PENICILLIUM STRAIN, COMPOSITIONS AND METHODS FOR PROMOTING PLANT GROWTH

REFERENCE TO A DEPOSIT OF BIOLOGICAL MATERIAL

This application contains a reference to a deposit of biological material in accordance with the Budapest Treaty, having the deposit accession number NRRL 68252, deposited with Agriculture Research Service Culture Collection (NRRL) on 18 January 2023, which is incorporated herein by reference.

FIELD OF INVENTION

The present invention generally relates to an isolated *Penicillium* strain, named *Penicillium olsonii* TLL1, having the deposit accession number NRRL 68252 and its use in promoting plant growth. In particular, the present invention provides compositions suitable for promoting plant growth comprising said isolated fungal strain that can alleviate Al stress and increase phosphorus availability, and methods of promoting plant growth thereof.

BACKGROUND

The availability of macro and micronutrients play a pivotal role in growth and development of plants. Among the macro nutrients, phosphorus is an inevitable element involved in metabolic processes and signal transduction pathways in plants. Even though phosphorus ranks 11th among the elements available in earth lithosphere, 29% of the arable land in the world suffers from phosphorus deficiency. The low diffusivity in soil, precipitation as inorganic metal oxides and fixation into organic compounds makes phosphorus inaccessible to plants. Phosphorus limitation is a major nutritional constraint as it affects plant growth development and compromises optimal yields. Phosphorus deficiency in agriculture sector was often addressed using the application of phosphate fertilizers, but the phosphorus use efficiency in plants is only 5-25% due to the rapid fixation into insoluble forms. Moreover, the over-application led to loss of soil fertility and associated environmental hazards. Therefore, there is a high demand to develop an environment-friendly strategy to increase phosphorus uptake in plants.

Phosphate-solubilizing microorganisms (PSM) helps in phosphate assimilation from both organic and inorganic forms of phosphates in soil, rendering its uptake by the plants. Soluble phosphate acquisition by PSM is attributed to several mechanisms such as chelation and ion exchange processes, production of organic acids, secretion of microbial-derived enzymes such as acid phosphatases, phytases, and production of phytohormones (Bergkemper et al.,

2016). In this regard, phosphate-solubilizing fungi (PSF) such as from the genera *Penicillium* (Li et al., 2016), *Aspergillus* (Yin et al., 2017) and *Trichoderma* (Lei and Zhang, 2015) have successfully been applied as microbial inoculants to a variety of crops for improved phosphorus uptake, resulting in increased biomass and yield. For example, *Penicillium oxalicum* and *Aspergillus niger* have been shown to increase the bioavailability of phosphate and promote maize growth in calcareous soil (Yin et al., 2015) and *Penicillium guanacastense* has been reported as a potent biological fertilizer, promoting the growth of *Pinus massoniana* under phosphorus-limiting conditions (Qiao et al., 2019). Clearly, the employment of efficient PSF is a promising eco-friendly strategy in the current agriculture scenario for improving phosphorus uptake in plants and plant growth promotion.

Apart from phosphorus deficiency, plant productivity is also significantly affected in acidic soils. While previously estimated in early 1995 to be as much as 50% of the world's potentially arable soils, the current world's total acidic soil area could have increased markedly due to the ever-increasing air pollution and rigorous industrialization. Aluminium (Al) toxicity in acidic soils is one of the most important causes of significant reduction in crop productivity worldwide and anthropogenic soil acidification caused by extensive use of acid-forming fertilizers has expedited the release the phytotoxic aluminium Al^{3+} ions as they are easily solubilized from the soil at pH < 5.0.

High concentrations of Al^{3+} ions in the soil affect plant growth and productivity in many ways, including inhibition of root growth, reduction of nutrient and water uptake. Primary root growth stunting is the primary symptom of Al toxicity in plants, which is caused mainly by the inhibition of cell division and cell elongation in apical meristematic zone. In particular, nitric oxide (NO), iron (Fe) and callose have been shown to accumulate in the root apex and aid in the inhibition of primary root growth upon Al toxicity.

