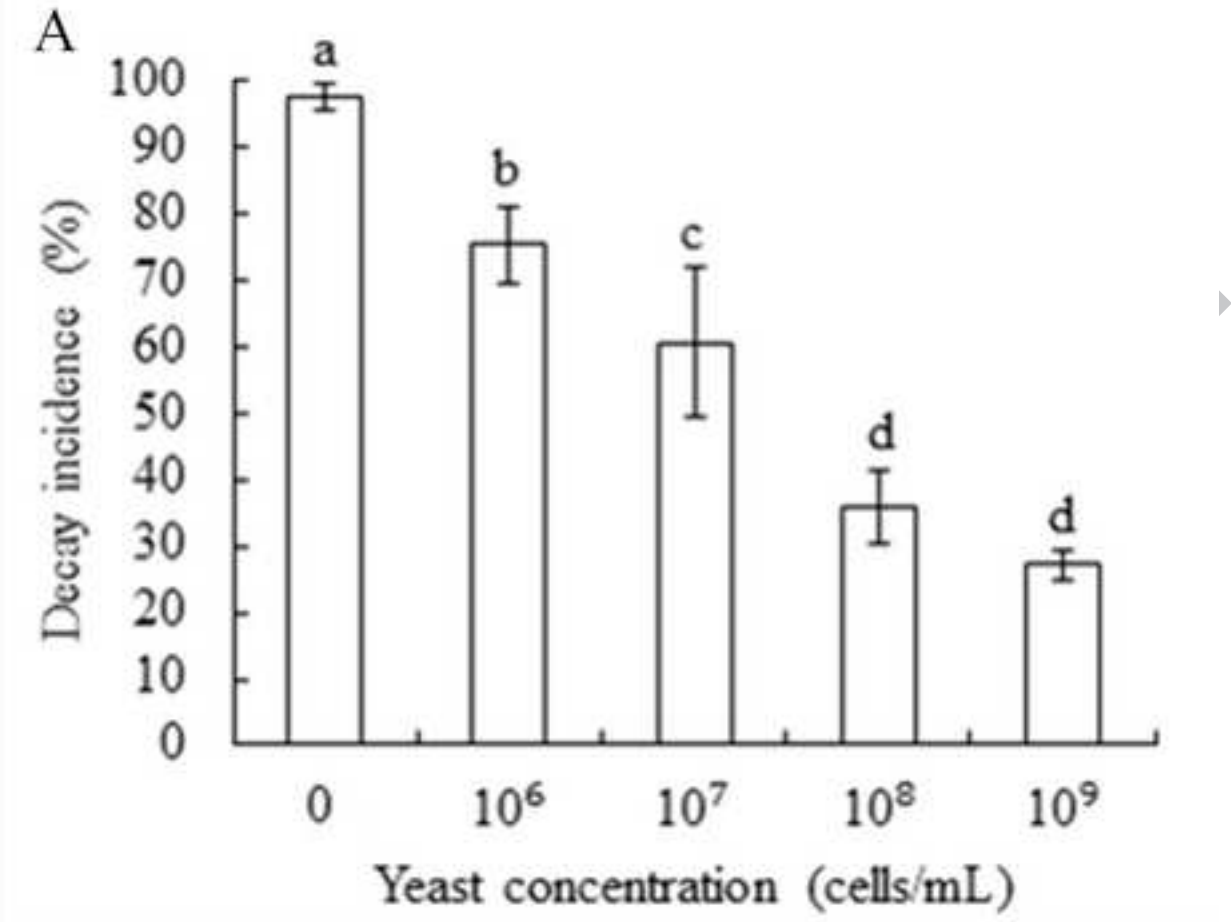
Fig. 5 Control of *P. digitatum* on oranges by *H. uvarum* Y3 in combination with phosphatidylcholine (1.5% w/v). CK: oranges treated with sterile distilled water as the control, 1.5%P: oranges treated with phosphatidylcholine (1.5%, w/v), Y: oranges treated with *H. uvarum* Y3, Y+1.5%P: oranges treated with H. uvarum Y3 combined with phosphatidylcholine (1.5%, w/v).

Fig. 6 Effect of phosphatidylcholine (1.5% w/v) on population dynamics of *H. uvarum* Y3 in wounds of oranges at 20°C. Bars represented standard errors. Data in columns with the different letters are significantly different according to Tukey's Test at $P<0.05$. Y: oranges treated with *H. uvarum* Y3, Y+1.5%P: oranges treated with H. uvarum Y3 combined with phosphatidylcholine (1.5%, w/v).

