



## Phosphate-solubilizing characteristics of yeasts

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### Abstract

Phosphorus (P) is an essential element for plant development and metabolism. Because of its low availability and mobility in soils, it is often a limiting nutrient of plant growth. When phosphorus content in soil is insufficient, symptoms of phosphorus deficiency can appear, such as purple spots on leaves and stems or inhibition of development and maturation. To provide adequate nutrients for plant growth, appropriate fertilizers should be applied. However, overuse of chemical fertilizers can cause unanticipated environmental effects. To decrease the negative environmental effects resulting from continued use of chemical fertilizers, we can inoculate plants with phosphate-solubilizing microorganisms. Phosphate-solubilizing microorganisms are crucial in dissolving fertilizer phosphorus and bound phosphorus in soil in a manner that is both environmentally friendly and sustainable. In this study, we selected yeasts with calcium-phosphate-solubilizing ability and found that this ability to be regulated by environmental factors (e.g. amount of soluble phosphate, liquid or solid agar plates condition, and type of inorganic phosphate). *Arabidopsis thaliana* was inoculated with selected yeasts; we found that *Cryptococcus laurentii* (JYC370) promoted plant growth in an inorganic phosphate (P<sub>i</sub>)-deficient medium supplemented with calcium phosphate dibasic dihydrate. The amount of cellular inorganic phosphate was also higher in yeast-treated plants than in control plants grown in the P<sub>i</sub>-deficient medium. This result reveals the potential of these strains for biofertilizer applications and commercial use as biofertilizer agents in the future.

**Keywords** – *Arabidopsis thaliana* – biofertilizer – calcium phosphate – phosphorus – phosphate-solubilizing microorganisms

### Introduction

Many elements are required for plant growth. According to the amount required by plants, elements are classified into major and trace elements. Major elements are chemical elements required by plants in large amounts and include nitrogen, phosphorus, and potassium. Trace elements are elements required by plants in small amounts and include iron, manganese, and boron. If plants lack these elements, symptoms of plant diseases can occur, and plant growth can be hindered. Although phosphorus (P) is abundant in soil in both inorganic and organic forms (Gyaneshwar et al. 2002), it is the least mobile and available major nutrient to plants in most soil conditions. Therefore, it is frequently the major or prime limiting factor of plant growth (Hinsinger 2001). Phosphorus is required for seed germination, protein formation, photosynthesis, and

metabolism, and it also promotes flowering and fruiting (Sultenfuss & Doyle 1999, Wrage et al. 2010). Depending on the environmental pH, phosphorus exists as several anions in soil ( $\text{PO}_4^{3-}$ ,  $\text{HPO}_4^{2-}$ , and  $\text{H}_2\text{PO}_4^-$ ); however, plants can only assimilate the inorganic phosphate ( $\text{P}_i$ ) forms of  $\text{HPO}_4^{2-}$  and  $\text{H}_2\text{PO}_4^-$ , which are present in soil at very low concentrations 0.1–10  $\mu\text{M}$  (Hinsinger 2001, Nussaume et al. 2011). Plants have evolved various developmental and biochemical mechanisms to overcome phosphorus deficiency. Developmentally, plants can invest more resources into development and allocate more carbon to the roots (Hermans et al. 2006), resulting in an increased root–shoot biomass ratio (Wrage et al. 2010). Biochemically, the hydrolysis of phospholipids is an important biochemical pathway that can lead to an increase in internal phosphate availability (Cruz-Ramírez et al. 2006). Plants can also improve their internal phosphatase activity or secretion of organic acids and phosphatases, which are required for the mineralization of organic P to release  $\text{P}_i$  into soil (Raghothama 1999, Belgaroui et al. 2016).

To provide adequate nutrients for plant growth, farmers intensively apply P fertilizers to prevent phosphorus deficiency and thus increase yields (Gilbert 2009). However, overuse of chemical fertilizers can cause unanticipated environmental effects. To reduce the negative environmental effects resulting from the overuse of chemical fertilizers, we can inoculate plants with plant-growth-promoting microorganisms, which benefit plant growth and development (Abhilash et al. 2016, Kumar 2016). Phosphate-solubilizing microorganisms, which are a type of plant-growth-promoting microorganisms, play a crucial role in supplying P to plants in a manner that is both environmentally friendly and sustainable (Gyaneshwar et al. 2002). Phosphate-solubilizing microorganisms include microorganisms that convert insoluble phosphates into soluble phosphates. These organisms utilize various mechanisms to solubilize phosphates. For example, (1) production of phosphatase: similar to plants, phosphate-solubilizing microorganisms produce phosphatase to catalyze the hydrolysis of phosphate (Ponmurugan & Gopi 2006). Phosphatases catalyze dephosphorylating reactions that involve the hydrolysis of phosphoester or phosphoanhydride bonds (Rodríguez & Fraga 1999). Depending on the environmental pH, phosphatases can be acidic or alkaline. Acidic phosphatases (EC 3.1.3.2) are a common class of enzymes that catalyze the hydrolysis of phosphomonoesters at acidic pH (Behera et al. 2017). Alkaline phosphatases (E.C.3.1.3.1) are hydrolases that function at alkaline pH (Nalini et al. 2015). (2) Secretion of organic acids: Studies have shown that microorganisms acidify the medium through the production of organic acids or the secretion of  $\text{H}^+$  during phosphate solubilization. Therefore, inorganic phosphate can be released through the substitution of protons for  $\text{Ca}^{2+}$  (Mardad et al. 2013, Meena et al. 2017). The secretion of various organic acids by phosphate-solubilizing bacteria is considered the main mechanism of phosphate solubilization (Chen et al. 2006, Patel et al. 2008). The type of acid produced depends on the microorganism. Many organic acids, including citric acid, gluconic acid, lactic acid, and succinic acid, are produced by phosphate-solubilizing bacteria. Among various organic acids, gluconic acid is the main organic acid secreted during solubilization by phosphate-solubilizing bacteria (Goldstein 1995, Vyas & Gulati 2009). Several researchers have reported that inoculation with phosphate-solubilizing bacteria result in improved growth, yield, and P uptake in several crops (Hameeda et al. 2008, Hu et al. 2010, Yu et al. 2012, Saxena et al. 2013). In addition to bacteria, phosphate-mineralizing fungi, such as *Absidia* spp. and *Penicillium purpurogenum*, have been reported to show potential as plant-growth-promoting agents (Gaiind & Nain 2015). The soil fungi belonging to genera *Aspergillus* and *Penicillium* have been shown to possess the ability to solubilize sparingly soluble phosphates in vitro by secreting inorganic or organic acids (Whitelaw 1999). However, most studies have investigated the phosphate-solubilizing ability of bacteria and filamentous fungi and have seldom focused on yeasts. The genome of yeasts is more stable than that of bacteria, and they are easier to culture than fungi. Therefore, yeasts may be a favorable candidate for biofertilizers. The aims of this study were as follows: (a) to select yeasts with calcium-phosphate-solubilizing ability, (b) to investigate whether this ability of yeasts is regulated by environmental factors (e.g. amount of soluble phosphate, liquid or solid agar plate condition, and type of inorganic phosphate), and (c) to

evaluate whether the inoculation of plants with calcium-phosphate-solubilizing yeasts improves their growth.