A number of Al-tolerant yeasts and fungi are capable of growing in concentrations as high as 100 mM. For instance, *Penicillium janthinellum* reduced aluminium toxicity by synthesizing citric acid (Zhang et al., 2002). In the presence of *P. janthinellum*, the Al-stressed *Solanum lycopersicum* extracted and translocated higher Al into the shoots and roots while maintaining their growth compared to the significantly growth-retarded control plants (Khan et al., 2015). Similarly, the employment of Al-tolerant fungi could thus also provide an effective strategy in mitigating aluminium stress and improve plant development.

Accordingly, there is a significant need to provide improved compositions and methods for the promotion and enhancement of plant growth that overcome, or at least ameliorate, one or more of the disadvantages described above.

SUMMARY OF INVENTION

The present disclosure relates to a newly isolated *Penicillium* strain, named *Penicillium olsonii* TLL1 ("POT1"), capable of promoting plant growth.

According to a first aspect, there is provided an isolated fungal strain of species *Penicillium olsonii*, having the deposit accession number NRRL 68252, deposited with Agriculture Research Service Culture Collection (NRRL) on 18 January 2023.

According to a second aspect, there is provided a plant growth-promoting composition comprising the isolated fungal strain of the first aspect, its mycelia, hyphae, spores and/or cell-free culture filtrate thereof.

According to a third aspect, there is provided a use of the growth-promoting composition according to the second aspect as a fertilizer.

According to a fourth aspect, there is provided a method of promoting plant growth, said method comprising applying an efficacious amount of a growth-promoting composition according to the second aspect to a medium used to grow the plant, wherein the application of the composition promotes plant growth compared to a plant grown in an untreated medium.

According to a fifth aspect, there is provided a plant seed coated with the isolated *Penicillium olsonii* strain of the first aspect.

Advantageously, the novel strain of the present disclosure is able to solubilize phosphates in soil and increase phosphorus availability for plant uptake. More advantageously, the strain of the present disclosure is also able to alleviate aluminium stress in plants and promote plant growth by inhibiting negative components of root development such as the accumulation of iron, callose and/or nitric oxide in root cell. These and other advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The accompanying drawings illustrate disclosed embodiments and serve to explain the principles of the disclosed embodiments. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

FIG. 1 depicts the identification of phosphate solubilizing fungus and growth promotion of Arabidopsis POT1 under P sufficient and P-limiting conditions. (**A**) Phylogenetic tree

generated from maximum likelihood (ML) analysis of phosphate-solubilizing fungus POT1 based on ITS sequence data. Boot strap values on 1000 replications are shown at the nodes of the tree. (**B**) Colony morphology of phosphate-solubilizing fungus POT1. POT1 was cultured on (left) PDA and (right) MEA for seven days. Scale bar represents 1cm. (**C**) Arabidopsis plants grown in full P conditions with and without POT1. Four-day-old plants were transferred to full P media pre-cultured with and without POT1 under *in vitro* conditions for 10 days. Scale bar represents 1 cm. The bar diagrams show primary root length (**D**), Shoot fresh weight (**E**), root fresh weight (**F**) of Arabidopsis Col-0 plants co-cultivated with POT1 in full P conditions for 10 days. The bar diagram shows combined data from three independent experiments (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). (**G**) Shoot P content of Arabidopsis plants co-cultivated with and without POT1 after four weeks. The concentration of total phosphorus content in Arabidopsis shoots were significantly increased after growth in the presence of POT1 in full P under *in vitro* conditions (n=3; unpaired two-tailed t-test, **P<0.01). (**H**) Arabidopsis plants grown in low P conditions with and without POT1. Four-day-old plants were transferred to full P media pre-cultured with and without POT1 under *in vitro* conditions for 10 days. Scale bar represents 1 cm. The bar diagrams show primary root length (**I**), Shoot fresh weight (**J**), Root fresh weight (**K**) of Arabidopsis WT plants co-cultivated with POT1 in low P conditions for 10 days. The bar diagram shows combined data from three independent experiments (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). (**L**) Shoot P content of Arabidopsis plants co-cultivated with and without POT1 after four weeks. The concentration of total phosphorus content in Arabidopsis shoots were significantly increased after growth in the presence of POT1 in low P under in vitro conditions (n=3 ; unpaired two-tailed t-test, **P<0.01).

