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Biological Control of *Pichia bruneiensis* AL3 against Green Mould Disease on Oranges

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ABSTRACT

Agriculture plays an essential role in Thailand economy. However, to control plant pathogens and maximize crop yield, pesticides are overused and misused. This results in toxic pollutants that are harmful to humans and the environment. Biological control offers an alternative to the use of synthetic chemicals. Mandarin orange (*Citrus reticulata*) is widely grown and consumed in Thailand, but it is threatened by green mould disease caused by *Penicillium digitatum,* which usually occurs at post-harvest storage. A total of 40 isolates of epiphytic yeasts were isolated from symptomless orange peels. They were screened for antagonistic activity against *P. digitatum* originally isolated from a diseased orange using a dual culture method. Five isolates exhibited at least 70% inhibition; therefore, they were further screened for their activity on orange using fruit inoculation. An isolate, AL3, reduced the disease incidence (79%) and lesion size (34.75±2.57 mm) caused by *P. digitatum* compared to the control treatments (disease incidence, 100% and lesion size, 50.00±1.03 mm, respectively). The yeast was analyzed using molecular data based on Internal Transcribed Spacers (ITS region) and it was identified as *Pichia bruneiensis* AL3. Its mode of action on anti-sporulation was studied. When tested on agar plates, living cells and culture broth of *P. bruneiensis* AL3 were able to inhibit spore germination of *P. digitatum* resulting in 33.2 and 38.2% germination, respectively, compared to the control treatment (76.8% germination). These results showed that *P. bruneiensis* AL3 has the potential to develop as a biological control agent (BCA).

Keywords: Biocontrol, Green mould disease, Mandarin orange, *Penicillium digitatum.*

INTRODUCTION

One-third of the total labor force covering six million households in Thailand is employed in the agricultural sector. Therefore, agribusiness plays an essential role in the Thai economy (BOT, 2019). Crop production has become a crucial engine contributing both directly and indirectly to Thailand's Gross Domestic Product (GDP). Mandarin orange (*Citrus reticulata*), also known as tangerines, is the most consumed orange within the edible *Citrus* species. In Thailand, it is widely grown and sold domestically and exported internationally. They are mainly consumed as fresh fruit because they contain various nutritional contents, especially vitamins and minerals.

Around 0.2 million tons of oranges were produced in Thailand in 2020 (OAE, 2022), however, they are prone to many diseases. One the most severe diseases in orange production is *Penicillium* rots, post-harvest diseases, which occurs during the storage period. There are two types of rots including blue mould and green mould diseases caused by *P. italicum* and *P. digitatum*, respectively, the pathogen, *P. digitatum,* can cause critical post-harvest losses (Papoutsis *et al*., 2019). Various fungicides such as Imazalil, pyrimethanil, fludioxonil, and tiabendazole are currently used to control the diseases. However, these chemicals are

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harmful to humans and the environments and some of the pathogens have developed resistance to these fungicides due to overuse and misuse of fungicides as well as spontaneous mutation of the fungal pathogens (Kanashiro *et al*., 2020). Applying fungicides is mostly restricted during the storage period because this can lead to toxic chemical residues to consumers. There is an urgent need to find an alternative approach to solve this problem.

Biological control is an approach using beneficial organisms to protect plants from diseases and damage caused by phytopathogens and pests. Numerous successful applications of biocontrol have been widely reported (Liu *et al*., 2013; Bakhshi *et al*., 2018; Przemieniecki *et al*., 2018; Vatankhah *et al*., 2019; Obeng *et al*., 2021; Bhatta, 2022; Tavallaie *et al*., 2022). Epiphytes are well known for their beneficial properties to control plant pathogens and triggering the plant immune system (Bruisson *et al*., 2019). In this study, yeasts are focused on because several antagonistic yeasts have been reported to effectively control post-harvest rot such as *Aureobasidium pullulans* (Ferrz *et al*., 2016), *Candida oleophila* (Liu *et al*., 2019), and *Pichia galeiformis* (Chen *et al*., 2020).

The main objectives of this research were the followings:

(1) To isolate epiphytic yeasts from orange peels

(2) To evaluate their antagonistic activity against *P. digitatum* using *in vitro* and *in vivo* tests

(3) To study the inhibition of spore germination by the living cells and culture broth of the candidate BCA.

MATERIALS AND METHODS

Isolation of Penicillium digitatum

\Mandarin oranges were collected from local markets in Bangkok. Both infected oranges with visible signs of green mould disease and healthy oranges without any sign of infection were collected. The isolation method is modified from Rungjindamai (2016). For the infected oranges, the fungus was isolated by sub-culturing onto Potato Dextrose Agar (PDA). The healthy oranges were incubated in moist chambers at 25°C for 5 days. They were checked daily, when the green mould disease occurred on the surface of oranges, the fungal pathogen was transferred to PDA and incubated at 25°C for 7 days. Koch's postulate was carried out to confirm the pathogenicity of this isolate.