Fig. 7 Effects of phosphatidylcholine and *H. uvarum* Y3 on spore germination (A) and mycelial development (B) of *P. digitatum*. Germination rate and mycelial development were measured by microscope and micrometer after 18 h incubation at 25°C in PDB. Bars represent standard errors. Data in columns with different letters are statistically different according to Duncan's Multiple Range Test at $P<0.05$

Fig.8 Scanned electron micrographs of the germination and growth of *Penicillium digitatum* in orange wounds treated with *H. uvarum* Y3 at 20°C for 6 days. Fruit treatments were as follows: A=CK (sterile distilled water), B= *H. uvarum* Y3 *and* C = *H. uvarum* Y3 combined with phosphatidylcholine

Figure1



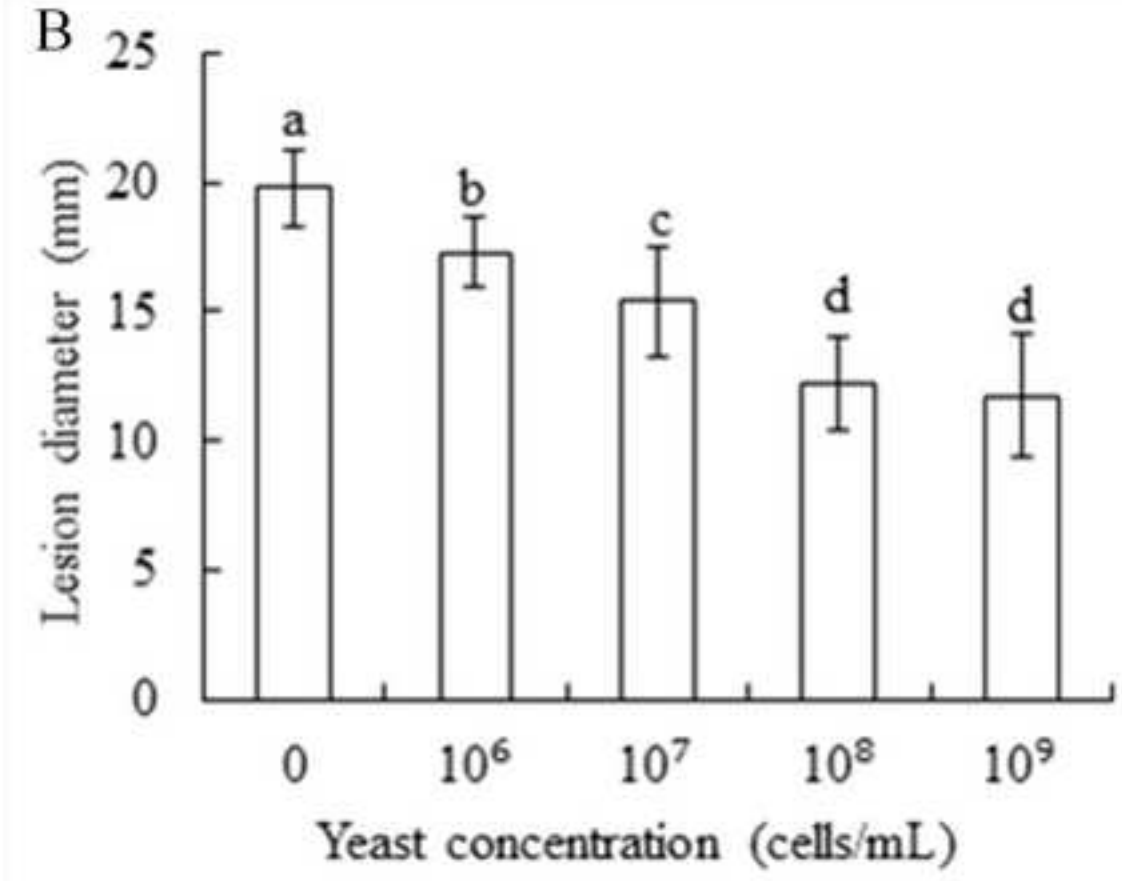
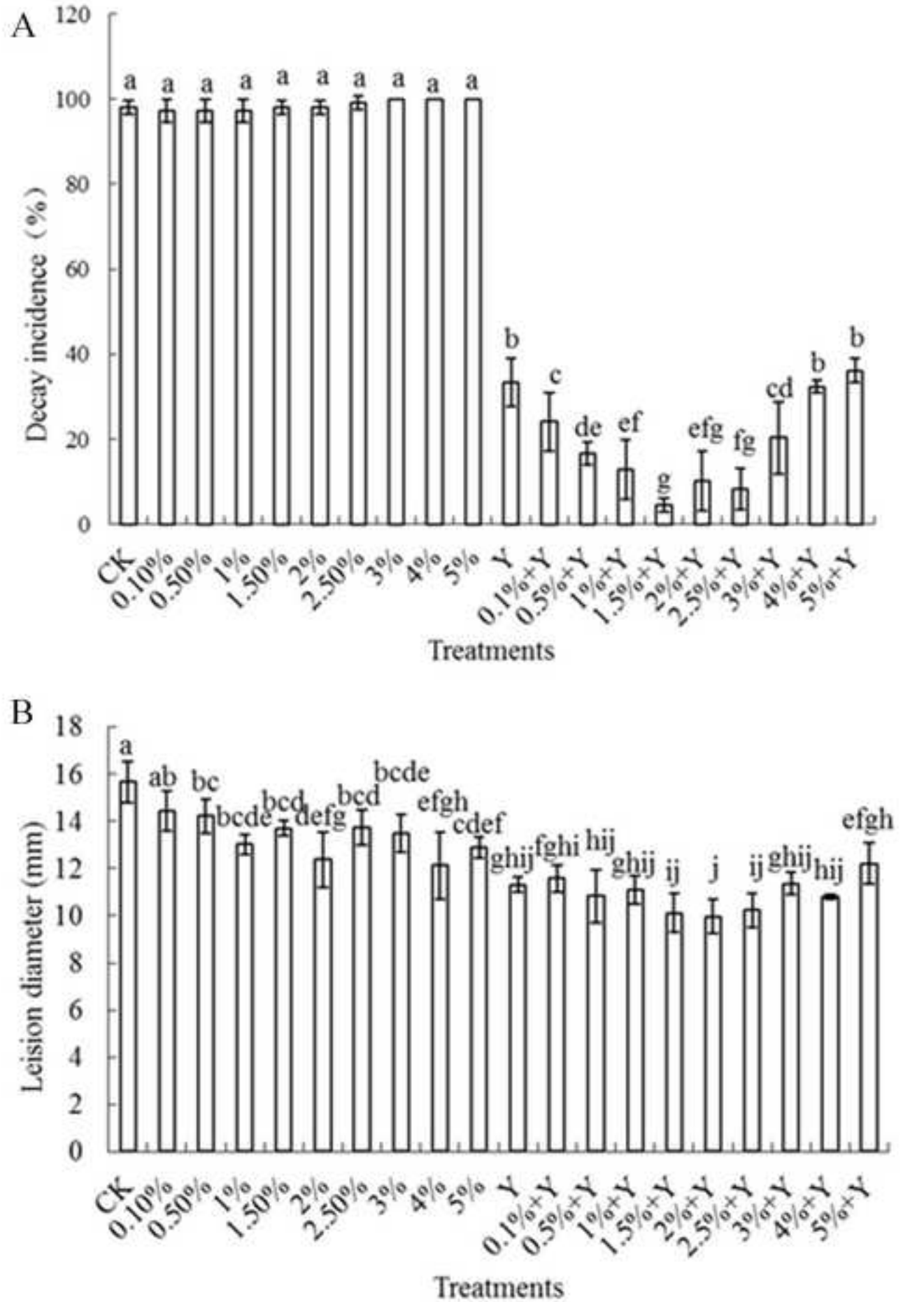