FIG. 2 depicts the reduction of callose and NO accumulation in POT1 treated roots. (**A**) Callose accumulation in Arabidopsis roots under full P and low P conditions inoculated and uninoculated conditions with POT1. Four-day-old plants were transferred to full P media pre-cultured with and without POT1 under *in vitro* conditions for 10 days. Callose accumulation was analyzed in 1.5 cm root tips using aniline blue staining. The white arrowhead points to callose accumulation in the stem cell niche in phosphate limiting conditions. Three independent experiments were performed, and one representative experiment is shown. Scale bar 100µm. (**B**) Nitric oxide (NO) accumulation in Arabidopsis roots under full P and low P conditions under inoculated and uninoculated conditions with POT1. Four-day-old plants were transferred to full P media pre-cultured with and without POT1 under *in vitro* conditions for 10 days. NO accumulation was analyzed in 1.5 cm root tips using DAF-FM DA staining. Inoculation with POT1 reduced NO accumulation in roots. Scale bar 100µm.

FIG. 3 shows the insoluble phosphate solubilisation activity of POT1 under different recalcitrant P sources. (**A**) POT1 producing dissolved circular zones of Phosphorus when cultured on Pikovskaya agar and NBRIP media amended with CaP for seven days. (**B**) A bar diagram showing phosphate solubility indices of POT1 when cultured in Pikovskaya agar and NBRIP media amended with CaP for seven days. (**C**) Phosphate-solubilizing capability of POT1 in different inorganic phosphorus compounds such as tricalcium phosphate (CaP), aluminium phosphate (AlP), Iron phosphate (FeP) and hydroxyapatite (HA) after fourteen days. Scale bar represents 1cm. (**D**) Soluble P concentration produced by POT1 in different inorganic insoluble phosphates such as CaP, AlP, FeP and HA. POT1 spore suspension was inoculated in 100 ml modified Pikovskaya media amended with CaP, AlP, FeP and HA for two weeks and the soluble phosphate concentration was measured using phosphomolybdenum spectrophotometry. Every treatment included three biological replicates, each of which contained three technical replicates (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). The error bars show standard deviation.

FIG. 4 shows that POT1 promotes growth of Arabidopsis under insoluble phosphate and phosphate limiting conditions. (**A**) A representative image of *A. thaliana* WT plants grown in full P, low P, CaP, AlP, FeP and HA as sole source of P with and without POT1. Ten-day-old plants were transferred to nutrient-poor vermiculite soil pre-inoculated with and without POT1 and were harvested after two weeks. The scale bar represents 1 cm. The bar diagrams represent Shoot fresh weight (**B**), Leaf area Index (**C**), Number of leaves (**D**), Shoot phosphate content (**E**) of *A. thaliana* WT plants grown in full P, low P, CaP, AlP, FeP and HA with and without POT1 in vermiculite for two weeks. The bar diagram shows combined data from fifteen independent experiments (n=15; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). The concentration of total phosphorus content in Arabidopsis shoots were significantly increased after growth in the presence of POT1 in vermiculite soil (n=3; unpaired two-tailed t-test, **P<0.01).

FIG. 5 shows that POT1 promotes growth of leafy vegetable Bok Choy under insoluble phosphate and phosphate limiting conditions. (**A**) A representative image of Bok Choy plants grown in full P, low P, CaP, AlP, FeP and HA as sole source of P with and without POT1. Ten-day-old Bok Choy plants were transferred to nutrient-poor vermiculite soil pre-inoculated with and without POT1 and were harvested after four weeks. Scale bar represents 5 cm. The bar diagrams show Shoot fresh weight (**B**), number of leaves (**C**), leaf area index of 4th leaf (**D**) of Bok Choy plants co-cultivated with POT1 in full P, low P, CaP, AlP, FeP and HA conditions after four weeks. The bar diagram shows combined data from nine independent experiments (n=9; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). (**E**) Shoot P

content of Bok Choy plants co-cultivated with and without POT1 after four weeks. The concentration of total phosphorus content in Bok Choy shoots were significantly increased after growth in the presence of POT1 in vermiculite soil (n=3; unpaired two-tailed t-test, **P<0.01).

FIG. 6 shows the results of pH and nutrient analysis of soil used for plant growth experiments. (**A**) Comparison of pH of commercial and Lim Chu Kang (LCK) soil used for plant growth promotion experiments using POT1. (**B**) A bar diagram showing comparison of phosphorus content in commercial as well as Lim Chu Kang soil. (**C**) A representative image of phosphate fixation at different soil pH levels.

