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Selection and characterization of a yeast strain for the suppression of brown spot on Tru Long pummelo (*Citrus maxima*)

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ABSTRACT

Tru Long pummelo is an endemic plant of Quang Nam province with high economic value. However, many different diseases, especially brown spot caused by Alternaria fungus on pummelos, have affected its quality. Pesticides are both overused and misused, adversely affecting the environment and human health. Therefore, there is an urgent need to reduce the use of synthetic chemicals. Biological control offers an alternative to the use of pesticides. Eight yeast strains have been isolated from healthy leaves, pummelo fruits, and healthy mulberries. They were screened for biological activity against Alternaria sp. by the dual culture method. The inhibitory potency ranged from 10.46% to 59.86%. The strain with the highest percent (59.86%) was identified as Candida tropicalis using sequence analysis of the ITS region.

1. INTRODUCTION

Pummelo (*Citrus maxima*) is a citrus fruit tree grown popularly in Asian countries such as China, India, Thailand, Malaysia, the Philippines, and Viet Nam. It has excellent nutritional and medicinal value for humans. A 100g edible portion, contains: 89g water, 0.5g protein, 0.4g fat, 9.3g starch, 49 IU vitamin A, 0.07mg vitamin B1, 0.02mg vitamin B2, 0.4 mg niacin, and 44 mg vitamin C (Vu, 1996). The leaves, flowers, and peels of Pummelo contain essential oils. Pummelo peel also has pectin, naringin, digestive enzymes peroxidase and amylase, ramoza sugar, and many digestive enzymes amylase and peroxidase.

Tru Long pummelo in Dai Binh village is an endemic tree species of Quang Nam province with high economic value and is favored on the market. Tru Long pummelo has thick segments, large cloves, a sweet taste, and a unique flavor. It is easy to plant a high yield with a total area of nearly 60 hectares, which is the primary source of income for thousands of households. However, pummelo growth activity faces many difficulties and obstacles. Pummelos are often susceptible to diseases caused by pathogenic microorganisms because they contain a lot of water and nutrients that provide an ideal substrate for the growth of pathogenic microorganisms. One of the reasons that limit the growth and reduce the yield of Tru Long pummelo is the disease caused by fungal *Alternaria* spp.

Alternaria brown spot, caused by *Alternaria* spp., is one of the significant diseases of pummelo and its hybrids worldwide (Woudenberg et al., 2015; Chitolina et al., 2019). It attacks browse, branches, and fruit, causing brown or black lesions surrounded by a yellow halo (Gai et al., 2021). Severely, infected leaves and fruit may drop, and entire buds may wilt and die (Timmer et al., 2003).

Although the application of synthetic fungicides in agriculture has an effect against fungi, they may trigger mycotoxin accumulation and causes the development of resistant fungal strains (Mernke et al., 2011; Cabral et al., 2019). The presence of toxic residues in agricultural products can also potentially adversely affect human health, the environment, and biodiversity (Alavanja et al., 2012; Casida et al., 2012; Geiger, 2012; Jordaan et al., 2012). Therefore, there is an urgent need to solve this problem. Epiphytic microorganisms that live on plant surfaces without causing any symptoms to plants are known to effectively inhibit plant pathogens (McGrath & Andrews, 2007; Janisiewicz et al., 2010; Janisiewicz et al., 2013). Among microbial agents, yeasts have several properties that make them ideal, including the ability to survive adverse environmental conditions and low nutritional requirements with a long shelf life.

Antagonistic yeasts exhibit several possible mechanisms against fungal pathogens, for example, competition for nutrients and space via biofilm formation by yeast for resistance, the release of hydrolytic enzymes, parasites, production of antifungal volatile organic compounds (VOCs), and stimulation of host defense pathways (induction of host resistance) (Konsue et al., 2020). Therefore, the objective of this study was to select yeast strains for the prevention of diseases caused by *Alternaria* spp. on the Tru Long pummelo tree.