Figure2



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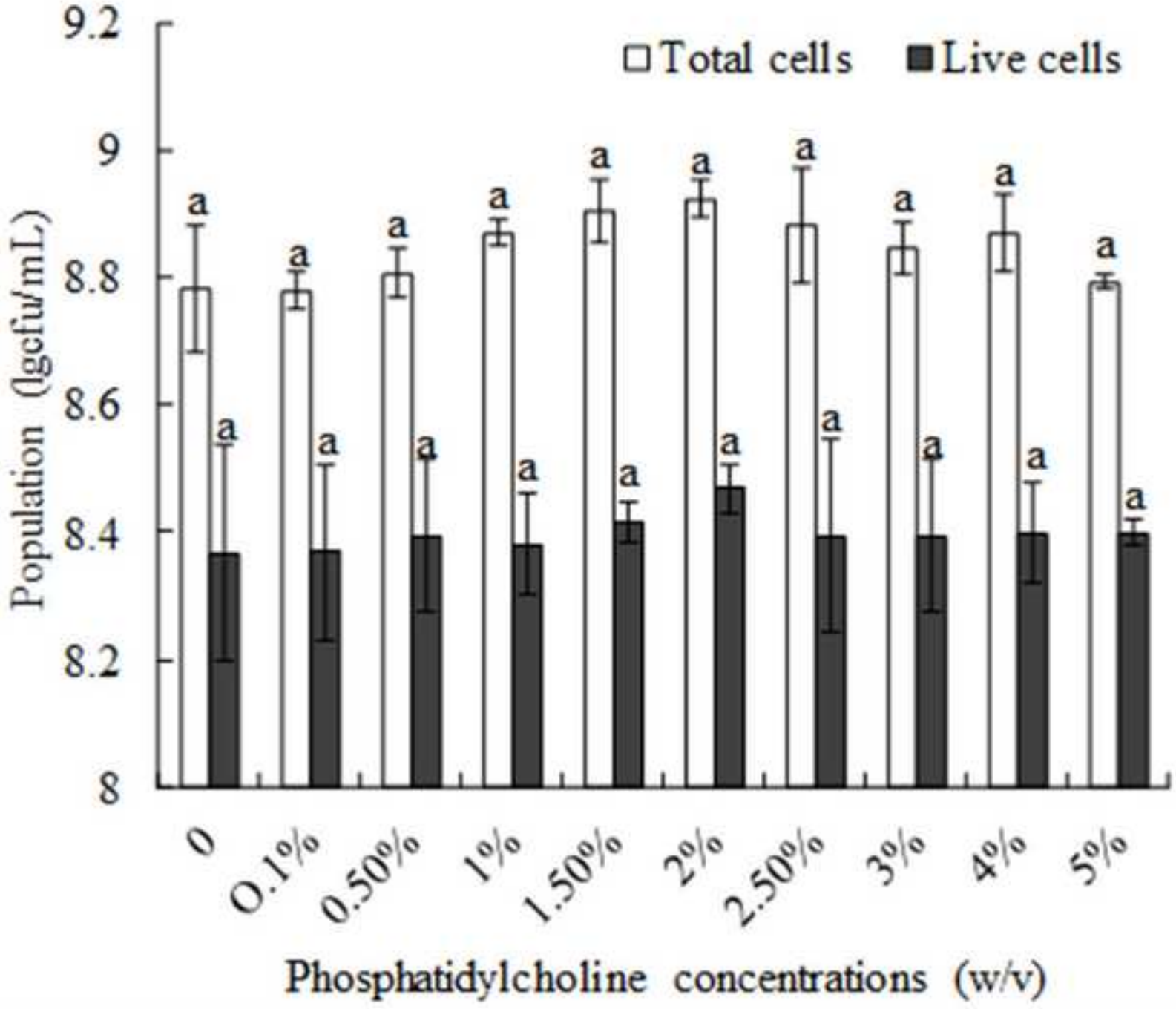
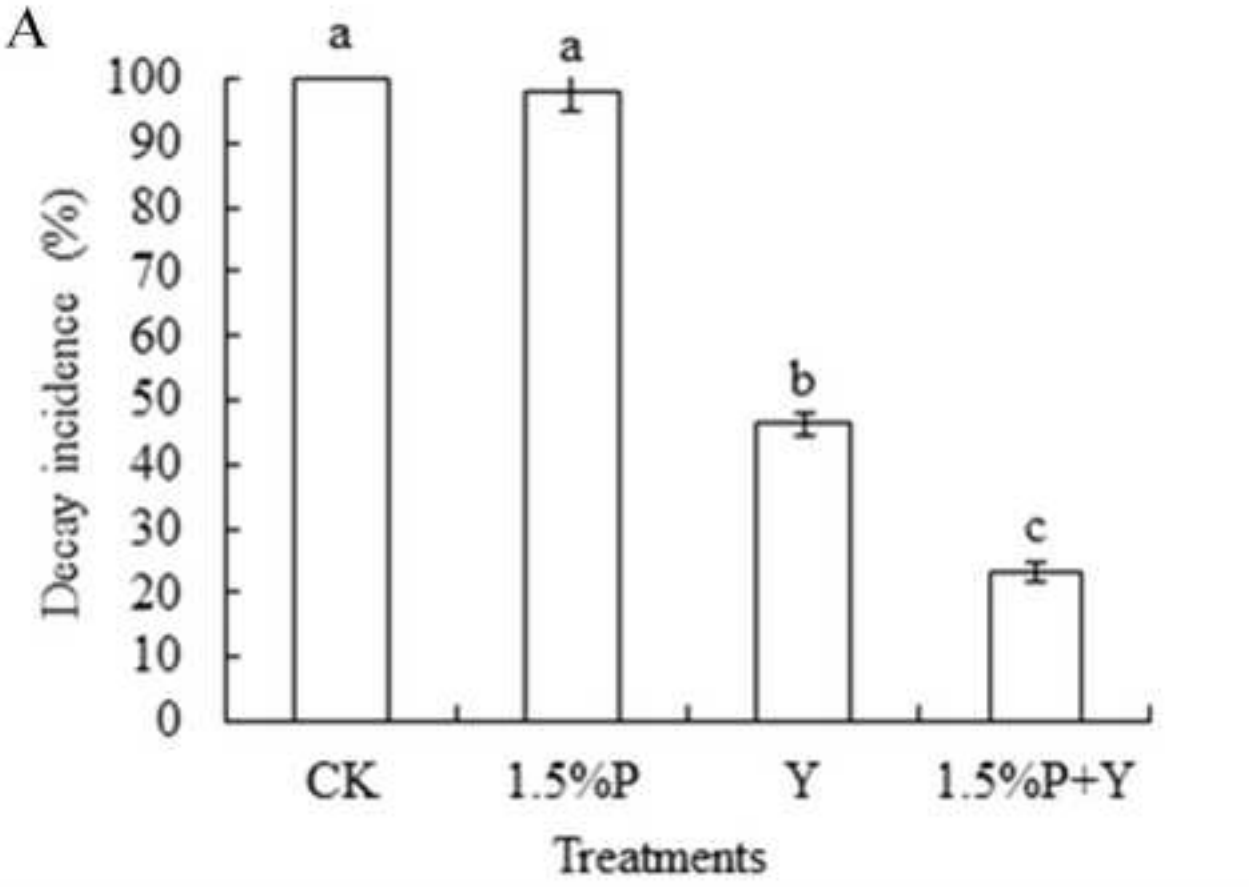