FIG. 7 shows that POT1 promotes plant growth index of leafy vegetables in Singapore local soil. (**A**) A representative image of Bok Choy plants grown in Lim Chu Kang soil with and without POT1. Ten-day-old Bok Choy plants were transferred to Lim Chu Kang soil pre-inoculated with and without POT1 and harvested after four weeks. Scale bar represents 5 cm. The bar diagrams show Shoot fresh weight (**B**), number of leaves (**C**), leaf area index of 3rd and 4th leaf (**D**), of Bok Choy plants co-cultivated with POT1 after four weeks. The bar diagram shows combined data from twelve independent experiments (n=12; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). (**E**) Shoot P content of Bok choy plants co-cultivated with and without POT1 after four weeks. The concentration of total Phosphorus content in Bok Choy shoots were significantly increased after growth in the presence of POT1 in LCK soil (n=3; unpaired two-tailed t-test, **P<0.01).

FIG. 8 shows that POT1 promotes plant growth index of rice in Singapore local soil. (**A**) A representative image of rice grown in Lim Chu Kang soil with and without POT1 after six weeks. Scale bar represents 10 cm. Fourteen-day old rice seedlings were transferred to LCK soil pre-inoculated with and without POT1 and were grown for three months. The bar diagrams show plant height (**B**), number of tillers (**C**), shoot phosphorus content after six weeks (**D**) and, seed weight per plant (**E**), and seed number per plant (**F**) of co-cultivated with and without POT1 in LCK soil. The bar diagram shows combined data from twelve independent experiments (n=12; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05).

FIG. 9 depicts the comparison of growth promotion activity of POT1 with other *Penicillium* strains under P-limiting conditions. (**A**) Arabidopsis plants grown in No P conditions with POT1 and other *Penicillium* strains. Four-day-old plants were transferred to No P media pre-cultured with and without POT1, *P. bilaiae, P. chrysogenum, P. janthinellum* and *P. simplicissimum* under *in vitro* conditions for 10 days. Scale bar represents 1 cm. The bar diagrams show primary root length (**B**), Shoot fresh weight (**C**), Root fresh weight (**D**) of

Arabidopsis WT plants co-cultivated with *P. bilaiae, P. chrysogenum, P. janthinellum* and *P. simplicissimum* in -P conditions for 10 days. The bar diagram shows combined data from three independent experiments (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05).

FIG. 10 depicts the comparison of growth promotion activity of POT1 with other *Penicillium* strains under insoluble phosphate conditions. (**A**) Arabidopsis plants grown in Full P, No P and CaP conditions with POT1 and other *Penicillium* strains such as *P. bilaiae*, *P. chrysogenum, P. janthinellum* and *P. simplicissimum*. Ten-day-old plants were transferred to nutrient-poor vermiculite soil pre-inoculated with and without POT1, *P. bilaiae*, *P. chrysogenum, P. janthinellum* and *P. simplicissimum* under Full P, No P and CaP and were harvested after four weeks. Scale bar represents 1cm. The bar diagrams show Shoot fresh weight (**B**), Anthocyanin content (**C**) of Arabidopsis WT plants co-cultivated with POT1, *P. bilaiae*, *P. chrysogenum*, *P. janthinellum* and *P. simplicissimum* in full P, No P and CaP conditions for 10 days. The bar diagram shows combined data from three independent experiments (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05).

FIG. 11 shows the POT1 mediated plant growth promotion of Arabidopsis under P sufficient and P-limiting conditions (related to FIGS. 1C and 1H). Four-day-old Arabidopsis WT, Col-0 seedlings were transferred to full P (**A**) and low P (**B**) media pre-cultured with and without POT1. Root lengths were measured after ten days of co-cultivation. POT1 treatment significantly improved primary root length. Scale bar represents 1 cm.

FIG. 12 shows that POT1 elongates primary roots of Arabidopsis without any soluble P source. (**A**) A representative image of Arabidopsis plants grown in -P conditions with and without POT1. Four-day-old Arabidopsis plants were transferred to -P media inoculated with and without POT1 under *in vitro* conditions for 10 days. The bar diagrams show primary root length (**B**), Shoot fresh weight (**C**), Root fresh weight (**D**), and shoot P content (**E**) of Arabidopsis WT plants co-cultivated with POT1 in no P conditions. The bar diagram shows combined data from ten independent experiments (n=10; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). Scale bar represents 1cm.