## Materials & Methods

### Yeast culture condition

The yeast strains used in this study were isolated from *Drosera spatulata* leaves in our previous study (Fu et al. 2016). As shown in Table 1, the yeasts selected in this study were strains with the ability to solubilize calcium phosphate dibasic dihydrate ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ ) or calcium phosphate tribasic ( $\text{Ca}_5(\text{OH})(\text{PO}_4)_3$ ) on Pikovskaya's agar plate, and some strains without solubilizing ability were selected as the control groups (Amprayn et al. 2012). Before each experiment, yeasts were cultured on Yeast Extract–Peptone–Dextrose (YPD) agar plates (1% Yeast extract, 2% Peptone, 2% Dextrose, and 2% (w/v) agar) at 28°C in an incubator. The voucher specimens of yeasts used in this study are deposited in the Bioresource Collection and Research Center, Hsinchu City, Taiwan (<http://www.bcrc.firdi.org.tw/>). Any requests should be addressed to the corresponding author.

**Table 1** Yeast strains used in this study.

Strains	calcium phosphate dibasic dehydrate <sup>1</sup>	calcium phosphate tribasic <sup>1</sup>	MS-calcium phosphate dibasic dehydrate	MS-calcium phosphate tribasic
<i>Meyerozyma caribbica</i> (JYC358)	No	Yes	Yes	Yes
<i>Candida</i> sp. (JYC363)	Yes	Yes	Yes	Yes
<i>Torulasporea</i> sp. (JYC369)	Yes	Yes	Yes	Yes
<i>Cryptococcus laurentii</i> (JYC370)	Yes	Yes	Yes	Yes
<i>Pseudozyma</i> sp. (JYC372)	Yes	Yes	Yes	Yes
<i>Aureobasidium pullulans</i> (JYC375)	Yes	Yes	Yes	Yes
<i>Kazachstania jainicus</i> (JYC361)	No	No	No	No
<i>Hanseniaspora uvarum</i> (JYC364)	No	Yes	No	No
<i>Hanseniaspora uvarum</i> (JYC365)	No	Yes	No	No

<sup>1</sup> Data from Fu et al. 2016

### Phosphate solubilization measurement

#### *In vitro* screening of yeast for calcium-phosphate-solubilizing activity

For the phosphate solubilization assay, the yeast strains selected were screened in a  $\text{P}_i$ -deficient (–P) quarter-strength Murashige–Skoog (MS) medium (pH 5.7) containing 10 g of sucrose, 0.5 g of 2-morpholinoethanesulfonic acid monohydrate, 25 mL of  $10 \times$  macronutrient salts (8.25 g of  $\text{NH}_4\text{NO}_3$ , 9.5 g of  $\text{KNO}_3$ , 2.2 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.85 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 500 mL of deionized water), 25 mL of  $10 \times$  minor nutrient salts (M0529, Sigma), 950 mL of deionized water, solid medium with 1.2% (w/v) Bacto-agar, and 5 g of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (04231, Sigma) or  $\text{Ca}_5(\text{OH})(\text{PO}_4)_3$  (CB0263, Bio Basic Inc.). Yeast suspensions (3  $\mu\text{L}$ ) were pipetted in the center of (–P) MS agar plates. After 5 (MS-calcium phosphate dibasic dihydrate) or 7 days (MS-calcium phosphate tribasic) of incubation at 28°C, a visible halo zone around the colonies was observed (caused by the solubilization of calcium phosphate by the yeast). The solubilization efficiency unit of each strain was calculated as the diameter of the entire visible halo zone divided by the diameter of the zone with yeast colonies. The strains that could dissolve calcium phosphate dibasic dihydrate or calcium phosphate tribasic on MS plates were then further tested for quantitative analysis of their calcium phosphate dibasic dihydrate or calcium phosphate tribasic solubilizing ability in liquid medium.

### ***Quantitative analysis of yeast for phosphate-solubilizing activity***

Quantitative analysis of phosphate-solubilizing yeasts was performed for their calcium phosphate dibasic dehydrate- and calcium phosphate tribasic- solubilizing abilities in liquid MS medium. Yeast strains were inoculated in 12 mL of MS medium and incubated for 3 days at 28°C. The yeast cultures were sampled every day to measure the concentration of soluble phosphate and the pH value. Yeast cultures (3 mL) were centrifuged at 13,000 rpm for 2 minutes, and the supernatant was transferred to a new microtube. The concentration of inorganic phosphate was measured through the ascorbate method (Ames 1966). The pH of the supernatant was measured using a pH meter (UltraBasic Benchtop pH Meter, Denver Instrument, Bohemia, NY, USA).

### **Influence of soluble phosphate on phosphate-solubilizing ability**

Media containing three concentrations of exogenous soluble phosphate (0, 5, and 20 mM) were prepared by using MS agar medium and 100 mM K<sub>2</sub>HPO<sub>4</sub> solution. A yeast suspension (3 µL) was inoculated into (-P) MS agar plates containing each of the three soluble phosphate concentrations in triplicate. The SE of each strain was calculated as the diameter of the visible halo zone divided by the diameter of the zone with yeast colonies.

### **Cocultivation of plants with yeasts**

#### ***Plant materials and growth conditions***

All plants used in this study were *Arabidopsis thaliana* ecotype Columbia (Col-0). The medium used in this experiment was the same as the P<sub>i</sub>-deficient medium but with the addition of 0.5 g of calcium phosphate dibasic dehydrate. Seeds were surface sterilized using 1% (v/v) sodium hypochlorite solution with a few drops of Tween 20 for 5 minutes. After washing four times in sterile distilled water, seeds were sown in plates containing the -P + calcium phosphate dibasic dehydrate medium. The plates were then placed vertically in a plant growth chamber with a photoperiod of 16-h light and 8-h dark at 22°C. Yeasts were inoculated at the opposite ends of agar plates containing 14-day-old germinated *Arabidopsis* seedlings (10 seedlings per plate). The culture condition was as follows: a photoperiod of 16-h light and 8-h dark and 22°C.