Isolation of Epiphytic Yeasts from Orange Peels

The samples of oranges for epiphytic yeasts were collected from two wholesale markets including Si-Moom-Mueng Market and Talad-Thai Market located in Pathum Thani Province, Central Thailand. The samples were collected in November and December 2021. Symptomless oranges were brought from wholesale markets in Thailand. They were brought back to the lab for isolation on the same day. The isolation method is modified from Rungjindamai (2016). The oranges were surface sterilized by soaking in 70% ethanol for 1 minutes. Afterward, the orange peel was taken from the fruits. The peel was cut into pieces (1×1 cm^2) using a sterile blade. The five pieces were transferred to a 20 mL-tube containing 9 mL of 0.1% tween 20 and the tube was mixed using a vortex mixer for three minutes. The sample was serially diluted (from 10^{-1} – 10^{-3}) and the samples of each dilution were spread onto tryptone glucose Yeast Extract Agar (TGY) supplemented with kanamycin (50 µg L^{-1}) to suppress bacterial growth. The isolation plates were incubated at 25°C for 5 days. Yeast colonies growing on the TGY agar were randomly picked and transferred to fresh PDA without antibiotics. The cultures of epiphytic yeasts were maintained in 20% glycerol solution at -20°C in a deep freezer (Pitt and Hocking, 2009).

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Molecular Identification

Identification of the pathogen (*P. digitatum*) and the epiphytic yeast (AL3) was carried out. Their genomic DNAs were extracted using a boiling method (Fang and Hedin, 2003). Molecular identification was based on sequence analyses of the Internal Transcribed Spacer region (ITS). Their ITS regions were amplified using primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3')/ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') using PCR condition described by Schoch *et al.* (2012) with some modifications following: (1) Preheat 96°C (2 minutes), (2) Denature 96 °C (1 minute), (3) Annealing 53°C (1 minute), (4) Extension 72°C (1 minute and 30 seconds), (5) Repeat of (2)-(4) for 35 cycles, (6) Final extension at 72°C (7 minutes), (7) Final hold 15 °C. PCR products were purified and sequenced by Marogen Inc, South Korea, using the same sets of primers for PCR amplification. The DNA sequences of AL3 and MK1 were compared with sequences deposited in the NCBI's GenBank DNA database using the BLASTn search tool. Sequences showing the highest similarity values were chosen and the dataset was aligned using BioEdit v.7.1.3 and MEGA v.11.0.13. Phylogenetic analyses based on Maximum Parsimony (MP) and Maximum Likelihood (ML) methods were performed using PAUP v4.0a. Statistical supports including MP bootstrap and ML bootstrap supports were calculated. The phylogenetic tree was generated and edited using TreeView v0.5.0. The sequences of these fungi were deposited at the GenBank Database, which provided accession numbers OQ798893 (*P. digitatum* MK1) and OQ798894 (*Pichia bruneiensis* AL3).

In vitro Test

The isolate of *P. digitatum* MK1 originally isolated in this study was used for *in vitro* test. A dual culture method was used to determine the bioactivity of yeasts against the pathogen using a method described by South *et al*. (2020) with some modifications. The epiphytic yeasts were inoculated 1 cm away from both sides of a plate containing PDA. An agar plug of actively growing *P. digitatum* was inoculated in the center of the PDA. The plates were incubated at 25°C for 7 days. There were three replicates per treatment. The control was the inoculation of the pathogen without epiphytic yeasts. Radii (R) of the fungal growth of *P. digitatum* were measured (in mm) and the percentage of inhibition was calculated using the following formula.

$$Percentage\ of\ inhibition\ (\%) = \frac{(R_{control} - R_{Dual\ plate})}{R_{control}} \times 100$$

In vivo Test on Oranges

Five isolates of epiphytic yeasts were selected from *in vitro* test that showed promising inhibitory effects against *P. digitatum*. They were screened for further activity on oranges. The *in vivo* test on oranges was adapted from Li *et al*. (2022). Single colonies of the selected epiphytic yeasts were transferred to 3 mL of Potato Dextrose Broth (PDB) and the cultures were incubated at 25°C for 24 hours. Mandarin oranges were bought from the Talad Thai, a wholesale fruit market in Thailand in December 2021. Fruits were brought back to the lab and stored at 4 °C and the experiment was performed within 24 hours to prevent the rot of fruits. Fruits were surface sterilized by dipping into 5 L of 0.5% sodium hypochlorite for 15 minutes and, subsequently, they were rinsed twice with 5 L of sterile distil water for 15 minutes. After air-drying at room temperature for 30 minutes, the oranges were wounded using a sterile 200-µL tip (2 mm depth, 2 wounds on the opposite sites per orange) and 10 µL of cell suspension of each epiphytic yeasts (a concentration of 10^7 CFU mL^{-1}) was pipetted in the wounds. The treated oranges were air-dried for 1 hour and then the same volume of spore suspension of *P. digitatum*

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(10^7 spores mL^{-1}) was inoculated on the same wound.