FIG. 13 depicts a representative image of *A. thaliana* WT leaves when grown in full P, low P, CaP, AlP, FeP and HA as sole source of P with and without POT1. FIG. 13 shows that POT1 improves the number of leaves of Arabidopsis WT Col-0 when cultured under insoluble phosphate sources, P-sufficient and P-limiting conditions (related to FIG. 4).

FIG. 14 shows that POT1 reduces anthocyanin accumulation in the leaves of Arabidopsis under P-sufficient and P-limiting conditions. Bar diagrams representing anthocyanin content in Arabidopsis thaliana plants when grown under different phosphate sources with and without POT1 inoculation in vermiculite soil. Relative anthocyanin content per gram fresh weight were represented in (1-4) leaves (**A**), (5-8) leaves (**B**), and (9-12) leaves (**C**) of Arabidopsis grown under full P, low P, No P, CaP, AlP, FeP and HA as sole source of P with and without POT1 in three independent experiments (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05).

FIG. 15 shows that POT1 promotes growth index of Arabidopsis when grown in vermiculite soil supplied with any soluble phosphate sources. (**A**) A representative image of Arabidopsis WT plants grown in full P and no P conditions with and without POT1. Ten-day-old plants were transferred to vermiculite pre-inoculated with POT1 for 14 days. Scale bar represents 1 cm. The bar diagrams represent Shoot fresh weight (**B**), number of leaves (**C**), and leaf area indices (**D**) of seventh and eighth leaf of Arabidopsis WT plants grown in full P and no P conditions with and without POT1 in vermiculite. The bar diagram shows combined data from fifteen independent experiments (n=15; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). Scale bar represents 5 cm. (**E**) The concentration of total phosphorus content in Arabidopsis shoots were significantly increased after growth in the presence of POT1 without any phosphate source (n=3; unpaired two-tailed t-test, **P<0.01).

FIG. 16 shows that POT1 promotes growth index of leafy vegetable when grown in vermiculite supplied with any soluble phosphate. (**A**) A representative image of Bok Choy grown in full P and no P conditions with and without POT1. Ten-day-old plants were transferred to vermiculite pre-inoculated with and without POT1 for seven days. Scale bar represents 5 cm. The bar diagrams represent Shoot fresh weight (**B**), number of leaves (**C**), leaf area indices (**D**), Shoot phosphorus content (**E**) of seventh and eighth leaves of Bok Choy plants grown in full P and no P conditions with and without POT1 in vermiculite. The bar diagram shows combined data from nine independent experiments (n=9; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05).

FIG. 17 depicts a representative image of leaves of Bok Choy grown in full P, low P, CaP, AlP, FeP and HA as sole source of P with and without POT1. Ten-day-old Bok Choy plants were grown in vermiculite pre-inoculated with POT1 for four weeks. Scale bar represents 1 cm. POT1 improves leaf number in Bok Choy plants in vermiculite soil test supplied with P-sufficient and P-limiting conditions (related to FIG. 5).

FIG. 18 shows soil nutrient profiling of commercial as well as Singapore local soil. Bar diagrams showing comparison of nutrient content N, P, K, Ca, Mg, Fe (**A**), and Na, Cu, Mn, Zn and B (**B**) in commercial as well as Lim Chu Kang soil from three independent experiments (n=3; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05).

FIG. 19 depicts a representative image of Bok Choy plants grown in Lim Chu Kang soil for four weeks with and without POT1.

FIG. 20 depicts a representative image of *Oryza sativa* var. Indica (Temasek™ rice) grown in Lim Chu Kang soil for six weeks with and without POT1.

FIG. 21 shows that POT1 promotes growth of leafy vegetables in Singapore local soil. (**A**) A representative image of Bok Choy plants grown in Lim Chu Kang soil with Live POT1, Heat killed POT1 and without POT1. Ten-day-old Bok Choy plants were grown in vermiculite pre-inoculated with POT1 for four weeks. The bar diagrams show increased Shoot fresh weight (**B**), number of leaves (**C**), leaf area indices of 5^{th} and 6^{th} leaves (**D**) of Bok Choy plants co-cultivated with POT1 after four weeks. The bar diagram shows combined data from nine independent experiments (n=9; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). Scale bar represents 5 cm.