#### ***Quantitative analysis of cellular P<sub>i</sub> in plants***

At 14 days after inoculation, inorganic phosphate was measured. Cellular P<sub>i</sub> content was determined through the ascorbate method (Ames 1966). Briefly, plant tissues were weighed and subsequently submerged in 1 mL of 1% glacial acetate. After eight freeze-thaw cycles, 100 µL of the extract was mixed with 200 µL of H<sub>2</sub>O and 700 µL of P<sub>i</sub> reaction buffer (A = 0.48% NH<sub>4</sub>MoO<sub>4</sub>, 2.86% (v/v) H<sub>2</sub>SO<sub>4</sub>; B = 10% (w/v) ascorbic acid; A:B (v/v) = 6:1). The reaction was allowed to proceed at 45°C for 20 min. P<sub>i</sub> content was determined from the standard curve of K<sub>2</sub>HPO<sub>4</sub> and is expressed as µmol·g<sup>-1</sup> fresh weight (Wang et al. 2012).

#### **Statistical analysis**

Data are expressed as mean ± standard deviation (SD). The calcium phosphate dibasic dihydrate – or calcium phosphate tribasic-solubilizing ability of different yeast strains and the biomass and cellular P<sub>i</sub> content of *Arabidopsis* were compared with controls through one-way ANOVA with the least significant difference (when variance was homogeneous) or Games-Howell (when variance was heterogeneous) post hoc test. A value of  $P < 0.05$  was considered statistically significant.

### **Results**

#### **Yeasts' *in vitro* calcium-phosphate-solubilizing activity on solid agar plates**

Phosphorus availability is subject to its chemical fixation in soil with other metal cations, which depends on environmental conditions. In this study, nine strains were used (Table 1); five

had exhibited in vitro calcium phosphate dibasic dehydrate-solubilizing ability on Pikovskaya's agar plate, and eight had exhibited calcium phosphate tribasic-solubilizing ability on Pikovskaya's agar plate, as reported in our previous study (Fu et al. 2016). The phosphate-solubilizing ability on MS-calcium phosphate dibasic dehydrate and MS-calcium phosphate tribasic plates was different from that on Pikovskaya's agar plate. Interestingly, *Meyerozyma caribbica* (JYC358) did not show calcium phosphate dibasic dehydrate-solubilizing activity on Pikovskaya's agar plate but showed the activity on MS-calcium phosphate dibasic dehydrate plates. Furthermore, *Hanseniaspora uvarum* (JYC364 and JYC 365) showed calcium phosphate tribasic-solubilizing activity on Pikovskaya's agar plate but not on MS-calcium phosphate tribasic plates. Among the strains tested, *Candida* sp. (JYC363) exhibited the strongest solubilizing activity for both calcium phosphate dibasic dehydrate and calcium phosphate tribasic, with solubilization efficiency of 1.72 and 1.9, respectively (Figs. 1, 2, Table 2). Because of the unstable phosphate-solubilizing activity of *Pseudozyma* sp. (JYC372) on the agar plate, this strain was not further analyzed in the following experiments.

### Phosphate-solubilizing ability with different amounts of soluble phosphate

In this study, for most strains, the diameters of the visible halo zones around the colonies decreased when yeasts were incubated with the highest soluble phosphate concentration used (20 mM) (Figs 1, 2). This finding suggested that the calcium phosphate dibasic dehydrate- or calcium phosphate tribasic-solubilizing ability of most yeast strains was affected by exposure to a high concentration of soluble phosphate. On the MS-calcium phosphate dibasic dehydrate plates, the solubilizing ability of *Torulasporea* sp. (JYC369) was induced by low levels of exogenous soluble phosphate (0 mM) and inhibited by high levels of exogenous soluble phosphate (5 and 20 mM) (Fig. 1C, Table 2). By contrast, *M. caribbica* (JYC358), *Cryptococcus laurentii* (JYC370), and *A. pullulans* (JYC375) showed the strongest solubilizing ability in the presence of 5 mM soluble phosphate but the weakest ability in the presence of 20 mM soluble phosphate (Fig. 1A, D, F, Table 2). Notably, *Candida* sp. (JYC363) still dissolved calcium phosphate dibasic dehydrate in the presence of a high concentration of soluble phosphate; *Candida* sp. (JYC363) may not as influenced by exogenous soluble phosphate as were the other strains (Fig. 1B, Table 2). On MS-calcium phosphate tribasic plates, the ratio of the visible halo zone to colony diameter generally showed a decreasing trend as the soluble phosphate concentration increased for all yeast strains used in this study (Fig. 2, Table 3). Both *Torulasporea* sp. (JYC369) and *C. laurentii* (JYC370) lost their phosphate-solubilizing ability in the presence of 20 mM soluble phosphate (Fig. 2C, D, Table 3). Among the strains used, the phosphate-solubilizing ability of *Candida* sp. (JYC363) remained the strongest on MS-calcium phosphate tribasic plates (Fig. 2B, Table 3).

### Quantitative analysis of yeasts' phosphate-solubilizing ability in liquid medium

After screening the calcium-phosphate-solubilizing yeasts on agar plates, we found slight differences in the phosphate-solubilizing ability of the yeast strains between Pikovskaya's agar plate and MS plates with calcium phosphate dibasic dihydrate or calcium phosphate tribasic. Furthermore, the phosphate solubilization efficiency of nine strains were measured through quantitative analysis of the available phosphorus in the MS liquid medium (Fig. 3). In our preliminary test, we cultured yeasts in MS medium with calcium phosphate dibasic dehydrate or calcium phosphate tribasic for 7 days. We found that the solubilizing ability of most strains peaked on the third day and remained steady until day 7 (data not shown). Therefore, the following experiments were conducted in 3 days. In the MS-calcium phosphate dibasic dehydrate liquid medium, *Candida* sp. (JYC363) exhibited high solubilizing ability ( $4.77 \pm 0.09 \mu\text{M}$ ), whereas *M. caribbica* (JYC358), *Torulasporea* sp. (JYC369), *C. laurentii* (JYC370), and *A. pullulans* (JYC375) showed moderate solubilizing ability ( $3.81 \pm 0.84$ ,  $1.85 \pm 0.34$ ,  $1.52 \pm 0.34$ , and  $2.5 \pm 0.42 \mu\text{M}$ , respectively). However, *Kazachstania jainicus* (JYC361), *Hanseniaspora uvarum* (JYC364 and JYC 365), and *Pseudozyma* sp. (JYC372) showed little or no solubilizing ability ( $0.30 \pm 0.11$ ,  $0.35 \pm 0.15$ ,  $0.34 \pm 0.07$ , and  $-0.29 \pm 0.04 \mu\text{M}$ , respectively). The negative value indicates that the