2. MATERIALS AND METHOD

2.1. Materials

The samples were collected from Dai Binh village, Quang Nam province.

Tru Long pummelo leaves and fruits with brown spot symptoms for disease isolation. Leaf samples (pummelo, oranges, and silkworms) are healthy, unblemished, and asymptomatic to isolate antagonistic strains.

2.1. Method

2.1.1. Isolation of fungal Alternaria spp.

The samples were kept in a humidified chamber and incubated at 25°C for 3 days. Diseased tissues were cut using a flame-sterilized surgical blade. Surface slices with infected tissues (1×1 cm^2) were inoculated into water agar (WA) medium and incubated at 28°C for 3-5 days. Mycelial tips grown from slices were transferred to Potato Dextrose Agar (PDA) medium. Pathogenicity of isolated *Alternaria* strain was confirmed by Koch's artificial inoculation method. Fungal strains were stored at –80°C.

2.1.2. Isolation of yeast

Leaf samples (pummelo, orange, mulberry) that were healthy, blemish-free, and disease-free were washed with tap water to remove surface dirt and allowed to dry at room temperature in an incubator for one hour. Samples were then crushed and added to 90 ml of sterile distilled water and diluted 10^3-10^6-fold. 100µl of each diluted sample was cultured on Hansen's medium at 28°C for 3 to 5 days. Yeast strains were purified and preliminary identification was carried out based on the classification key of Kurtzman and Fell (1998), Luong Duc Pham (2006) and biochemical reactions (ability to ferment glucose and sucrose, ability to urea breakdown) for preliminary identification of species.

2.2. Selection of yeasts with strong antagonistic ability to *Alternaria* sp.

Using the dual culture method to investigate the antagonistic ability of *Alternaria* fungus compared with isolated yeast strains. The petri dish containing the PDA medium was divided into two parts, fungal *Alternaria* sp. is inoculated in the center of the plate, and the yeast is inoculated in a straight line to the side corner about 2.5cm from the position of the fungal.

Using a plate containing only *Alternaria* sp. as a control. After two days of incubation at 30°C, observe and evaluate the antifungal zone according to the following formula Han et al. (2015)

$$I = \frac{(R - r)100}{R}\%$$

In which, I is the antagonistic effect (inhibiting fungal growth by yeast), R is the radius of the control mycelium, and r is the radius of the mycelium with the yeast strain (cm).

The growth inhibition rate of mycelium was evaluated as follows:

Less than 30%: low antifungal activity

Between 30 - 50%: moderate antifungal activity

Between 50 - 70%: high antifungal activity

Above 70%: very high antifungal activity

2.3. Identification of selected yeast strains by ITS sequence analysis

Yeast was cultured for 96 hours in Hansen's medium on a shaker at 180 rpm to obtain biomass.

Total DNA was extracted from biomass according to CTAB method. Amplification of the ITS gene region by PCR reaction was carried out in 20 µl volume, including 2X PCR master mix (Phusa Biochem Ltd); 1pmol primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3'), 1pmol primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3'); 80-100ng DNA (Zhao et al., 2019).

The PCR conditions were as follows: 94°C for 10 min; followed by 30 cycles, 92°C for 1 min, 52 °C for 1 min, 72 °C for 1 min, and final synthesis at 72°C for 5 min.

PCR products were purified and sent for sequencing at NEXT GENE Science and Technology Co., Ltd. ITS comparison to described fungi species was obtained by using the Basic Logarithmic Alignment Search Tool (BLAST) algorithm in the National Centre for Biotechnology Information (NCBI) database.

2.4. Statistical Analysis

Data were analyzed using ANOVA. Multiple comparison tests (Duncan's and Tukey's-tests) were carried out using SPSS Statistic 16.0 software. Data were presented as Mean ± Standard. $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Isolation of *Alternaria* spp.