FIG. 22 shows that POT1 promotes growth of rice in Singapore local soil. (**A**) A representative image of rice grown in Lim Chu Kang soil with Live POT1, Heat killed POT1 and without POT1. Two-week-old *Oryza sativa* var. Indica (Temasek™ rice) seedlings were grown in LCK soil for three months. The bar diagrams show Plant height (**B**), number of tillers after six weeks (**C**), total seed weight per plant (**D**), and seed number per plant (**E**) of rice co-cultivated with live POT1, heat killed POT1 and without POT1. The bar diagram shows combined data from nine independent experiments (n=9; unpaired two-tailed t-test, ***P<0.001; **P<0.01; *P<0.05). Scale bar represents 5 cm.

FIG. 23 shows a bar diagram comparing soluble P concentration produced by POT1, *P. bilaiae*, *P. chrysogenum*, *P. janthinellum* and *P. simplicissimum* in modified Pikovskaya broth amended with CaP. The error bars show standard deviation. Every treatment included three biological replicates, each of which contained three technical replicates (n=3: unpaired two-tailed t-test, ***P<0.001;**P<0.01; *P<0.05).

FIG. 24 shows an agar plate containing CAS-blue agar and MEA media inoculated with POT1. POT1 secreted siderophores, which turned the color of the media from blue to

purplish red, indicated by arrows. FIG. 24 shows that POT1 secretes siderophores to the culture media.

FIG. 25 shows the effect of POT1 treatment on Arabidopsis plants under Al stress on agar plates. (**a**) Images of the Arabidopsis seedlings that were untreated (control), those grown on 100 μM $AlCl_3$ (Al) and 100 μM $AlCl_3$ along with POT1 colonies (Al+POT1). Red lines indicate the maximum root length. Comparison of the primary root length (**b**), root fresh weight (**c**), shoot fresh weight (**d**), leaf areas of the two largest leaves (**e, f**) of Arabidopsis seedlings grown under control, Al and Al + POT1 conditions at pH 4 and pH 5.6. Data represent mean ± standard deviation (SD) of 9 replicates. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIG. 26 shows the effect of POT1 treatment on endogenous aluminium, nitric oxide and callose concentration in Arabidopsis roots under Al stress. Roots of Arabidopsis grown under control, control + POT1, 100 μM $AlCl_3$ (Al), and 100 μM $AlCl_3$ along with POT1 inoculation (Al + POT1) were stained with morin (**a**), DAF-FM DA (**b**), and aniline blue (**c**) and observed by confocal laser scanning microscopy to visualize aluminium (**a**), nitric oxide (**b**) and callose (**c**) contents, respectively. All images are representative of 9 seedlings per treatment. Bars, 100 μm.