There were seven treatments including five BCAs, one fungicide and sterile distilled water, the latter two served as negative and positive controls, respectively. The fungicide, azoxystrobin (Toscana, Sinon Corporation, Taiwan), was prepared at the concentrations indicated by the manufacturers' recommendation. The final concentration was 0.1%. The artificially infected oranges were placed into plastic boxes (1 orange per box). The boxes were incubated at 25°C for 5 days. The experiments were performed in 7 replicates (seven oranges per treatment and 14 wounds in total). A rot symptom caused by green mould disease was assessed within 5 days. The data were expressed in disease incidence (% of infection) and lesion sizes (diameter in mm). The disease incidence was calculated from the numbers of infected wounds and numbers of total wounds. The lesion diameter was measured using a Vernier caliper and was the average of its vertical and horizontal diameters.

Anti-Spore Germination Test

The effect of yeast AL3 on spore germination of *P. digitatum* was assessed using a method modified by Zhang *et al*. (2019) and Li *et al*. (2022). A yeast, AL3, was grown in PDB at 25°C for 4 days. The culture of AL3 was centrifuged and the supernatant was transferred to a new tube, then, the cell pellet was washed twice with Normal Saline Solution (NSS) and resuspended in 5 mL of NSS. The concentration of the cell suspension was adjusted to 2×10^7 cells mL^{-1}. Then, 0.5 mL of spore suspension of *P. digitatum* (1×10^5 spore mL^{-1}) was mixed with either 0.5 mL of living cell suspension or culture filtrate in a test tube. Sterile PDB was used as the control treatment. The tubes were mixed with a vortex mixer. The mixture of each treatment (10 µL) was pipetted onto Water Agar (WA) and the plates were sealed with parafilm. The WA plates were incubated at 25°C for 36 hours. The germination of spores of *P. digitatum* was determined by counting 100 spores per replicate. There were 5 replicates (five plates) per treatment. Spores were considered to have germinated if the germ tube was equal to, or longer than, the diameter of the spores.

Statistical Analysis

Data were analyzed using SPSS for Windows v.28. One-way Analysis Of Variance (ANOVA) was used to compare the significant difference of positive results among treatments. The level of significance was set at a P-value< 0.05. The data are presented as means±Etandard Error (SE).

RESULTS AND DICUSSION

The Pathogen: Penicillium digitatum

The pathogen (isolate MK1) was isolated from an infected orange collected from a supermarket in Central Bangkok. The macro- and microscopic observations of this isolate confirmed that it belonged to the genus *Penicillium*, and it produced conidiophores and conidia [Figure 1 (A and B)]. Koch's postulate was performed, and it confirmed its pathogenicity (Figure 1 C). Eight species of *Penicillium* including *P. aurantiogriseum, P. citrinum, P. digitatum, P. expansum, P. griseofulvum, P. italicum, P. polonicum,* and *P. sclerotigenum* DNA sequence analysis showed that our isolate MK1 had the closest relationship with numerous sequences of *P. digitatum* with high statistical supports (100% Maximum Parsimony Bootstrap Support, MPBS and 100% Maximum Likelihood Bootstrap Support, MLBS). This confirmed the identity of an isolate MK1 as *P. digitatum*. This study focused on this pathogen because of its devastating effect on citrus fruits. This fungus can cause post-harvest rot in oranges,