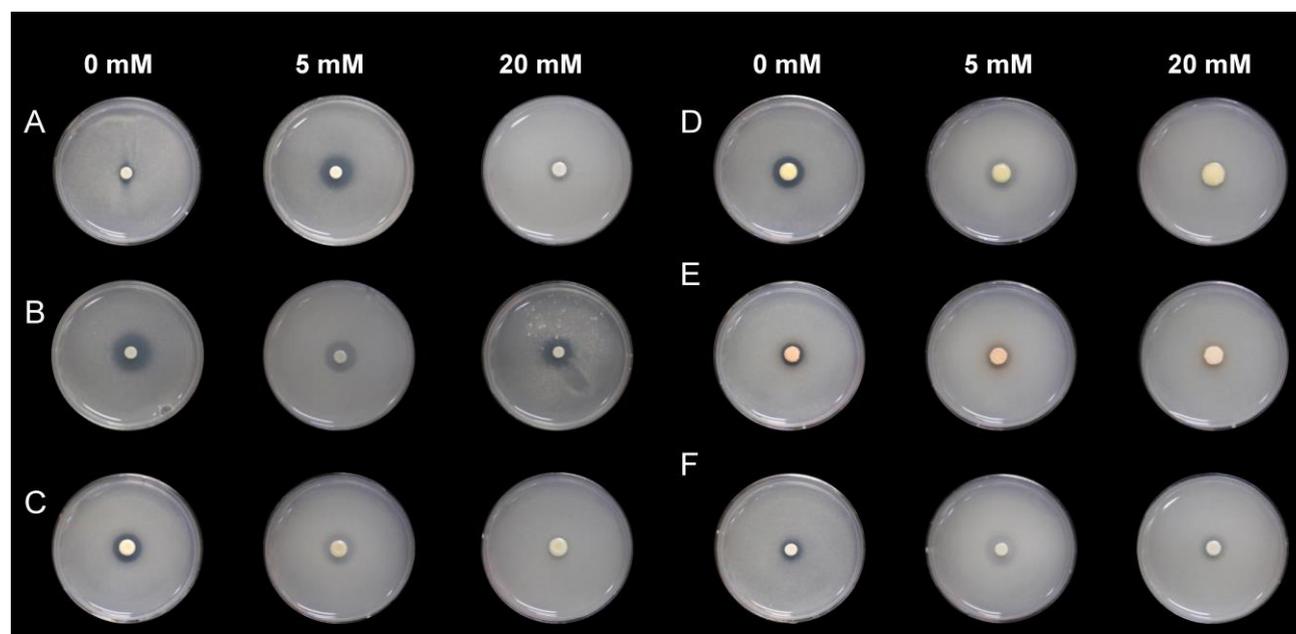
phosphate dissolved by the yeast was less than that in the medium without yeast inoculation, because the phosphate dissolved was entirely consumed by the yeast. The yeast growth condition of *Pseudozyma* sp. (JYC372) was similar to other yeasts; therefore, *Pseudozyma* sp. (JYC372) did not exhibit solubilizing ability in MS-calcium phosphate dibasic dehydrate.

**Table 2** Solubilization efficiency of different yeasts on MS-calcium phosphate dibasic dihydrate plates. Data are expressed as mean  $\pm$  SD.

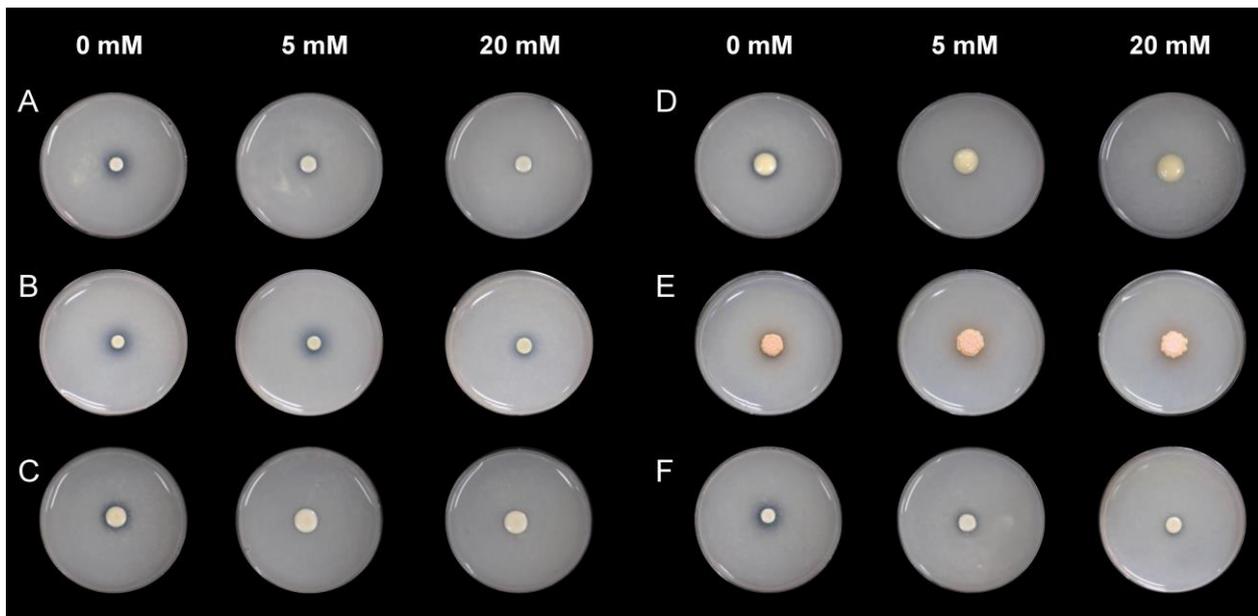
Soluble phosphate level (mM)	0 mM	5 mM	20 mM
<i>Meyerozyma caribbica</i> (JYC358)	1.6 $\pm$ 0.31	2.06 $\pm$ 0.08	1.44 $\pm$ 0.08
<i>Candida</i> sp. (JYC363)	1.72 $\pm$ 0.2	2.16 $\pm$ 0.71	2.05 $\pm$ 0.08
<i>Torulasporea</i> sp. (JYC369)	1.36 $\pm$ 0.06	1.25 $\pm$ 0	1.125 $\pm$ 0
<i>Cryptococcus laurentii</i> (JYC370)	1.19 $\pm$ 0.07	1.31 $\pm$ 0.13	1.11 $\pm$ 0
<i>Aureobasidium pullulans</i> (JYC375)	1.64 $\pm$ 0.14	2.07 $\pm$ 0.09	1.33 $\pm$ 0

**Table 3** Solubilization efficiency of different yeasts on MS-calcium phosphate tribasic plates. Data are expressed as mean  $\pm$  SD.

