ANTIFUNGAL ACTIVITY OF SOIL YEAST (*LACHANCEA KLUYVERI* SP132) AGAINST RICE PATHOGENIC FUNGI AND ITS PLANT GROWTH PROMOTING ACTIVITY

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ABSTRACT

A soil yeast, *Lachancea kluyveri* SP132, was isolated from rice paddy field soil in Nakhon Pathom province, Thailand, and was evaluated for its antifungal activity and plant growth promoting activity at Kasetsart University, Kamphaeng Saen Campus, Thailand, from 2016-2017. SP132 displayed potent *in vitro* inhibitory activity on mycelial growth against *Rhizoctonia solani*, a rice sheath blight fungal pathogen, using the dual culture method. The potent yeast also exhibited antifungal activity against *Curvularia lunata*, a rice dirty panicle fungal pathogen. Cell-free culture of SP132 displayed an effect on hyphal morphology and mycelial growth of pathogenic fungi. Based on the inhibition activity values, cell-free culture of SP132 showed the best effect on *R. solani* growth. The highest inhibition activity against *R. solani* (87.67%, compared with the control) was achieved using 30% cell-free culture. The ability of SP132 to produce extracellular antifungal enzymes (chitinase, cellulase and amylase) suggested that these enzymes may be partly correlated with the antagonistic activity against rice pathogenic fungi. Study on plant growth promoting activities revealed that this effective yeast antagonist produces indole-3-acetic acid (IAA) and ammonia and can generate phosphate solubilization. The treated rice seed with SP132 had improved seed germination and seedling growth. These results suggested that the *L. kluyveri* SP132 isolated in this work may be further used as a biocontrol agent and plant growth promoting agent.

Key words: rice sheath blight, rice dirty panicle, biocontrol, seed germination, seedling growth

INTRODUCTION

Sheath blight caused by *Rhizoctonia solani* is one of the most common and destructive diseases of rice, which occurs throughout temperate and tropical rice production countries, including Thailand. Rice sheath blight disease causes 10% and 20% annually yield loss in India and Thailand, respectively (Boukaew and Prasetsan, 2014). Under outbreak and favorable environmental conditions, the yield loss can reach over 50% (Qingzhong et al., 2001; Richa et al., 2016). *R. solani*, a sclerotium-forming plant pathogenic fungus, is a universal soil saprotrophic and facultative plant parasite. Apart from rice, this pathogen also infects several important plant species, for example, sugar beet, cucumber and potato (El-Tarabily, 2004; Huang et al., 2012; Ben Khedher et al., 2015). Dirty panicle is also a serious disease of rice, causing great losses in grain and seed production. The Southern part of Thailand had 92.2% of disease occurrence (Bubpha et al., 2016). Balgude and Gaikwad (2016) reported that this disease was found to be very severe all over Maharahtra, India, causing 20-40% yield loss. *Curvularia lunata* is one

of the primary causal fungal pathogens of dirty panicle disease. This fungus is also a pathogen on various plant species, such as lotus, sweet sorghum and mulberry (Cui and Sun, 2012; Tong et al., 2015; Bussaban et al., 2017).

Fungicides are widely used for controlling these fungal plant pathogens. However, chemical control has a significant impact on human health and environment. Biological control has been described as an attractive alternative and environmentally friendly strategy for controlling plant diseases. Yeasts are one of the promising antagonistic microorganisms, since they are effective against a wide range of pathogens, are easily produced in large scale production using inexpensive substrates and do not produce toxins or metabolites harmful to human health (Qing and Shiping, 2000; Nally et al., 2015). Several yeast species have been shown to be effective biological control agents in protecting plants against fungal diseases. For example, yeasts isolated from the sour and grey rots, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, showed biocontrol activity against *Botrytis cinerea* which causes grey rot disease (Nally et al., 2013). *Pichia guilliermondii* and *Metschnikowia pulcherrima* showed potential antagonistic activity against *Fusarium fujiikuroi* which causes bakanae disease of rice (Matić et al., 2014).