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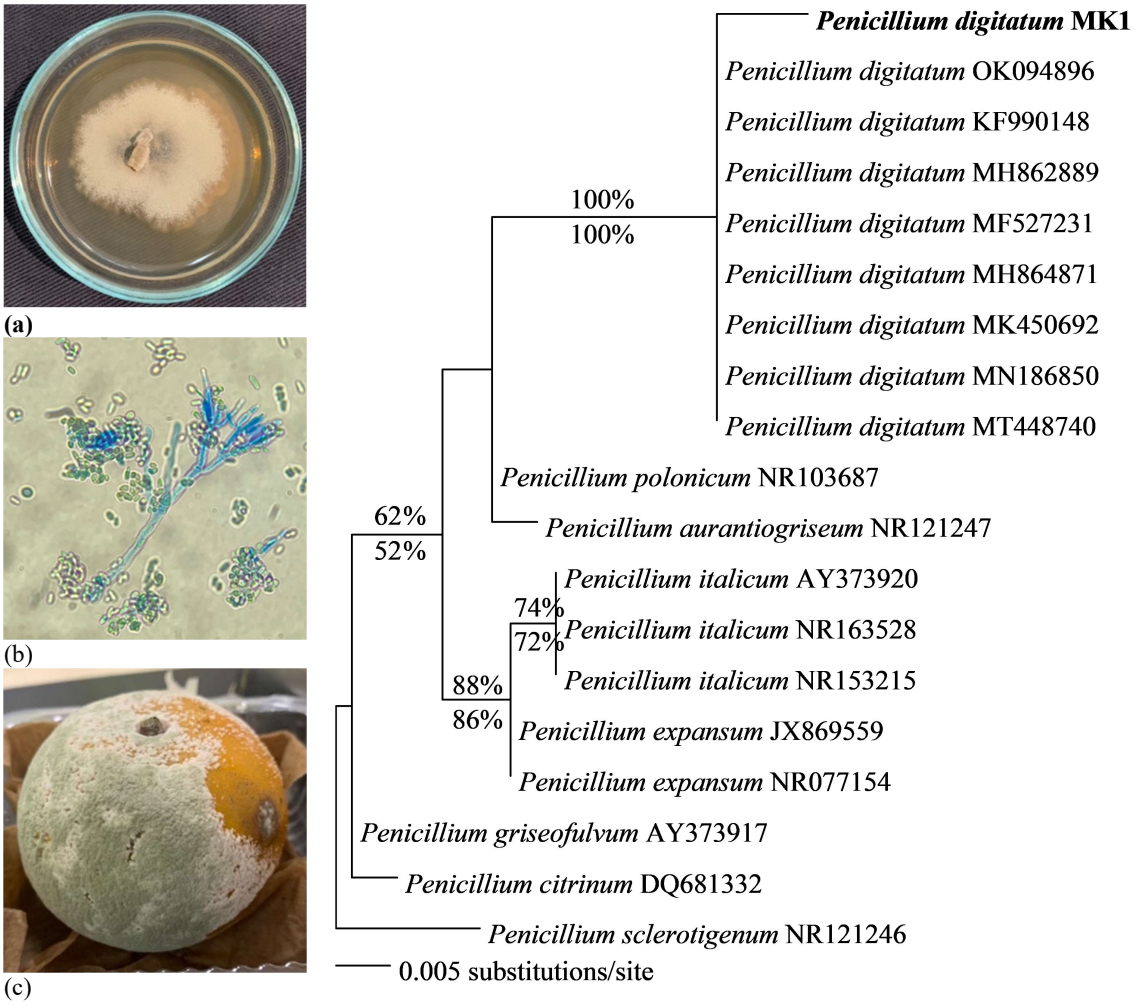

Figure 1. Identification of the green mold pathogen on a mandarin orange. (A) A colony of *Penicillium digitatum* MK1 on PDA incubated at 25 °C for 14 days, (B) Microscopic observation of the fungus depicting a conidiophore and conidia, (C) Reinfection of an orange inoculated with the pathogen showing the green mold rot, and (D) Molecular identification of *P. digitatum* MK1 by ITS sequence analyses. Bootstrap supports of MP (above) and ML (below) are shown on the tree.

tangerines, lemons, and grapefruit indicating its global economic importance and the management needed to minimize the losses in citrus production (Li *et al*., 2022; Costa *et al*., 2019).

In vitro Test on PDA

A total of 40 isolates of epiphytic yeasts were recovered from these samples. All of them were screened for their activity against *P. digitatum* MK1. Most research on biocontrol against green mould disease on oranges mainly focused on using medicinal plant extracts (Wisittawong and Nalumpang, 2017; Rahmati-Joneidabad and Behbahai, 2021) and bacterial (biological control agents), BCAs, especially *Bacillus subtilis* and *B. pumilus,* were widely reported as promising BCAs (Thonglem *et al*., 2007; Intha *et al*., 2013). Our result expands the knowledge of microbial bio-control by

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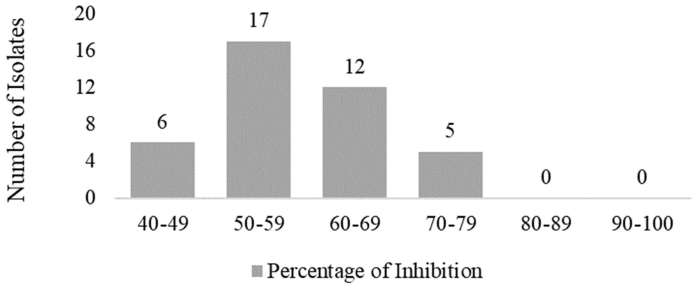

Figure 2. A histogram of percentage of inhibition and number of epiphytic yeasts tested against *Penicillium digitatum*. The experiment was conducted on PDA incubated at 25°C for 7 days.

showing that yeasts also have the potential as BCAs on oranges.