Based on the microscopic observation of spore-forming stalks, spores, and mycelium and using the taxonomy key of Simmons (2002), and Woudenberg et al. (2013), we isolated 02 *Alternaria* strains AL1, AL2 from 11 diseased samples of pummelo trees (Table 1, Figure 1).

Artificial inoculation by Koch's method showed that disease symptoms on infected leaves and fruits were consistent with the results of field investigation. Furthermore, re-isolated fungal strains from infected samples had morphological characteristics similar to the original strain.

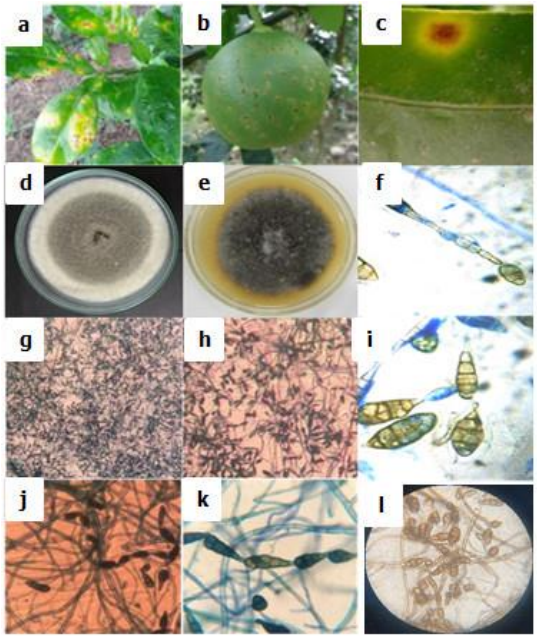

Figure 1. Characteristics of *Alternaria* spp. a-c: symptoms of the brown spot on leaves and fruits; d, e: colonies of AL1, AL2 on PDA. g-i: spores of AL1 strain, j-l: spores of AL2 strain.

3.2. Yeast isolation results

Eight yeast strains LB1, LB2, NB, D1, D2, C1, C2, and CH were isolated from plant samples in this study (Table 2, Figure 2-5). According to the physiological and biochemical (Kurtzman & Fell, 1998) results, the eight yeast strains belonged to the 4 general *Rhodotorula, Saccharomyces, Pichia,* and *Candida.*

Table 1: The results of fungal *Alternaria* isolation from Tru Long pummelo samples

Strain symbol	Plant sample	Morphological features	
		Colonies, colors	Spore
AL1	Fruit	Colonies were initially white, then turned grey. The mycelium radiates thin, grows, and spreads on the surface of the medium.	The length of unbranched spore chains is up to 12 spores with one or two branches on the surface of PDA plate. Spores are short, arising singly. The number of transverse and longitudinal septa of the spore cell varies from 1 to 6 and from 0 to 2, respectively.
AL2	Leaves	Dark gray to black colonies are dense with sparse aerial mycelium and produce cells simple compatriots bring	Polyspore cell division with 9–11 transverse septa and 1-2 longitudinal septa.