In recent years, a large array of yeasts, including species of *Galactomyces*, *Barnettozyma*, *Aureobasidium* and *Rhodotorula*, have been shown to act as biocontrol agents and plant growth promoting agents (Ignatova et al., 2015; Fu et al., 2016). Plant growth-promoting microorganisms have a number of beneficial effects on plant growth, for example, nitrogen fixation, phytohormones production, solubilization of mineral phosphates and other nutrients and cyanide, siderophore and antibiotic production (de Souza et al., 2015).

In this study, we isolated yeast strains from rice leaves and rice field soil samples and screened the potent yeast against *R. solani*. The selected yeast antagonist, *Lachancea kluyveri* (Syn. *Saccharomyces kluyveri*) SP132, which showed the best activity to inhibit *R. solani*, was also evaluated for its inhibition activity against *C. lunata*. Cell-free culture was also investigated for its ability to inhibit fungal growth. To our knowledge, this is the first report of *L. kluyveri* acting as a biocontrol agent to inhibit the rice fungal pathogens, *R. solani* and *C. lunata*, and also as a plant growth promoting agent to improve seed germination and seedling growth of rice.

MATERIALS AND METHODS

Pathogenic fungi. The rice pathogenic fungi, *R. solani* KPK00289 and *C. lunata* KPK00290, were obtained from the Plant Health Clinic, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. The pathogenic fungi were maintained at 4 °C on potato dextrose agar (PDA) medium.

Isolation and screening of antagonistic yeasts. The yeast antagonists were isolated from rice leaves, and rice field soils in Nakhon Pathom province and Suphanburi province, Thailand. Clay loam soil samples were collected at a dept below 10 cm. Leaf samples were collected from healthy rice plant. A 10 g of each samples was added to 100 ml of yeast extract peptone dextrose broth (YPD) supplemented with 100 µg/mL streptomycin and incubated at 30 °C for 24 h with shaking at 150 rpm/min. After serial dilution, the suspension was plated on YPD agar and incubated at 30 °C for 24 h. All yeast isolates were screened to select the antagonistic yeast displaying the strongest growth inhibitory activity against mycelial growth of *R. solani* using dual culture assay. Then, the selected yeast antagonist was also evaluated for its ability to inhibit mycelial growth of *C. lunata*. A 5 mm plug of 5 day-old pathogenic fungus was placed 2.5 cm away from the edge in a Petri dish containing PDA and incubated at 30 °C in an incubator without light for 24 h. The isolated yeast was streaked on the same Petri dish 4.5 cm away from the plug of pathogenic fungus (Saechow et al., 2018). After 3 days (*R. solani*) and 7 days (*C. lunata*) incubation at 30 °C, the percentage of inhibition of radial growth (PIRG) was calculated

using the following formula: PIRG (%) = [(R1 – R2)/R1)] × 100, where, R1 is the radial diameter of the control colony and R2 is the radial diameter of the treatment colony (Rahman et al., 2009). Experiments were performed in triplicates.

Yeast identification. Identification of yeast antagonist was performed based on its genetic material using molecular biology technique. The amplification of the internal transcribed spacer (ITS) of the rDNA including the 5.8S gene was performed using PCR with the universal primers ITS1 and ITS4, according to White et al. (1990). The DNA sequence was compared to those previously published in GenBank using the BLASTN program.

Phylogenetic analysis. The sequences were multiple aligned using Clustal W and phylogenetic tree was generated with the neighbour joining method using MEGA X program (Kumar et al. 2018). Bootstrapping was performed for 1,000 replicates.

Effect of SP132 on hyphal morphology. Hyphal strands at the edge of the fungal colony nearest to the inhibition zone after incubation for 3 days (*R. solani*) and 5 days (*C. lunata*) were removed and examined under a light microscope (Olympus CX31, Japan).