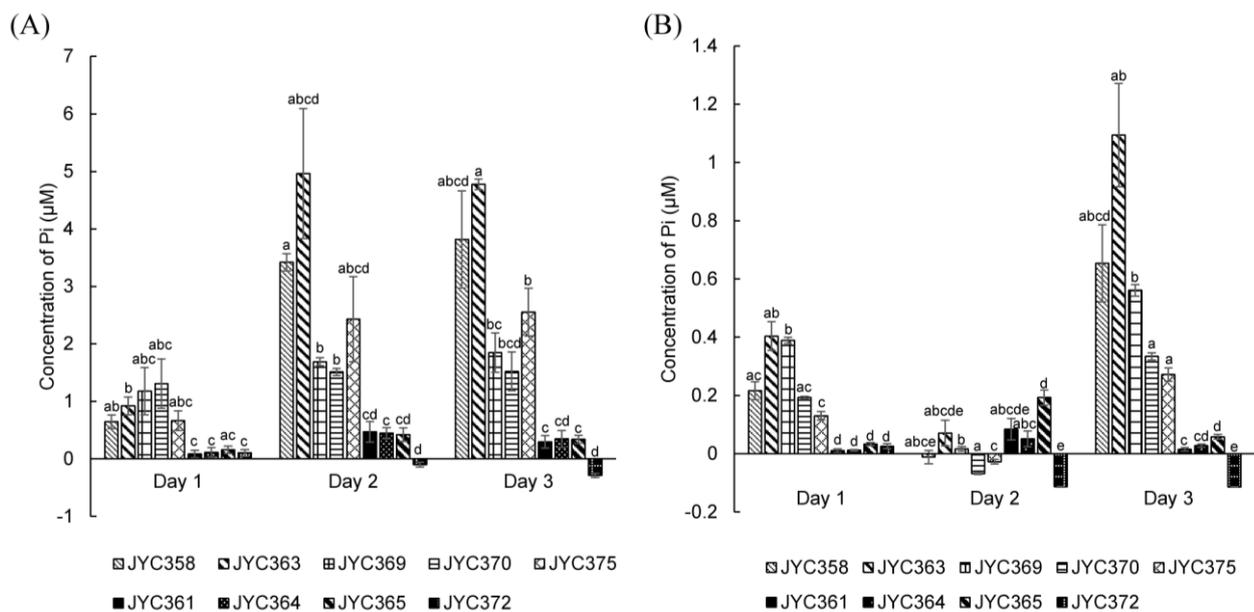
Soluble phosphate level (mM)	0 mM	5 mM	20 mM
<i>Meyerozyma caribbica</i> (JYC358)	1.78 $\pm$ 0.17	1.56 $\pm$ 0.08	1.36 $\pm$ 0.1
<i>Candida</i> sp. (JYC363)	1.9 $\pm$ 0.07	1.73 $\pm$ 0.07	1.64 $\pm$ 0.17
<i>Torulasporea</i> sp. (JYC369)	1.38 $\pm$ 0.01	1.12 $\pm$ 0.01	0
<i>Cryptococcus laurentii</i> (JYC370)	1.34 $\pm$ 0.01	1.13 $\pm$ 0.05	0
<i>Aureobasidium pullulans</i> (JYC375)	1.67 $\pm$ 0	1.33 $\pm$ 0.08	1.3 $\pm$ 0.01



**Figure 1** – Colony and phosphate solubilization characteristics of yeast strains on MS plate with calcium phosphate dibasic dihydrate. A *Meyerozyma caribbica* (JYC358). B *Candida* sp. (JYC363). C *Torulasporea* sp. (JYC369). D *Cryptococcus laurentii* (JYC370). E *Pseudozyma* sp. (JYC372). F *Aureobasidium pullulans* (JYC375).



**Figure 2** – Colony and phosphate solubilization characteristics of yeast strains on MS plate with calcium phosphate tribasic. A *Meyerozyma caribbica* (JYC358). B *Candida* sp. (JYC363). C *Torulaspora* sp. (JYC369). D *Cryptococcus laurentii* (JYC370). E *Pseudozyma* sp. (JYC372). F *Aureobasidium pullulans* (JYC375).

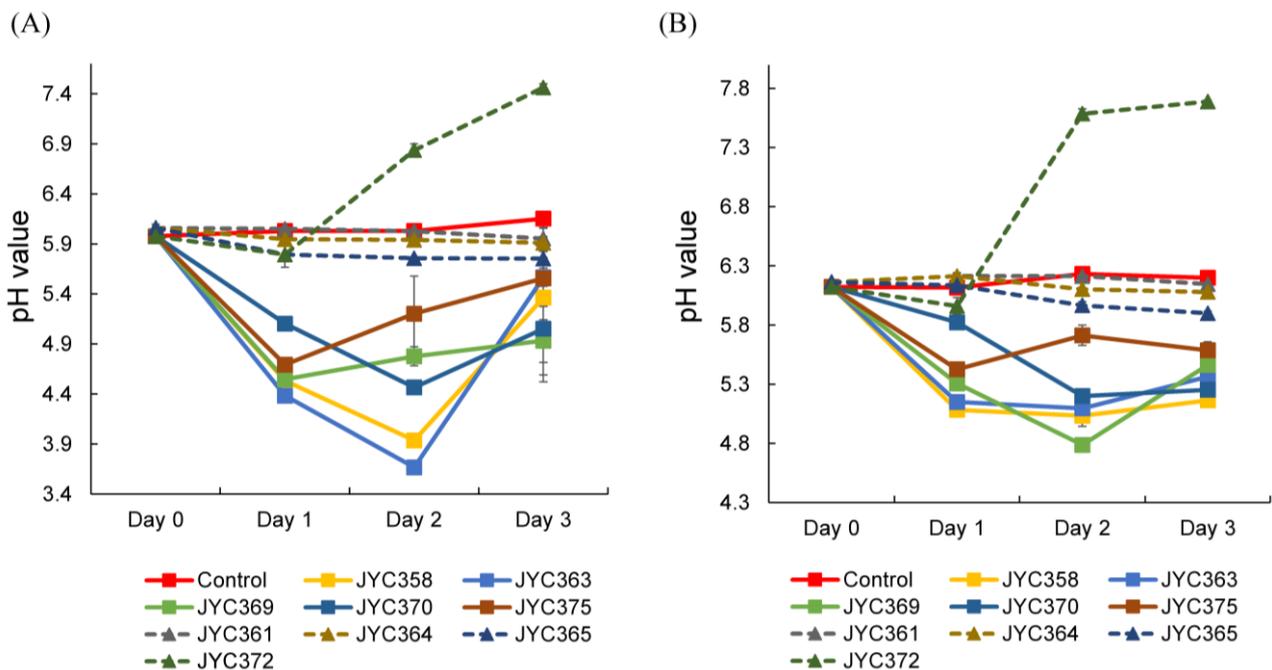


**Figure 3** – Changes of phosphorus content in MS medium. A MS medium with calcium phosphate dibasic dehydrate. B MS medium with calcium phosphate tribasic. Data are the mean of three independent experiments  $\pm$  SD. Means in the same group with the same letter are not significantly different from each other in one-way ANOVA with the Games–Howell post hoc test.