Table 2. Morphological and biochemical characteristics of 8 yeast isolates

Strain symbol	Morphological features		Sugar fermenting capacity			Ability to degrade urea	Genus
	Colony characteristics	Cell morphology	Budding cells	Glucose	Sucrose		
LB1	Colonies have a light pink color, large round, smooth surfaces, shiny, toothless edge saw	Small oval cells, with a width ranging from 3-5 µm and a length is about 11-13 µm.	+	-	-	+	*Rhodotorula*
LB2	Colonies are pink-red or pink-orange, round, glossy surface	Large oval cells, width ranging in size from about 2 – 4 µm and length is 8 - 10 µm.	+	-	-	+	*Rhodotorula*
NB	Colonies have a pinkish-orange color, smooth, uniformly convex, at least 7-11 mm	Elliptic cells, the size is about 5-6 µm in width and length is 13-15 µm.	+	-	-	+	*Rhodotorula*
CH	Colonies are white, round, shiny spores	Large globular cell, with a width ranging from 5-7 µm and a length is about 9-11 µm	+	+	+	-	*Saccharomyces*
C1	Colonies grow fast, have a milky white color, smooth, and glossy.	Pointed elliptical cells size is about 2-5 µm in width and length is 5-10 µm.	+	+	+	-	*Saccharomyces*
C2	Colonies are white	Long elliptical cells, about 13-19 µm	+	+	-	+	*Pichia*
D1	Milky white colonies, spores round, ovoid, smooth surface glossy	Large globular cells, about 10-12 µm in width, from 16 in µm length.	+	+	+	-	*Saccharomyces*
D2	Milky white colonies, round, glossy surface, Smooth surrounding edge	Oval or egg-shaped cells, the average size ranges from 1.5-4×4-7 µm.	+	+	+	-	*Candida*

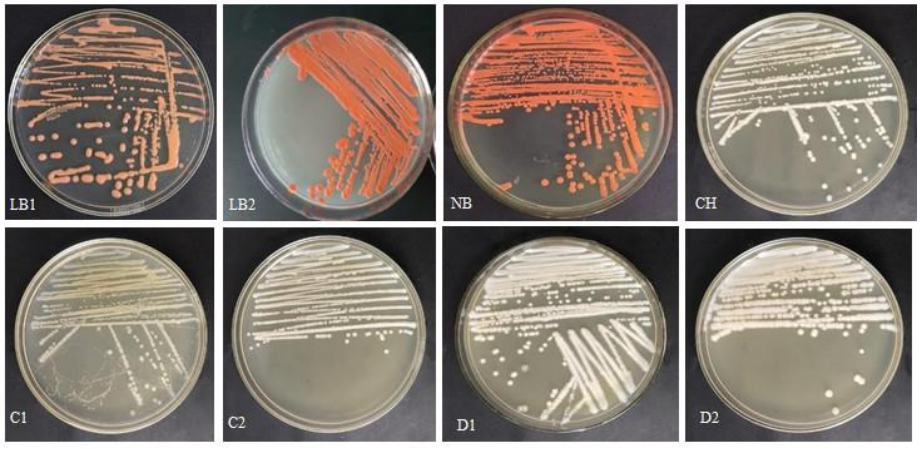

Figure 2. Colony morphology of 8 isolated yeast strains

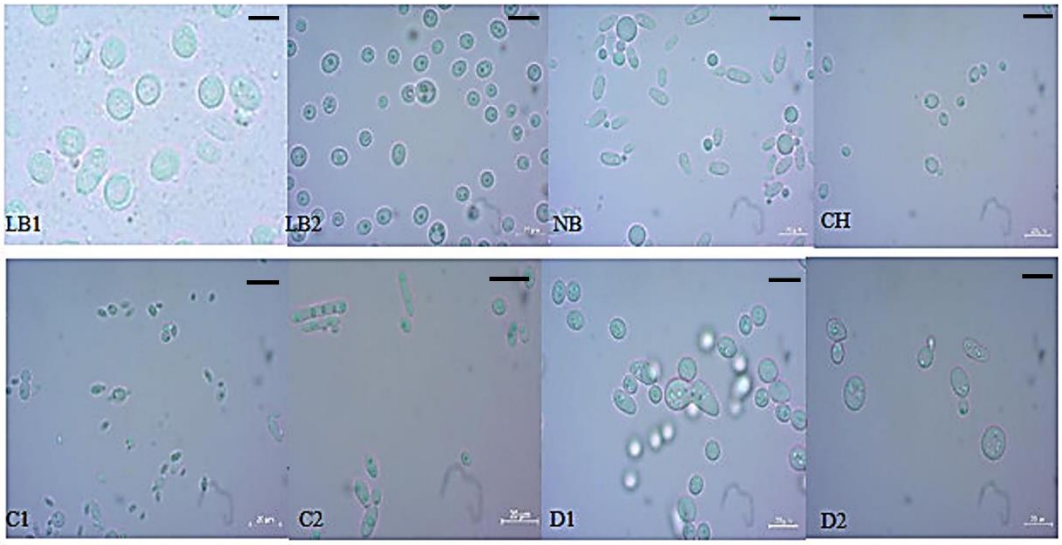

Figure 3. Cell morphology of the 8 isolated yeast strains. Scale bar, 20 µm.