Figure4



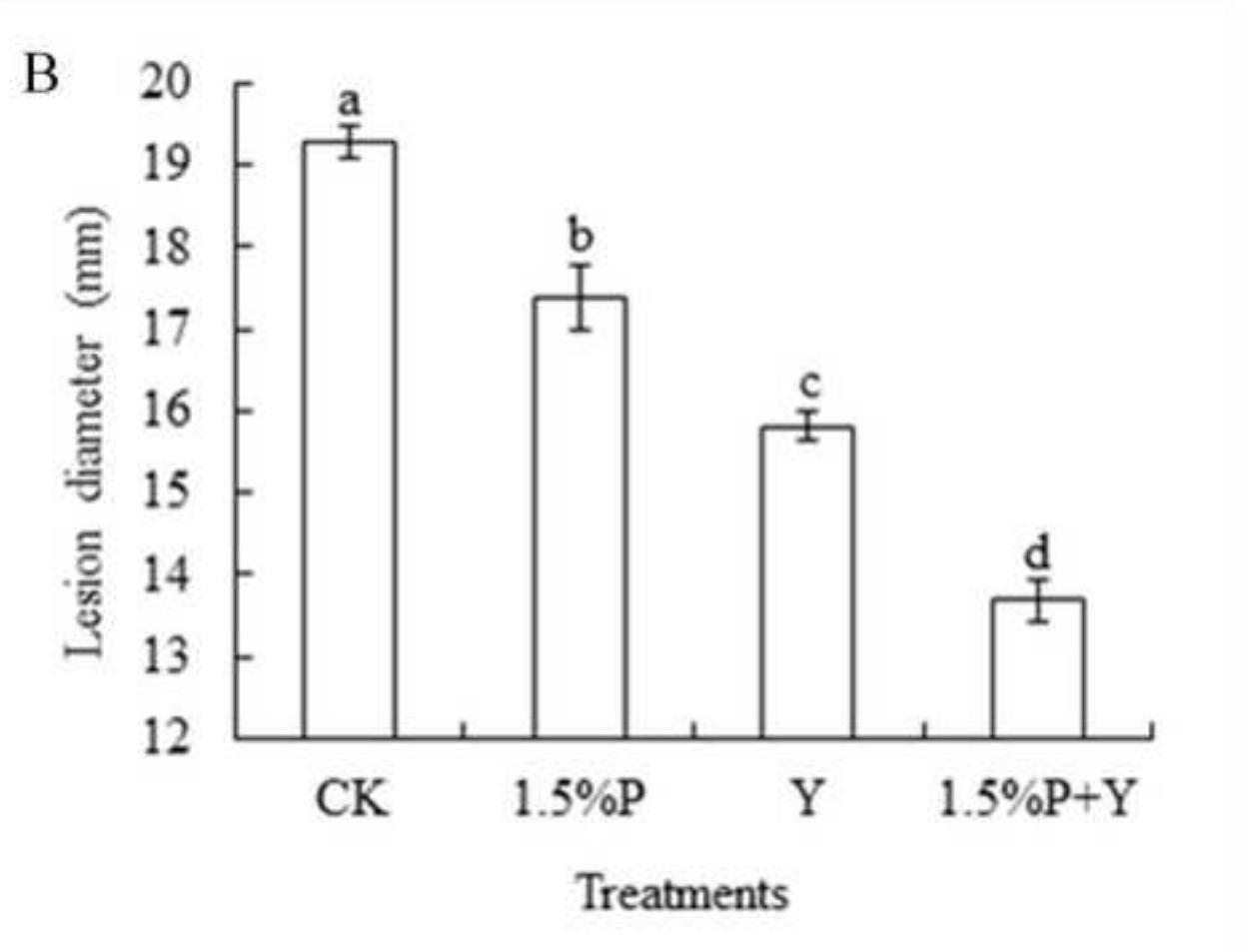


Figure5

Figure6

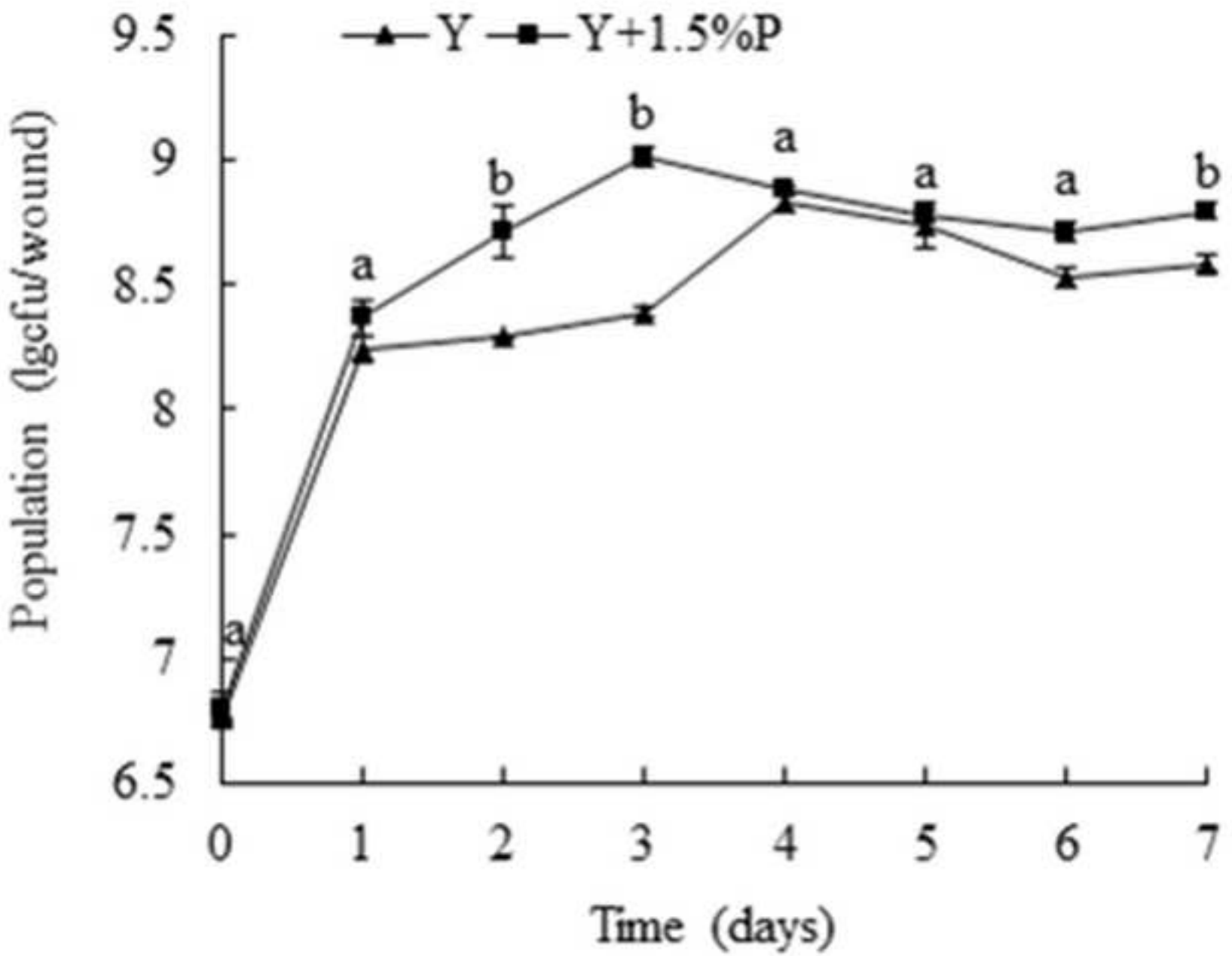


Figure7

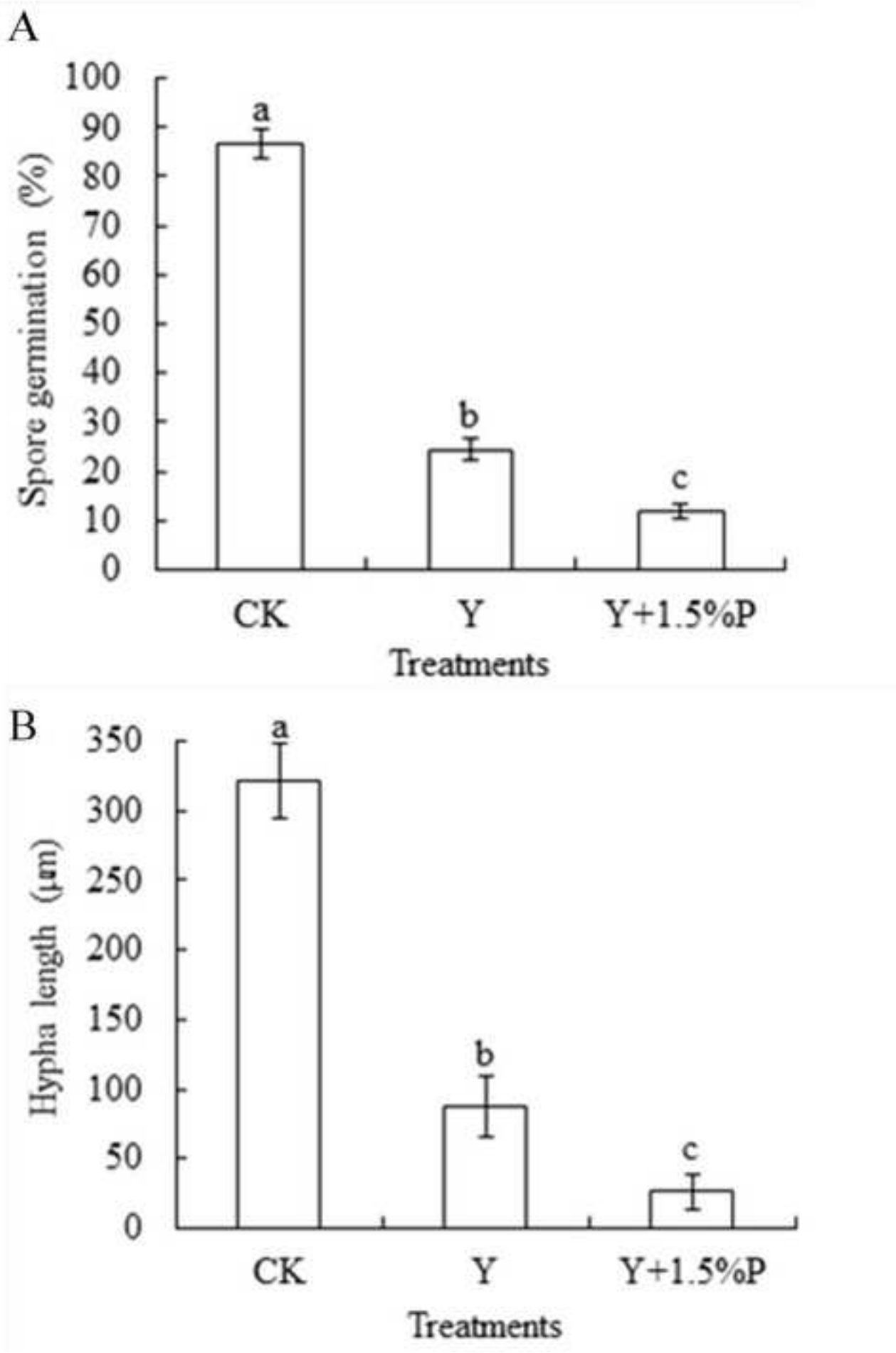