In the MS-calcium phosphate tribasic liquid medium, *Candida* sp. (JYC363) again exhibited high solubilizing ability ( $1.09 \pm 0.18 \mu\text{M}$ ), whereas *M. caribbica* (JYC358), *Torulaspora* sp. (JYC369), *C. laurentii* (JYC370), and *A. pullulans* (JYC375) showed moderate solubilizing ability ( $0.65 \pm 0.13$ ,  $0.56 \pm 0.02$ ,  $0.33 \pm 0.01$ , and  $0.27 \pm 0.02 \mu\text{M}$ , respectively). However, *K. jainicus* (JYC361), *Hanseniaspora uvarum* (JYC364 and JYC 365), and *Pseudozyma* sp. (JYC372) showed almost no solubilizing ability ( $0.01 \pm 0.01$ ,  $0.03 \pm 0.01$ ,  $0.06 \pm 0.01$ , and  $-0.11 \pm 0.00 \mu\text{M}$ , respectively). Again, the yeast growth condition of *Pseudozyma* sp. (JYC372) was not higher than

that of other yeasts used in this study; therefore, *Pseudozyma* sp. (JYC372) did not exhibit solubilizing ability in MS-calcium phosphate tribasic medium. We also found that more phosphates were dissolved by the yeasts in MS-calcium phosphate dibasic dehydrate than in MS-calcium phosphate tribasic.

Organic acid production by yeasts led to a decrease in the pH of the medium. The pH in most treatments dropped greatly compared with the initial pH ( $5.97 \pm 0.01$  in calcium phosphate dibasic dihydrate,  $6.12 \pm 0.02$  in calcium phosphate tribasic). The change in the pH of the medium was closely related to the phosphate-solubilizing ability of the yeasts. More soluble phosphate was dissolved in the medium with low pH (Fig. 4).



**Figure 4** – Changes of pH in MS medium. A MS medium with calcium phosphate dibasic dehydrate. B MS medium with calcium phosphate tribasic.

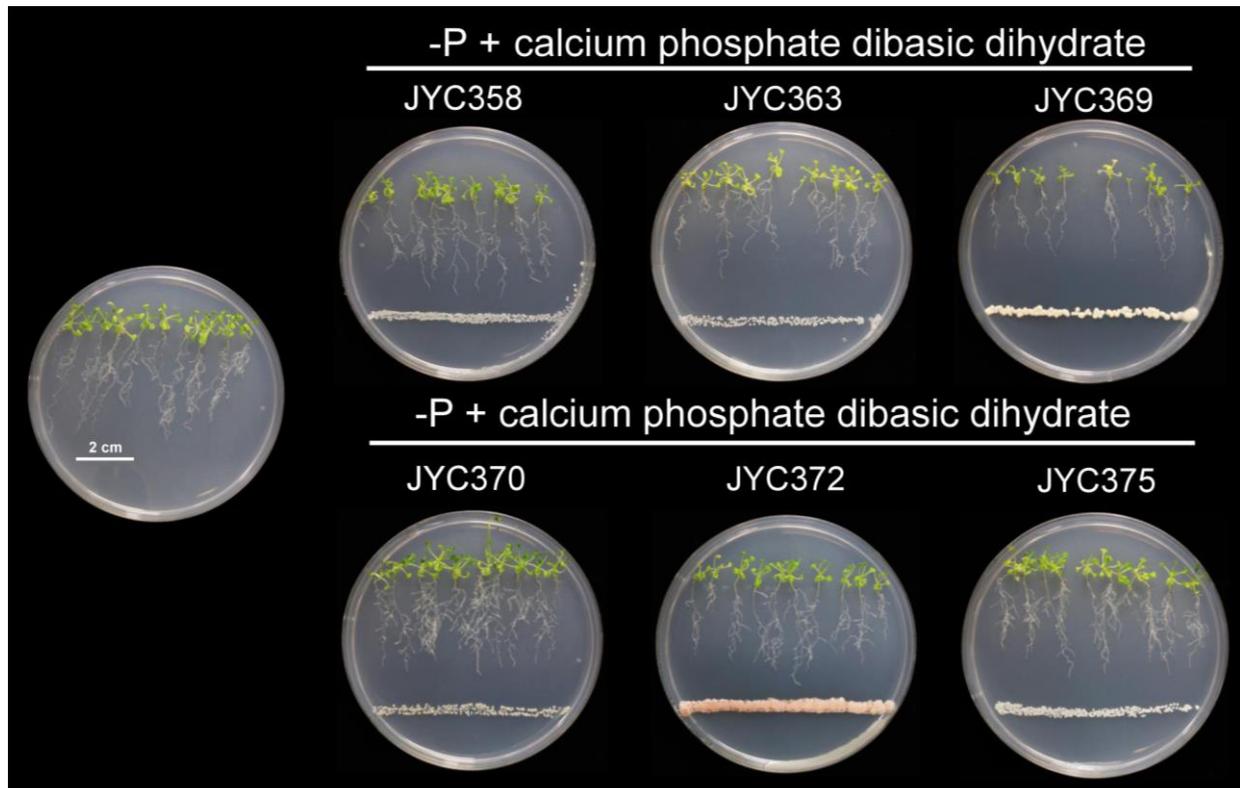
### Effects of phosphate-solubilizing yeasts on *A. thaliana* growth

To understand the effects of phosphate-solubilizing yeasts on plant growth and development, we used *A. thaliana* as a model system. Wild-type *A. thaliana* seedlings were germinated and grown on MS-calcium phosphate dibasic dehydrate plates. At 2 weeks after germination, yeast strains, including three phosphate-solubilizing strains, were inoculated 3 cm from the root tips, and one plate was not inoculated to serve as a negative control. *M. caribbica* (JYC358), *Candida* sp. (JYC363), and *A. pullulans* (JYC375) strains exhibited high phosphate-solubilizing ability, whereas *Torulasporea* sp. (JYC369), *C. laurentii* (JYC370), and *Pseudozyma* sp. (JYC372) strains exhibited low phosphate-solubilizing ability (Fig. 5). After 2 weeks of cocultivation of *A. thaliana* and *C. laurentii* (JYC370), remarkable increases in fresh weight were observed compared with controls (Fig. 6A). In particular, the cellular  $P_i$  content of *A. thaliana* cocultivated with *C. laurentii* (JYC370) was much higher than that of *A. thaliana* cocultivated with other strains or the control group (Fig. 6B, C). Although the cellular  $P_i$  content of *A. thaliana* cocultivated with *Pseudozyma* sp. (JYC372) or *A. pullulans* (JYC375) strains was higher than that of the control group, cellular  $P_i$  content, fresh weight, and biomass were not significantly increased compared with those of the control group. The results suggested phosphate-solubilizing yeasts exerted beneficial effects on plant growth and development. Notably, *Candida* sp. (JYC363), which showed the strongest phosphate-solubilizing ability, did not show plant-development-promoting behavior in our plant–yeast cocultivation assay.