There were six isolates that produced the lowest inhibition for between 40-49% of inhibition (Figure 2). Most epiphytic yeasts (29 isolates) inhibited *P. digitatum* with moderate activity (50-69% inhibition). There were five isolates that strongly inhibited the pathogen with 70-79% of inhibition (Figures 2 and 3). However, there were no epiphytes that were able to completely inhibit the growth of *P. digitatum* with > 80% inhibition. These five isolates of epiphytes including AL3, AL15, AL7, JK8, and JK15 were selected and further screened for antagonistic activity on orange fruits. Our results showed that epiphytic microbes naturally present on the surface of fruits were a good source of microbial BCAs. Among various kinds of epiphytic microbes, yeasts were considered the most interesting BCAs because they possessed various

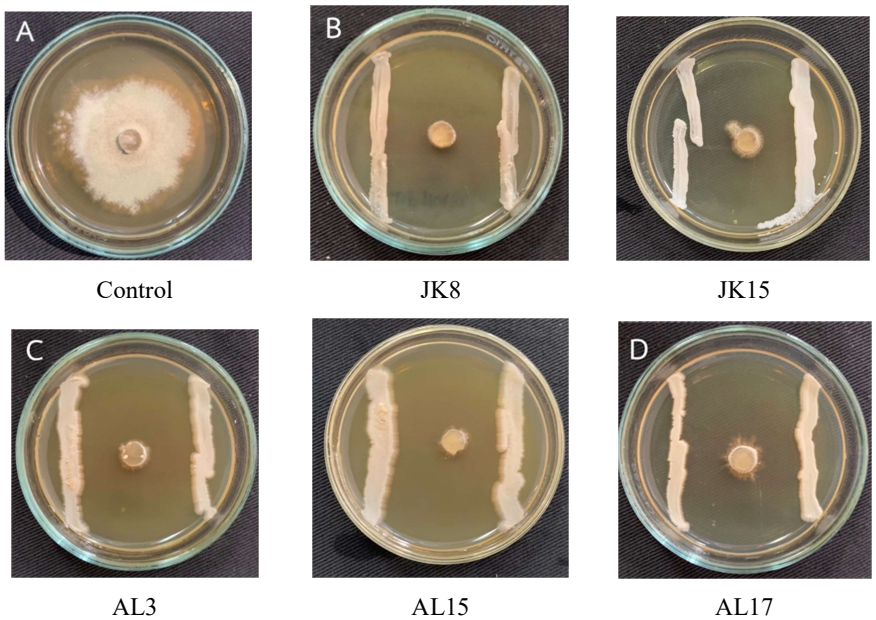

Figure 3. *In vitro* test of five candidate epiphytic yeasts against *P. digitatum* on PDA incubated at 25°C for 7 days. The pathogen was inoculated in the center and the yeasts were inoculated in the left and right of the PDA.

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characteristics, including: (1) Basic requirement on simple nutrients, (2) Rapid growth, (3) Capability to colonize and strive on fruit surfaces, and (4) Compatibility for space and nutrients (Hammami *et al*., 2022).

In vivo Test on Oranges

The control treatment oranges were treated with sterile Normal Saline Solution (NSS); all wounds were infected resulting in 100% infection (Figure 4). For the fungicide tested in this study, azoxystrobin completely inhibited the pathogen resulting in no infection (0%). Although four isolates (AL15, AL17, JK8, and JK18) of epiphytic yeasts were unable to reduce percentage of infection with 93-100%, one isolate AL3 reduced infection to 79%.

Lesion of green mould disease showing the degree of severity was expressed as the mean of lesion size (in mm, Figures 5 and 6). A lesion size of control treatment NSS was 50.0 mm. Fungicide, Azoxystrobin (AZ) completely inhibited the pathogen, so there was no lesion in all test fruits. Among five isolates of epiphytic yeasts, an isolate, AL3, the best candidate, produced the smallest lesion size at 34.75±2.57 mm, while lesion sizes treated with the other four isolates were between 40.6-44.8 mm. Based on these findings, AL3 was selected for further investigation on its possible mode of action. Although isolate AL3 did not completely inhibit *P. digitatum* at the same level as the synthetic fungicide, it showed some promising results by reducing disease incidence and lesion size caused by *P. digitatum*.