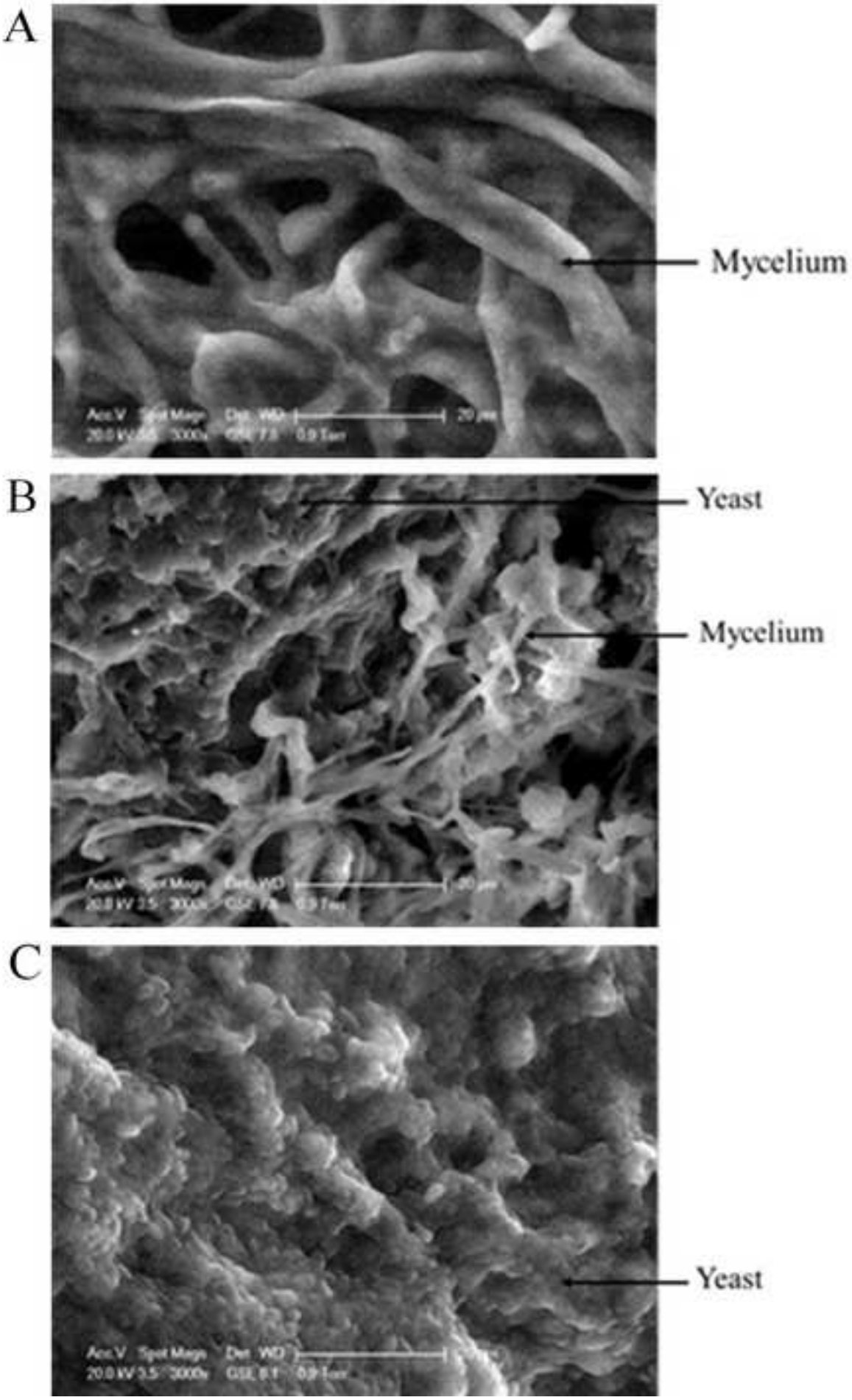


Figure8



Highlights:

- *H. uvarum* significantly inhibited green mold of oranges.
- Phosphatidylcholine significantly improved the biocontrol efficacy of *H. uvarum.*
- Phosphatidylcholine increased the population of *H. uvarum* in orange wounds.
- Phosphatidylcholine and *H. uvarum* controlled germination of *P. digitatum*.