## Discussion

### Factors influencing phosphate-solubilizing ability of yeasts

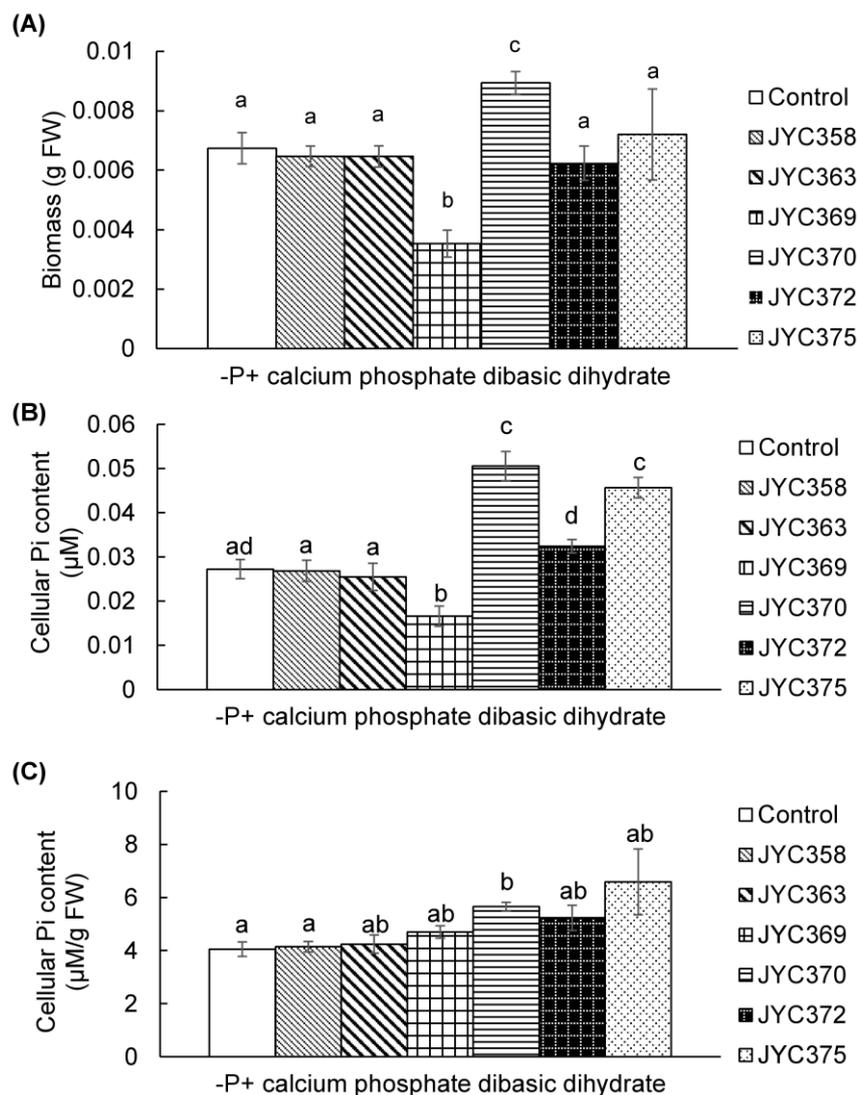
P is vital for plant growth and development. Phosphate-solubilizing microorganisms play a crucial role in enhancing phosphorus availability for plants in a manner that is both environmentally friendly and sustainable (Gyaneshwar et al. 2002). The criteria used to select yeast strains for this study were their ability to solubilize calcium phosphate dibasic dihydrate and calcium phosphate tribasic on MS plates. Therefore, we selected strains that could dissolve calcium phosphate dibasic dihydrate or calcium phosphate tribasic, and some strains that could dissolve any type of calcium phosphate were used as controls. According to a previous study (Fu et al. 2016), most yeast strains can dissolve calcium phosphate dibasic dihydrate on both Pikovskaya's agar plate and MS agar plates. Surprisingly, *M. caribbica* (JYC358), which could not solubilize calcium phosphate dibasic dihydrate on a Pikovskaya's agar plate, dissolved calcium phosphate dibasic dihydrate on an MS agar plate. By contrast, *Hanseniaspora uvarum* (JYC364 and JYC 365) showed calcium phosphate tribasic -solubilizing activity on Pikovskaya's agar plate, but the ability was lost on MS- calcium phosphate tribasic plates. Therefore, the components of the medium might influence the phosphate-solubilizing ability of yeasts. Nautiyal found that some bacteria cannot solubilize tricalcium phosphate on a Pikovskaya's agar plate but can do so on a plate containing the National Botanical Research Institute's phosphate growth medium. He analyzed the components of both Pikovskaya medium and National Botanical Research Institute's phosphate growth medium to identify which component has more influence on phosphate solubilization. He found that yeast extract, which is only present in Pikovskaya medium, is the major factor reducing the phosphate-solubilizing activity of bacteria and suggested that a medium without yeast extract may be a more efficient growth medium for screening Phosphate-solubilizing microorganisms (Nautiyal 1999).



**Figure 5** – Effects of phosphate-solubilizing yeasts on *A. thaliana* growth on –P + calcium phosphate dibasic dihydrate plates. Plants were inoculated with *Meyerozyma caribbica* (JYC358), *Candida* sp. (JYC363), and *Torulaspora* sp. (JYC369) strains exhibiting high phosphate-solubilizing ability and *Cryptococcus laurentii* (JYC370), *Pseudozyma* sp. (JYC372), and *Aureobasidium pullulans* (JYC375) strains exhibiting low phosphate-solubilizing ability.

The MS medium prepared in this study also lacked yeast extract. Therefore, the absence of yeast extract in the medium may explain why the phosphate-solubilizing ability of yeasts on MS-calcium phosphate dibasic dihydrate and MS-calcium phosphate tribasic differed from their phosphate-solubilizing ability on Pikovskaya's agar plate.

In this study, the phosphate-solubilizing ability of yeast strains also differed between liquid and solid media. *K. jainicus* (JYC361), *H. uvarum* (JYC364), and *H. uvarum* (JYC365) solubilized phosphate in a liquid medium but did not produce a visible halo zone on MS agar plates. In previous studies, no visible halo zone on agar plates was observed for many microorganisms, but they could still solubilize insoluble inorganic phosphates in liquid media (Leyval & Berthelin 1989, Nautiyal 1999). This can be explained by the low diffusion of the acids produced by microorganisms during growth in the solid medium (Cherif-Silini et al. 2013). These results indicate that the formation of a visible halo on agar plates is not a reliable criterion for isolating phosphate-solubilizing strains, because no visible halo zone on agar plates is detected for many isolates, but they still solubilize insoluble inorganic phosphates in liquid media.



**Figure 6** – Biomass and cellular P<sub>i</sub> content in *A. thaliana* on –P + calcium phosphate dibasic dihydrate plates. A Biomass. B cellular P<sub>i</sub> content in µM. C cellular P<sub>i</sub> content in µM/g fresh weight of *A. thaliana* in control and yeast-treated groups. Data are the mean of three independent experiments ± SD. Means in the same group with the same letter are not significantly different from each other in one-way ANOVA with Fisher's least significant difference (A, B) or Games–Howell post hoc (C) tests.