P. digitatum is a wound pathogen, therefore, it invades and infects fruits through wounds. These wounds are caused by environmental and human factors. The wounds can occur at any time during pre-

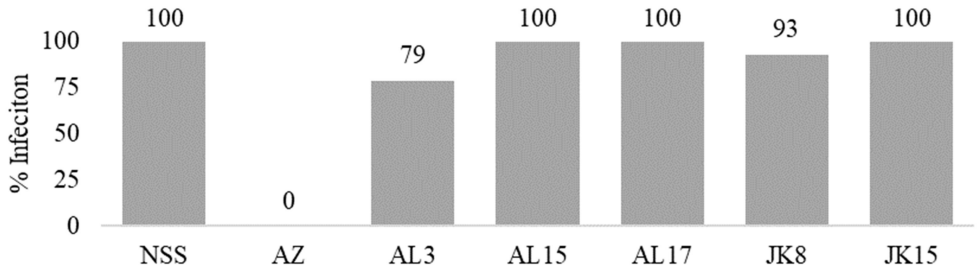

Figure 4. Disease incidence of oranges artificially inoculated with *P. digitatum.*

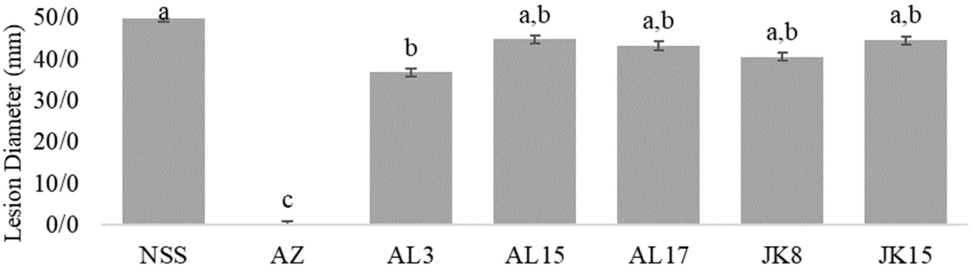

Figure 5. Mean values of lesion diameters on oranges caused by artificial inoculation of *P. digitatum* (1×10^5 spore mL^{-1}). The wounds are then treated with cell suspension of five candidate BCAs. Control treatments were sterile Normal Saline Solution (NSS), and a fungicide AZ= 0.1% Azoxystrobin.

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harvest, transport and storage at post-harvest (Costa *et al*., 2019). Candidate BCAs must be able to inhibit the pathogen on the fruit surface to ensure that they are effective when they are applied to fruits. Therefore, the *in vivo* test on citrus fruits is a crucial part of research in bio-control because it resembles an infection scenario of *P. digitatum* that causes post-harvest rot on mature citrus fruits.

Yeasts from natural sources have been widely reported as good candidates for BCAs against *P. digitatum*. Indratmi *et al.* (2021) showed that two yeast species, namely, *Debaryomyces hansenii* and *Aureobasidum pullulans,* originally isolated from the surface of apples inhibited *P. digitatum* when tested on oranges. While Delali *et al.* (2021) demonstrated that three yeasts species i.e. *Pichia kudriazevii*, *Kluyveromyces marxianus,* and *Yarrowia lipolytica* isolated from kimchi, a fermented food, effectively inhibited green mould pathogen in fruit experiment. Our results also support this statement by showing that yeasts from natural origins can be a good source of BCAs.

Figure 6. *In vivo* test on mandarin oranges. Fruits were treated with either fungicides or epiphytic yeasts (1×10^7 cells mL^{-1}). The fruits were then infected with *Penicillium digitatum* (1×10^6 spores mL^{-1}). (A) Control treatment with sterile Normal Saline Solution (NSS), (B) 0.1% AZ, Azoxystrobin, (C)–(G) Are five candidate epiphytic yeasts. The artificially infected fruits were incubated at 25°C for five days.

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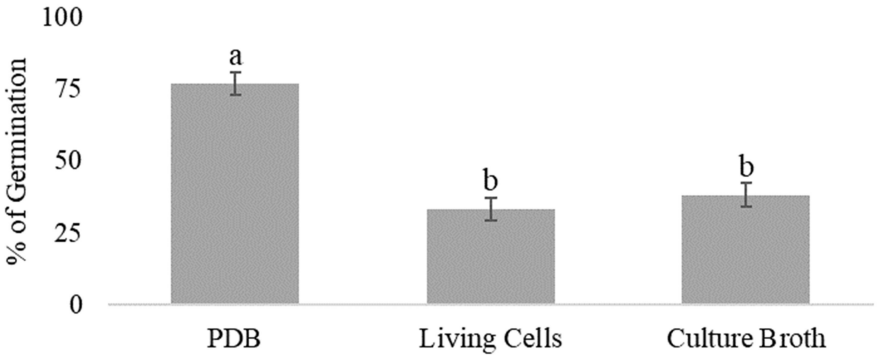

Figure 7. Percentage of spore germination of *P. digitatum* when treated with living cells and culture broth of *Pichia bruneiensis* AL3. The spores were placed onto Water Agar plates (WA) incubated at 25°C for 36 hours. There were five replicates (5 plates) with 100 spores per replicate.