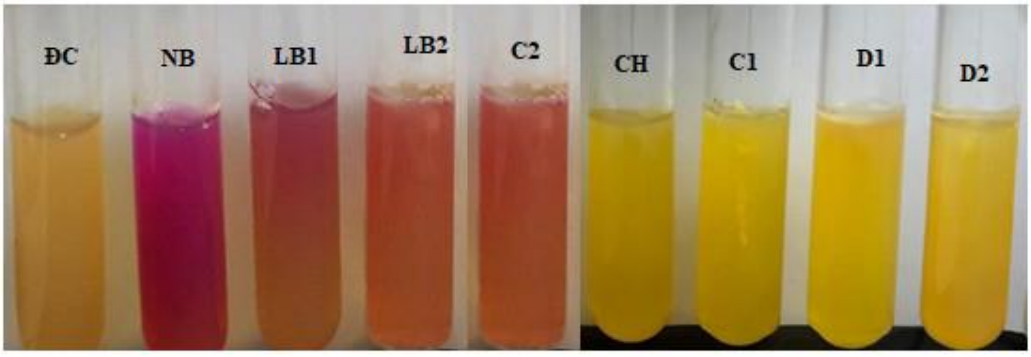

Figure 4. The ability of the eight yeast isolates to degrade urea on Christensen's medium after 7 days

3.1. Selection of yeasts with strong antagonistic ability to *Alternaria* sp.

The results showed that all eight isolates have the antagonistic ability to *Alternaria* sp., which ranged from 10.46% to 59.86%. In which, isolate D2 showed the strongest ability at 59.86%. This inhibition rate was higher than the *Trichosporon asahii* strain of 2.39% in the study reported by Bosqueiro et al. (2020).

Table 3. Resistance of yeasts against *Alternaria* sp.

Isolate	Radius of fungal colony (cm) 4 days	Inhibitory efficiency after 4 days (%)	Radius of fungal colony (cm) 8 days	Inhibitory efficiency after 8 days (%)
Control	3.81±0.3^{b}	-	7±0.00^{f}	-
LB1 + Alternaria sp.	3.78±0.22^{b}	0.73± 0.05^{b}	4.7±0.1^{c}	32.8±0.04^{c}
LB2 + Alternaria sp.	3.68±0.17^{b}	3.3±0.06^{b}	4.56±0.5^{c}	34.69±0.11^{c}
NB + Alternaria sp.	3.8±0.2^{b}	0.33±0.01^{b}	6.26±0.7^{e}	10.46±0.008^{e}
CH + Alternaria sp.	3.75±0.25^{b}	1.69±0.07^{b}	6.13±0.32^{e}	12.37±0.007^{e}
D1 + Alternaria sp.	2.7±0.3^{a}	29.08±0.09^{a}	4.21±0.27^{bc}	39.49±0.46^{bc}
D2 + Alternaria sp.	2.65±0.31^{a}	30.2±0.31^{a}	2.85±0.05^{a}	59.86±0.52^{a}
C1 + Alternaria sp.	2.66±0.03^{a}	30.14±0.17^{a}	3.73±0.25^{b}	46.8±0.24^{b}
C2 + Alternaria sp.	3.8±0.2^{b}	0.35±0.01^{b}	5.48±0.45^{d}	21.3±0.05^{d}