Effect of SP132 cell-free culture on mycelial growth and spore germination. SP132 was grown in potato dextrose broth (PDB) with continuous shaking at 150 rpm and 30 °C for 84 h. Cell-free supernatants were collected by centrifugation at 15,000 rpm for 10 min at 4 °C, and then filtered through a 0.45 µm Millipore® membrane. The yeast cell-free culture was added to PDB containing spore suspension ($1x10^5$ spores/mL) of each fungal pathogen to yield a final concentration of 5%, 15% and 30% (Saechow et al., 2018). For the control, each fungal pathogen was grown in PDB medium without a yeast cell-free culture. After culture at 30 °C with shaking at 150 rpm for 7 days, the mycelia were filtered and dried at 55 °C until constant weight.

Extracellular hydrolytic enzyme productions. The qualitative assay for extracellular hydrolytic enzyme productions was carried out using the agar well diffusion method. The ability of SP132 to produce chitinase and cellulase was examined on colloidal chitin agar (colloidal chitin 10 g/L, yeast extract 0.5 g/L, $(NH_4)_2SO_4$ 0.1 g/L, $MgSO_4{\cdot}7H_2O$ 0.3 g/L, KH_2PO_4 1.36 g/L, agar 15 g/L) and carboxymethyl cellulose (CMC) agar (CMC 5 g/L, yeast extract 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, KCl 1 g/L, KH_2PO_4 1 g/L, agar 15 g/L), respectively. Protease, amylase and lipase productions were examined on skimmed milk agar (skimmed milk 10 g/L, glucose 1 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, K_2HPO_4 0.2 g/L, agar 15 g/L), starch agar (soluble starch 2 g/L, yeast extract 5 g/L, beef extract 3 g/L, peptone 5 g/L, agar 15 g/L) and Tween 80 agar (Tween 80 10 mL/L, peptone 10 g/L, NaCl 5 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L, agar 15 g/L). A 200 µL sample of cell-free culture prepared as described above was added to the well. An equal volume of culture medium (PDB) served as the control. After incubation at 30 °C for 24 h, the colloidal chitin agar, CMC agar and starch agar were flooded with 1.5% iodine solution for 5 min (Kasana et al., 2008; Richa, 2016). Enzyme production on skimmed milk agar and Tween 80 agar were detected without staining. For chitinase, cellulase, protease and amylase enzyme detections, positive tests were indicated by a clear halo zone around wells. A precipitation around well indicated the positive reaction for lipase enzyme detection.

Indole-3-acetic acid (IAA) production. IAA production was determined using the method of Loper and Schroth (1986). SP132 was cultured in PDB broth containing 500 µg/mL of L-tryptophan at 30 °C with shaking at 150 rpm. After 48 h of incubation, a 2 mL sample of supernatant was mixed with 2 drops of orthophosphoric acid and 4 mL of the Salkowski reagent (35% of perchloric acid 50 mL, 0.5M $FeCl_3$ solution 1 mL) and incubated at room temperature for 30 min. Optical density of the developing pink color was measured spectrophotometrically at 530 nm.

Phosphate solubilization. Qualitative determination of phosphate solubilization was performed on a National Botanical Research Institute's phosphate (NBRIP) agar plate, containing 0.5% tricalcium

phosphate, by observing the clear halo zone around the bacterial colony. The quantitative bioassay was performed by growing SP132 in NBRIP broth for 7 days at 30 °C on a shaker at 150 rpm. The amount of soluble phosphate was measured following the ascorbic acid method (Ruangsanka, 2014).

Ammonia production. SP132 was cultured in 10 mL peptone water and incubated at 30 °C for 48-72 h. The ammonia production was detected by adding 0.5 mL/tube of Nessler's reagent. The appearance of brown to yellow was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Effect of SP132 on rice seed germination and seedling growth. Effect of SP132 on rice seed germination and seedling growth was investigated using a slightly modified method reported by Saechow et al. (2018). Rice seeds (cultivar Pathumthani 1, soft texture cultivar having a distinctive aroma and high cooking quality) were soaked in distilled water for 15 h, sterilized in 10% Clorox for 10 min and washed five times with sterilized distilled water. The surface sterilized seeds were soaked for 1 h in a cell-suspension of SP132 (1×10^8 CFU/mL) and then blotted dry. The seeds soaked in sterilized distilled water were used as the control. The treated seeds (100 seeds) were incubated in a tray with moist filter paper and incubated at 30 °C in a growth chamber. The percentage of seed germination and length of shoot and root were measured after 36 h and 7 days of incubation, respectively.