The pH of the medium inoculated with each calcium-phosphate-solubilizing yeast strain decreased. These results were probably attributed to the excretion of H<sup>+</sup> or the production of organic acids by the yeasts. A previous study found that *Arthrobacter* sp. CC-BC03, which dissolved the most phosphorus, acidified the pH of the medium the most (Chen et al. 2006), similar to our result. In addition, many studies have shown that organic acids can solubilize more phosphate than can inorganic acids at the same pH possibly because of chelation. Illmer and Schinner found that artificial acidification of culture media with HCl solubilized less calcium phosphate than was solubilized by either *Penicillium* sp. or *Pseudomonas* sp. (Illmer & Schinner 1992). However, the detailed mechanisms of yeast phosphate solubilization found in the present study require further investigation through high-performance liquid chromatography to analyze which organic acids are produced by the candidate strains.

### **Phosphate solubilization at different levels of soluble phosphate**

The growth rate of microorganisms is related to the level of environmental soluble phosphate (Thomas et al. 2011). Zeng et al. found that the phosphate-solubilizing ability of *Burkholderia multivorans* WS-FJ9 bacteria gradually decreased with a further increase in soluble phosphate levels, and the phosphate-solubilizing activity of the strain was completely inhibited in the presence of 20 mM exogenous soluble phosphate (Zeng et al. 2017). Through transcriptome profiling of *B. multivorans* WS-FJ9 grown in the presence of three levels of exogenous soluble phosphate, they determined that some genes related to cell growth were continuously upregulated, which would account for the improved growth of WS-FJ9 in the presence of high levels of soluble phosphate. Interestingly, they found that the genes related to glucose metabolism, including glycerate kinase, 2-oxoglutarate dehydrogenase, and sugar ABC-type transporter, were continuously downregulated, indicating that metabolic channeling of glucose toward the phosphorylative pathway is negatively regulated by soluble phosphate. In this study, the phosphate-solubilizing activity of some yeasts was also completely inhibited in the presence of high concentrations of exogenous soluble phosphate, suggesting that some yeasts can sense the concentration of exogenous soluble phosphate and can regulate their phosphate-solubilizing ability through metabolic rewiring. Thus, these strains are superior candidates for biofertilizer because they do not expend energy to produce organic acids and may instead expend energy to increase their population in soils.

### **Enhanced growth of plants by the yeasts**

Studies have shown that plants inoculated with phosphate-solubilizing bacteria demonstrate higher development than un-inoculated plants under soluble phosphate limiting conditions (Gurdeep & Reddy 2015, Otieno et al. 2015). Our study similarly showed that *C. laurentii* (JYC370) enhanced the growth and cellular P<sub>i</sub> content of plants on MS-calcium phosphate dibasic dihydrate plates. This finding suggests that plants can absorb soluble phosphate dissolved by *C. laurentii* (JYC370) and can convert it into biomass. We also found *C. laurentii* (JYC370) exhibited calcium phosphate tribasic-solubilizing activity. Most importantly, the phosphate-solubilizing activity of *C. laurentii* (JYC370) was flexible and was negatively regulated by soluble phosphate. In our previous study, *C. laurentii* (JYC370) showed other plant-growth-promoting characteristics, such as production of indole-3-acetic acid, solubilization of zinc oxide, production of NH<sub>3</sub>, catalase activity, and chitinase activity (Fu et al. 2016). *C. laurentii* is often associated with plants and is common in soil samples from fields (Sláviková & Vadkertiová 2003). *C. laurentii* can grow oligotrophically and is adequately adapted for survival in soil (Kimura et al. 1998). Thus, *C. laurentii* has been suggested to be competitive soil yeasts and a candidate biofertilizer. Cloete et al. (2009) reported that *C. laurentii* increased root growth by 51% when inoculated into seedlings of *Agathosma betulina*, a medicinal plant adapted to low-nutrient soils. *C. laurentii* has also been reported to have great commercial potential as a biological control agent against the postharvest diseases blue mold and brown rot, which are caused by *Penicillium expansum* and *Monilinia fructicola*, respectively, in sweet cherry fruit (Qin & Tian 2005) and postharvest diseases in other fruits, such as strawberries, kiwifruit, and table grapes (Lima et al. 1998). Benbow & Sugar (1999)

identified the potential of *C. laurentii* for biocontrol of postharvest diseases in pears through preharvest application.

### **Correlation between phosphate-solubilizing ability and plant growth promotion**

Phosphate-solubilizing microorganisms can promote plant growth and increase crop yield. Comparisons of the characteristics of phosphate-solubilizing microorganisms isolated from one ecological niche have shown their differences in phosphate-solubilizing ability (Taurian et al. 2010, Zhang et al. 2017), raising the question of whether higher phosphate-solubilizing ability is correlated with higher plant growth promotion. The growth of *Pisum sativum* was promoted by phosphate-solubilizing endophytic *Pseudomonas* isolates (Otieno et al. 2015) capable of solubilizing inorganic phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ). Among these isolates, the L132 strain exhibited higher phosphate-solubilizing ability than L321. However, the biomass of *P. sativum* plants inoculated with the strain L321 was higher than that of plants inoculated L132 (Otieno et al. 2015). Additionally, five of the *Pseudomonas* rhizobacteria strains naturally associated with *A. thaliana* were isolated, and their phosphate-solubilizing ability was characterized (Schwachtje et al. 2012). The *Pseudomonas* G53 strain exhibited higher phosphate-solubilizing ability than G62. The G53 and G62 strains had contrasting effects on plant growth; the growth of *Arabidopsis* plants was promoted by G62 and inhibited by G53 (Schwachtje et al. 2012). In this study, the yeast strain JYC363 displayed higher phosphate-solubilizing ability, whereas JYC370 showed moderate solubilizing ability (Fig. 3). The yeast strain JYC370 enhanced the growth of *A. thaliana* more than JYC363 did, indicating that this phosphate-solubilizing property is a characteristic promoting plant growth (Fig. 5). These results suggest that the higher phosphate-solubilizing ability of phosphate-solubilizing microorganisms may not be attributed to plant growth promotion. Integration of multiple microbial strains as a single consortium can offer different benefits to crop plants.

In summary, some yeast strains can regulate phosphate solubilization according to exogenous soluble phosphate. Among the yeast strains in this study, *C. laurentii* (JYC370) significantly enhanced the growth of *A. thaliana*. These results revealed the potential of *C. laurentii* (JYC370) for biofertilizer applications and commercial use in the field. However, field trials should be conducted, and the mechanisms underlying the calcium-phosphate-solubilizing activity of *C. laurentii* (JYC370) require additional study to verify the practical and effective use of *C. laurentii* as biofertilizer.

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