$^{a, b, c, d, e, f}$ *Means with different superscripts in a column differ significantly ($p<0.05$).*

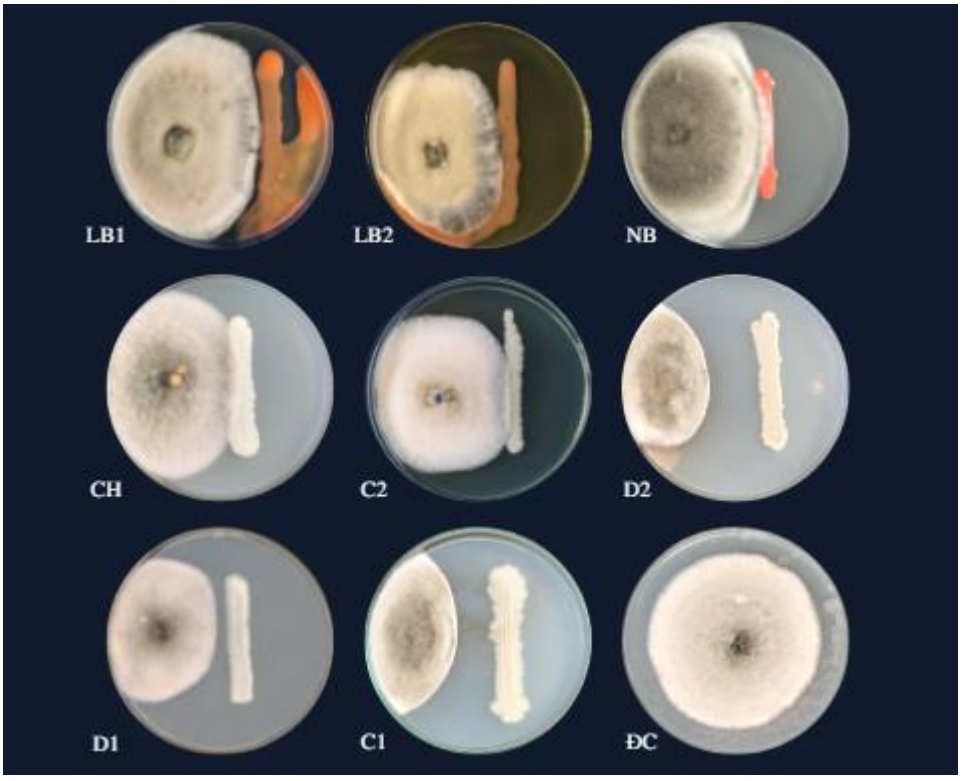

Figure 6. Antagonism of the eight yeast strains to *Alternaria* sp. after 8 days

The effectiveness of the D2 yeast strain against the fungus Alternaria sp. may be related to its ability to produce proteolytic enzymes to break down fungal cell walls , and its ability to produce volatile organic compounds, β-1,3-glucanase and competition for nutrients, which are detrimental to the germination of *Alternaria sp*. (Kowalska et al., 2022). Yeasts can secrete such enzymes as chitinases, glucanases, lipases, or proteases. Chitinases allow efficient degradation of the cell wall of plant pathogens, and their secretion is considered useful for biocontrol agents. Among yeasts ,this activity has been described for such genera as Candida, Metschnikowia, and Saccharomyces. Besides, β-1,3-glucanase, which is produced by such yeasts as Candida famata (Zopf) Lodder & Kreger, Rhodotorula mucilaginosa (A. Jorg.) F.C. Harrison and W. anomalus, is effective in reducing pathogen growth.