RESULTS AND DISCUSSION

Screening and identification of antagonistic yeast. Several previous reports demonstrated the potential value of yeast antagonists for controlling plant diseases, for example, bakanae disease of rice (Matić et al., 2014), grey rot disease and sour rot disease (Nally et al., 2013; Nally et al., 2015). Among the 86 yeast isolates obtained in this study, SP132 showed the highest inhibitory activity against colonies growth of *R. solani* (PIRG; 59%) using the dual culture method (Fig. 1A and B). This isolate was then selected to test its antifungal activity against the dirty panicle fungal pathogen of rice, *C. lunata*. The results revealed that SP132 could also inhibit colonies growth of *C. lunata* (PIRG; 29%) (Fig. 2A and B). SP132 was identified based on 5.8S-ITS region sequence analysis as *Lachancea kluyveri*, and its sequence was deposited in the GenBank database under the accession number MH333067. The result of phylogenetic analysis based on 5.8S-ITS sequence data is shown in Fig. 2.

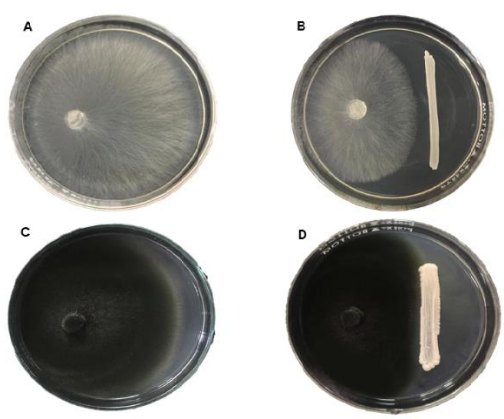

Fig. 1. Antifungal activity of SP132 against rice pathogenic fungi, *R. solani* (A); *R. solani* co-inoculated with SP132 (B); *C. lunata* (C); and *C. lunata* co-inoculated with SP132 (D).

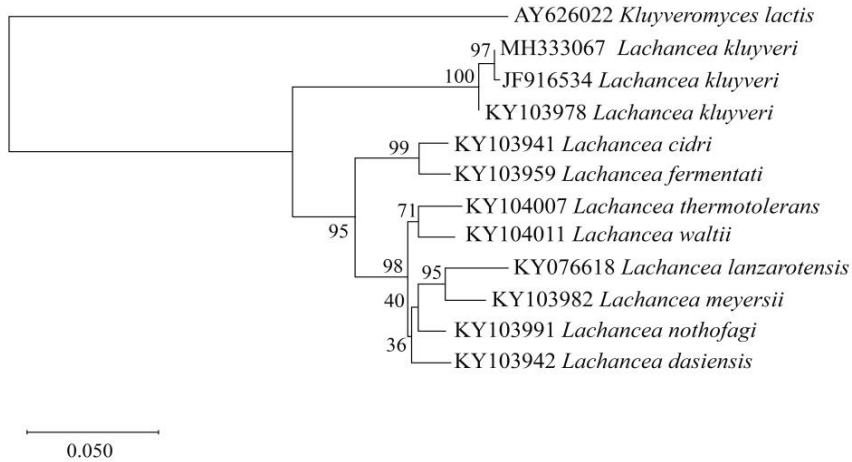

Fig. 2. Neighbour-joining tree based on 5.8S-ITS region showing the relationship between *L. kluyveri* SP132 (MH333067) and members of genus *Lachancea*.

Effect of SP132 on hyphal morphology of pathogenic fungi. The pathogenic fungi hyphae from the edge of the inhibitory halo were observed under a light microscope as shown in Fig. 3. SP132 caused cytoplasmic coagulation of *R. solani* hyphae (Fig. 3A) and enlargement of the cytoplasmic vacuoles of *C. lunata* hyphae (Fig. 3C), whereas the fungal hyphae from the untreated control samples showed normal and intact morphology (Fig. 3B and D).