Anti-Spore Germination

The percentage of spore germination for control treatment (with PDB) was 76.8%, while both treatments (living cell and culture broth of *Pichia bruneiensis* AL3) reduced spore germination to 33.2 and 38.2%, respectively (Figure 7). This suggests that the bioactivity of *P. bruneiensis* AL3 might be caused by the presence of living cells or bioactive substances secreted into culture broth.

Spore germination is considered a crucial step for disease development caused by *P. digitatum* on citrus fruit (Chen *et al*., 2020). Therefore, anti-spore germination is focused on this study. Sangwanich *et al*. (2013) showed that *Pichia guilliermondii* completely inhibited spore germination of *P. digitatum* by 100%. Likewise Perez *et al.* (2019) reported that two yeast species i.e. *Clavispora lusitaniae* and *Pichia fermentans,* inhibited spore germination with more than 90% inhibition. Agirman and Erten (2020) demonstrated that two yeast biocontrol agents (*Aureobasidium pullulans* and *Meyerozyma guilliermondii*) inhibited green and blue mold pathogens by various mechanisms including inhibition of spore germination. To prevent the green mould disease and to protect fruits, potential bio-control agents must be able to inhibit spore germination. Our study confirms that *P. bruneiensis* AL3 possesses this important mode of action.

Identification of the Candidate BCA AL3

AL3, the candidate BCA, was identified based on ITS region. *Candida sylvatica* NR111303 was used as the out-group. AL3 is closely related to *Pichia* spp. (Figure 8). Various species of *Pichia* were included in the dataset. Isolate AL3 has the closest relationship with six sequences of *P. bruneiensis* with high statistical supports (100% MP Bootstrap value and 100% ML Bootstrap value) and they form a sister clade with another six sequences of *P. fermentans* with high supports (100% MP Bootstrap value and 93% ML Bootstrap value), while other species of *Pichia* are placed distantly in lower clades. This clearly shows that Isolate AL3 is *P. bruneiensis*.

Application of yeasts as BCAs is widely studied and they are considered unharmful BCAs for humans because they have been used by food industries for centuries, especially for wine, beer, and bread production (Di Canito *et al*., 2021). Numerous yeast genera, in particular *Aureobasidum*, *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Pichia, Rhodotorula, Wickerhamomyces,* and

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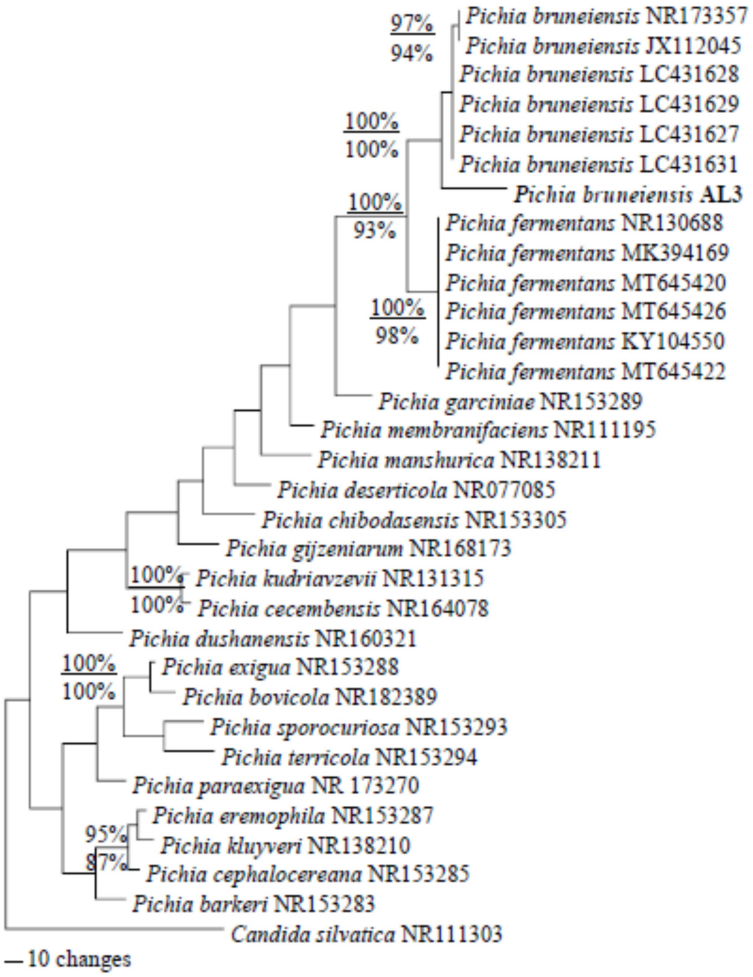

Figure 8. A phylogenetic tree of *Pichia bruneiensis* AL3 based on molecular analyses of ITS region. Bootstrap supports for MP (above) and ML (below) are shown on the tree.