The production of extracellular enzymes has been reported to be an important feature of the biological control of phytopathogenic fungi (Spadaro et al., 2016). Since the cell wall of fungi is composed of glycoproteins, polysaccharides, glucan manly, and chitin (Bowman et al., 2006), disruption of such structures requires antagonistic microorganisms using different enzymes, which can cause malformations, cell damage, mycelium lysis, and changes in membrane permeability (Dukare et al., 2019). Da Cunha et al. (2018) evaluated strains of *Rhodotorula minuta* and *Saccharomyces cerevisiae* for hydrolytic enzymes and found that one of the main mechanisms of antifungal activity may be related to the production of β-1,3-glucanase, yielding 0.004 g L-1 and 0.039 g L-1 dextrose for *R. minuta* and *S. cerevisiae*, respectively, after 24 h incubation. Competition for nutrients is one of the most important mechanisms reported for yeast (Dukare et al., 2019; Nunes, 2012; Spadaro et al., 2016). According to the author, in the presence of T. asahii, the germination rate of conidia of Alternaria sp. was significantly lower. Yeast can utilize a variety of carbohydrates, including disaccharides and monosaccharides (Zhang et al., 2011). One of the mechanisms used by yeasts to compete for iron is the production of intermediate compounds. These iron-chelating compounds, secreted to form a stable complex with iron ions, but are unusable by other microorganisms (including pathogenic fungi) (Dukare et al., 2019).

3.2. Identification of yeasts by molecular biology techniques

After the chemical characterization of all eight isolates, isolate D2 was selected because it presented a very high performance in antoganism test to *Alternaria* sp. Result of ITS gene analysis by using BLASTn tool showed, the yeast line of D2 is Candida tropicalis with sequence 521bp (5'-TTCCTCCGCTTATTGATATGCTTAAGTTCAG CGGGTAGTCCTACCTGATTTGAGGTCAAAG TTATGAAATAAATTGTGGTGGCCACTAGCA AAATAAGCGTTTTGGATAAACCTAAGTCGC TTAAAATAAGTTTCCACGTTAAATTCTTTCA AACAAACCTAGCGTATTGCTCAACACCAAA CCCGGGGGTTTGAGGGAGAAATGACGCTC AAACAGGCATGCCCTTTGGAATACCAAAG GGCGCAATGTGCGTTCAAAGATTCGATGAT TCACGAATATCTGCAATTCATATTACGTAT CGCATTTCGCTGCGTTCTTCATCGATGCGA GAACCAAGAGATCCGTTGTTGAAAGTTTTG ACTATTGTAATAATAAATCAAGTTTGACTG TAAATAAAAAGTTTGGTTTAGTTATAACCT CTGGCGGTAGGATTGCTCCCGCCACCAAAG AAATTTGTTCAATAAAAAACACATGTGGTG CAATTAAGCAAATCAGTAATGATCCTTCCG CAGGTTCACCTA CGGA-3'). *Candida tropicalis* is a valuable yeast species in the wine industry (Van Thuoc et al, 2015, it not only ferments fruit juice or high sugar content but also produces fermented products. Yeast with a characteristic flavor. Sugar fermenting capacity strains *C. tropicalis* has many potential applications in the wine industry or bio-alcohol. The efficacy of the antagonistic yeast (*Candida tropicalis* YZ27) as a biological control agent against postharvest natural decay and quality retention of litchi (cv. Bombai) was studied (Zhimo et al, 2018). Application of the yeast antagonist led to the rapid colonization on the surface of the fruit and significantly reduced the natural decay incidence and severity following storage at ambient condition (28 ± 2°C, 78 ± 1% RH) for six days compared to the control fruit.

4. CONCLUSION

In this study, 8 strains of yeast were isolated and selected with the antagonistic ability to *Alternaria* sp. causing disease on Tru Long pummelo trees. Test results of the inhibitory effect ranged from 10.46% to 59.86%. Among them, strain D2 had 59.86% the highest antagonistic effect to *Alternaria* sp. The results of D2 identification by ITS gene sequencing showed that this strain belongs to *Candida tropicalis*. The results of this study confirm that epiphytic yeast strains are a good source of biological control for plant diseases.

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