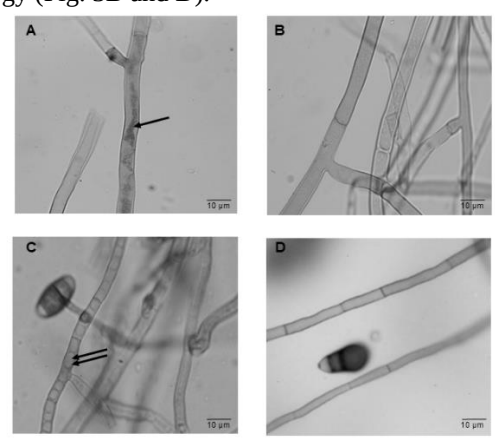

Fig. 3. Morphological changes of rice pathogenic fungi. Hyphae of *R. solani* from co-inoculation with SP132 (A); and normal hyphae of *R. solani* (B); hyphae of *C. lunata* from co-inoculation with SP132 (C); and normal hyphae of *C. lunata* (D). Arrow indicates cytoplasmic coaglulation and double arrows indicate vacuolization hyphae.

Effect of SP132 cell-free culture on fungal growth. The effect of SP132 cell-free culture on fungal growth was studied using the dry weight determination method. The pathogenic fungi were treated with different concentrations (5%, 15% and 30%) of SP132 cell free-culture. As shown in Table 1, the mycelial growth of *R. solani* and *C. lunata* were inhibited by cell free-culture. The highest dry weight reduction values of *R. solani* and *C. lunata* were 87.67% and 37.00%, respectively, after treating with 30% cell-free culture. Therefore, this concentration was selected to examine its effect on the spore

germination and germ tube elongation of the rice fungal pathogen. Regarding to the sclerotium-forming fungal pathogen of *R. solani*, spore germination and germ tube elongation were only examined in *C. lunata* using a light microscope. In the presence of SP132 cell-free culture, spore germination and germ tube elongation occurred but the hyphae showed abnormal vacuolization (Fig. 4A). In contrast to the untreated control, spores of *C. lunata* germinated normally and the germ tubes developed into normal hyphae (Fig. 4B).

Table 1. Effect of SP132 cell-free culture on dry weight of *R. solani* and *C. lunata.*

Concentration of cell-free culture (%)	Dry weight reduction (%)	
	R. solani	***C. lunata***
5	37.33 ± 2.88^{c}	10.67 ± 0.58^{c}
15	50.00 ± 2.00^{b}	24.67 ± 1.53^{b}
30	87.67 ± 2.52^{a}	37.00 ± 1.73^{a}

Data represented as mean±standard deviation. Means in each column with the same lowercase superscript letter are not significantly (p<0.05) different.

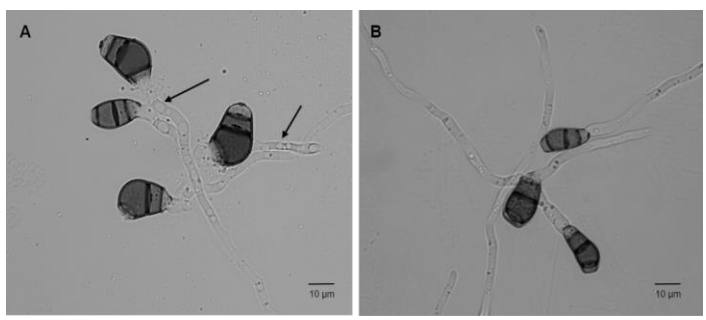

Fig. 4. Effect of SP132 cell-free culture (30%) on spore germination and germ tube elongation of *C. lunata* (A); and untreated spores of *C. lunata* (B). Arrows indicate abnormal vacuolization hyphae.