Yarrowia are reported to effectively inhibit *P. digitatum* and *P. italicum,* which cause postharvest decay of citrus fruits (Hammami *et al*., 2022)

Pichia is also widely known as promising BCAs against various plant diseases, for example, *P. angusta* (Fiori *et al*., 2008), *P. anomala* (Izgu *et al*., 2011; Haissam, 2011) *P. carribbica* (Zhao *et al*., 2012), *P. membranaefaciens* (Zhang *et al*., 2019, Santos and Marquina 2004) *P. guillermondii* (Pacheco *et al*., 2008). While *P. bruneiensis* was first reported as a novel yeast species isolated from flowers in Brunei (Sipiczki, 2012). In this study, the knowledge of *Pichia* as bio-control has been expanded by showing that *P. bruneiensis* has potential to be developed as a candidate BCA to control green mould disease.

CONCLUSIONS

This study shows that epiphytic yeasts are a good source of Biological Control Agents (BCAs) against the green mould pathogen, *P. digitatum*. The most promising BCA, AL3, was identified based on ITS region as

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Pichia bruneiensis. This BCA can inhibit the pathogen when it was tested *in vitro* and *in vivo* experiments, which were conducted on PDA and oranges, respectively. The mode of action was determined: both of the living cells and culture broth were able to inhibit spore germination of *P. digitatum*. Therefore, this demonstrates that it has the potential to be developed into commercial products.

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مهار بیولوژیکی *Pichia bruneiensis* AL3 در برابر بیماری کپک سبز روی پرتقال

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چکیده

کشاورزی نقش بنیادی در اقتصاد تایلند دارد. با این همه، برای مبارزه وجلوگیری از آفت‌های گیاهی و به حداکثر رساندن عملکرد محصول، آفت کش ها بیش از حد مورد سوء استفاده و کاربرد بیش از حد قرار می گیرند. این کار منجر به تولید آلاینده های سمی می شود که برای انسان و محیط زیست مضر است. مبارزه

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بیولوژیکی جایگزینی برای استفاده از مواد شیمیایی صنعتی ارائه می‌دهد. پرتقال ماندارین (Citrus reticulata) به طور گسترده در تایلند کشت و مصرف می شود، اما به دلیل بیماری کپک سبز(green mould) ناشی از *Penicillium digitatum*، که معمولاً در انبار پس از برداشت رخ می دهد، تهدید می شود. در مجموع ۴۰ جدایه از مخمرهای اپی فیتیک (epiphytic yeasts) از پوست پرتقال بدون علائم مرض جدا شد. این جدایه ها برای فعالیت آنتاگونیستی علیه *P. digitatum* که در اصل از یک پرتقال بیمار جدا شده بود با استفاده از روش کشت دوگانه غربالگری شدند. پنج جدایه حداقل ۷۰% مهار را نشان داد. بنابراین، آنها بیشتر برای فعالیت خود در پرتقال با استفاده از تلقیح میوه غربالگری شدند. جدایه AL3، بروز بیماری (۷۹٪) و اندازه ضایعه (۳۴.۷۵±۲.۵۷ میلی متر) ناشی از *P. digitatum* را در مقایسه با تیمارهای شاهد (به ترتیب ۱۰۰٪ و ۵۰.۰۰±۱.۰۳ میلی متر) را کاهش داد. مخمر با استفاده از داده های مولکولی بر اساس فاصله های رونویسی داخلی (منطقه internal transcribed spacers: ITS) تجزیه و تحلیل شد و به عنوان *Pichia bruneiensis* AL3 شناسایی گردید. نحوه عملکرد آن بر ضد اسپورزایی بررسی شد. هنگامی که آزمایش بر روی صفحه‌های آگار انجام شد، سلول‌های زنده و براث کشت(culture broth) *P. bruneiensis* AL3 توانستند جوانه‌زنی اسپور *P. digitatum* را مهار کنند که منجر به جوانه‌زنی به ترتیب ۳۳/۲٪ و ۳۸/۲٪ نسبت به تیمار شاهد (۷۶/۸ ٪ جوانه‌زنی) شد. این نتایج نشان داد که *P. bruneiensis* AL3 دارای توانایی توسعه به عنوان یک عامل کنترل بیولوژیکی (BCA) است.

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