These results indicated that cell-free culture of SP132 showed an inhibitory effect on the growth and cellular changes of rice pathogenic fungi. Study on the extracellular enzyme production revealed that SP132 was able to produce chitinase, cellulase and amylase (Table 2). Khedher et al. (2015) suggested that protease and chitinase activities in cell-free culture of *Bacillus subtilis* V26 can act on *R. solani* growth by antibiosis. Melent'ev et al. (2001) reported that crude chitinase from *Bacillus* sp. 739 and pure chitinase caused cellular change, hyphal swelling and vacuolization, which affected the growth and caused dry weight reduction of *Fusarium* and *Helminthosporium*. Calistru et al. (1997) reported that extracellular enzymes (amylase, cellulase, pectinase, lipase and protease) from *Trichoderma* spp. may play an important role in antibiosis against *Aspergillus flavus* and *Fusarium moniliforme*. Fan et al. (2002) reported that *Pichia membranifaciens* and *Candida guilliermondii*, producing chitinase and β-1,3-glucanase, exhibited some effects on controlling *Rhizopus stolonifer*. The ability of yeast antagonists to produce and secrete lytic enzymes is suggested to be associated with the attachment of yeast cells to fungal hyphae and the partial degradation of fungal mycelia (Bar-Shimon et al., 2004; Fu et al., 2016). The hydrolytic enzymes from SP132 are suggested to be involved in the antagonist activity of biological control agents against fungal pathogens as described above. With an increase in the concentration of the cell-free culture of SP132, the inhibition value increased. These results may relate to the increased extracellular enzyme concentrations in cell-free culture.

Table 2. Extracellular enzymes production by SP132.

Extracellular hydrolytic enzyme				
Chitinase	**Cellulase**	**Protease**	**Amylase**	**Lipase**
+	+	-	+	-

+ corresponds to enzymatic activity detected.
- corresponds to no detectable enzymatic activity.

There are few reports demonstrating the ability of *L. kluyveri* as a biocontrol agent. Souza et al. (2017) reported that *L. kluyveri* CCMA0151 showed the ability to inhibit *Aspergillus ochraceus* and *Aspergillus caebonarius* growth. Nally et al. (2013) demonstrated that *L. kluyveri* BSk11 significantly reduced *Fusarium oxysporum* and *Rhi. stolonifer* growth. However, no biocontrol mechanism of this yeast antagonist has been reported. Here, we first report on the inhibitory effects of *L. kluyveri* on the rice fungal pathogens, *R. solani* (sheath blight disease) and *C. lunata* (dirty panicle disease). Based on the results in this investigation, the antifungal activity against rice pathogens may be partly explained by the production of extracellular hydrolytic enzymes.

Effective suppression of plant diseases by microbial biocontrol agents may also be influenced by the specific pathogen, host commodity and particularly by environmental conditions (Zahavi et al., 2000; Tian et al., 2002). Survival and antagonistic activity of yeast antagonists in the environmental conditions depend on yeast species. The yeast suspensions of *Trichosporon pullulans*, *Cryptococcus laurentii* and *Rhodotorula glutinis* were sprayed at concentration of 1×10^8 CFU/mL onto sweet cherry fruit in two orchards located in Beijing and Jinzhou district of Liaoning Province, China. The results revealed that only *Cryp. laurentii* and *Rho. glutinis* remained at high and stable population levels on the surface of sweet cherry fruit under field conditions which the temperature ranged from 10.8-32.2 °C (Shi-Ping et al., 2004). Calvo-Garrido et al. (2014) reported that under Mediterranean climate in vineyard located in Spain (relative humidity 61.8-61.9%, temperature 22.1-22.8 °C and rainfall 21.7-25.0 mm), yeast suspension of *Candida sake* at 1×10^7 - 5×10^7 CFU/ml was effective at reducing *Bacillus cinerea* secondary inoculum on necrotic grapevine tissues. Therefore, future work is needed to evaluate the effectiveness of SP132 in reducing rice disease under greenhouse and field conditions.

Plant growth promoting analysis and effect of SP132 on rice seed germination and seedling growth. In the past few decades, the plant growth-promoting characteristics of various microorganisms, including yeast, have been reported (Amprayn et al., 2012; Ait Kaki et al., 2013; Ignatova et al., 2015; Fu et al., 2016). In the present investigation, SP132 was able to solubilize phosphate (13.80 mg/L) at 30 °C after 7 days in the NBRIP liquid medium (Table 3). The production of IAA and ammonia was also positive in SP132.

As shown in Table 4 and Fig. 5, the treated rice seeds with a cell-suspension of SP132 had improved seed germination, with the germination values enhanced from 73.33% to 92.66%. Shoot and root lengths were significantly higher when the seeds were treated with the cell-suspension of SP132. At 7 days of rice seeding incubation, the shoot and root lengths of rice treated with the cell-suspension were enhanced by 56.93% and 25.00%, respectively, compared to the untreated seeds. These results indicated that the cell-suspension of SP132 was able to enhance rice seed germination and seedling growth compared with the untreated control. IAA is a phytohormone that has a positive effect on plant growth by stimulating plant cell elongation (Vessey, 2003). The low level of IAA produced by SP132 suggested that IAA production is not the main mechanism for enhancing rice seedling growth. Similar results were obtained by Amprayn et al. (2012), who reported that IAA production was not the main mechanism for increasing rice seedling growth using a cell-suspension of *Candida tropicalis* as a result of the low level of IAA production. The current results indicated that SP132 can potentially be used as an alternative agent to improve seed germination and plant growth. The improved rice seedling growth

caused by SP132 may be due to the involvement of IAA production and other plant growth promoting agents acting synergistically to promote plant growth.

Table 3. *In vitro* production of plant growth-promoting metabolites.

Phosphate solubilization (mg/L)	IAA at 500 µg/mL of tryptophan (µg/mL)	NH_3
13.80±0.11	0.70±0.07	+

+ corresponds to positive test.

Table 4. Effect of SP132 cell-suspension on rice seed germination and seedling growth.

Treatment	Germination (%)*	Shoot length (cm) **	Root length (cm) **
Untreated control	73.33±6.24[b]	3.97±0.05[b]	2.40±0.08 [b]
SP132	92.66±2.05[a]	6.23±0.17[a]	3.00±0.01[a]

* Germination values were measured at 36 h of incubation.
** Shoot and root lengths were measured at 7 days of incubation.
Data represented as mean±standard deviation. Means in each column with the same lowercase superscript letter are not significantly (p<0.05) different.

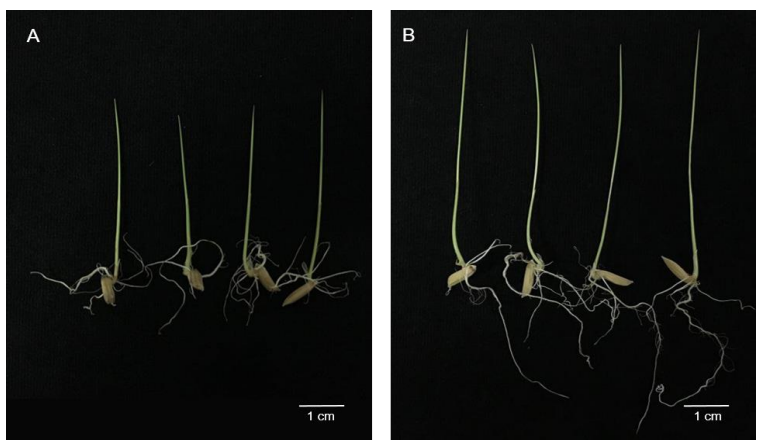

Fig. 5. Growth of rice seedlings (7 days of inoculation) from untreated control (A); and treated with SP132 (B)

CONCLUSION

L. kluyveri SP132, isolated from rice paddy field soil, exhibited potent *in vitro* inhibitory activity against rice fungal pathogens, especially, *R. solani*. Additionally, SP132 displayed multiple plant growth-promoting traits. This yeast antagonist can potentially be used as an alternative plant growth promoting agent to improve rice seed germination and seedling growth. Future work is needed to evaluate the effectiveness of SP132 in reducing rice disease and enhancing rice growth under greenhouse and field conditions.

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