FIG. 27 shows the effect of POT1 inoculation on morphological parameters of Arabidopsis plants grown on soil under Al stress. (**a**) Image of Arabidopsis plants grown under 0, 10, 20, 30, 40, 50 and 100 mM of $AlCl_3$. (**b** and **c**) Images of the Arabidopsis plants grown under 0 (control), 50 mM $AlCl_3$ (Al) and 50 mM $AlCl_3$ along with POT1 inoculation (Al + POT1). Scale bars: 100 mm. Comparison of shoot fresh weight (**d**), shoot Al content (**e**), total anthocyanin content (**f**) and total chlorophyll content (**g**) in Arabidopsis plants grown on soil under control, Al, and Al + POT1 conditions. Data represent mean ± SD of 6 replicates for d, 3 replicates for e, and 5 replicates for f and g. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIG. 28 shows the effect of POT1 inoculation on morphological parameters of Bok Choy plants grown on soil under Al stress. (**a**) Image of Bok Choy plants grown under 0, 10, 20, 30, 40, 50 and 100 mM of $AlCl_3$. (**b**) Image of Bok Choy plants grown under 0 (control), 20 mM $AlCl_3$ (Al) and 20 mM $AlCl_3$ along with POT1 inoculation (Al + POT1). Scale bar: 100 mm. Comparison of shoot fresh weight (**c**) and shoot Al content (**d**) of Bok Choy plants grown on soil under control, Al, and Al + POT1 conditions. Data represent mean ± SD of 15 replicates for c; 3 replicates for d. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIG. 29 shows the comparison of the effects of treatment of different *Penicillium* strains on Arabidopsis plants under Al stress on agar plates. (**a**) Images of the Arabidopsis seedlings that were grown on 100 μM $AlCl_3$ in the absence of a *Penicillium* strain (Control) or in the presence of the following *Penicillium* strains: POT1, *P. bilaiae*, *P. chrysogenum*, *P. janthinellum,* and *P. simplicissimum*. Comparison of the primary root length (**b**), root fresh weight (**c**), and shoot fresh weight (**d**) of Arabidopsis seedlings grown without or with *Penicillium* strains under Al stress. Data represent mean ± SD of 3 replicates. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIG. 30 shows the comparison of the effects of treatment of different *Penicillium* strains on Arabidopsis plants grown on soil under Al stress. (**a**) Image of the Arabidopsis plants that were grown on 100 μM $AlCl_3$ in the absence of a *Penicillium* strain (Control) or in the presence of the following Penicillium strains: POT1, *P. bilaiae*, *P. chrysogenum*, *P. janthinellum,* and *P. simplicissimum*. Comparison of total anthocyanin (**b**) and total chlorophyll contents (**c**) of Arabidopsis plants grown under Al stress without or with *Penicillium* strains. Data represent mean ± SD of 3 replicates. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIG. 31 shows the comparison of two experimental conditions of POT1 treatment on Arabidopsis seedlings under Al stress on agar plates. (**a**) Images of the Arabidopsis seedlings that were transferred to plates containing 0 μM $AlCl_3$ (control), 100 μM $AlCl_3$ (Al), 1-week-old POT1 inoculation growing on 100 μM $AlCl_3$ (Al + POT1 #1), and POT1 cells just inoculated on 100 μM $AlCl_3$ (Al + POT1 #2). (**b**) Comparison of the primary root length of Arabidopsis seedlings grown under control, Al, Al + POT1 #1 and Al + POT1 #2 conditions. Data represent mean ± standard deviation (SD) of 3 replicates. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

FIG. 32 shows the effect of POT1 treatment on Al contents in roots of Arabidopsis plants under Al stress. Al content in the roots were measured using Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES). Data are means ± SD from measurements of at least 50 roots. Statistical significance of the measurements was determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with Agricultural Research Service Culture Collection (NRRL), 1815 N. University Street, Peoria, Illinois, 61604, USA and given the following accession number:

Deposit	Accession Number	Date of Deposit
Penicillium olsonii TLL1	NRRL 68252	18 January 2023

Definitions

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

In general, technical, scientific and medical terminologies used herein has the same meaning as understood by those skilled in the art to which this invention belongs. Further, the following technical comments and definitions are provided. These definitions should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

As used herein, “a” or “an” may mean one or more than one unless indicated to the contrary or otherwise evident from the context.

As used herein, the term “comprising” or “including” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term “comprising” or “including” also includes “consisting of”. The variations of the word “comprising”, such as “comprise” and “comprises”, and “including”, such as “include” and “includes”, have correspondingly varied meanings.

As used herein, the terms “an efficacious amount” and “an effective amount” are used interchangeably and refer to an amount of the fungal strain and/or the composition comprising the fungal strain which is sufficient to effect the beneficial or desired results (i.e.

promoting plant growth) in a statistically significant manner. Used in this context for example, an effective amount of the composition of the present disclosure may result in a wide range of improved plant growth properties such as, but not limited to, improved root development, increased leaf area, increased plant yield, increased seed germination, increased photosynthesis, and/or an increased in biomass of the plant.

As used herein, the term" improving quality of growth medium" refers to increasing the amount and/or availability of nutrients such as phosphorus, required by, or beneficial to plants, in the medium for its growth and development. In addition, the term also refers to reducing or minimizing the amount and/or effect of an element such as aluminium in said medium, which may be detrimental to plant growth and development. Accordingly, improving the quality of the growth medium by the application of the fungal strain and/or compositions of the present disclosure thereby assists and promotes the growth of plants in said medium.

As used herein, the terms "low phosphorus content", "phosphorus-deficient", "phosphorus limiting", "P-deficient" and "P-limiting" may be used interchangeably and refer to the quantity of bioavailable phosphorus in the medium in which the plant is grown in, which is below the optimal amount required for said plant to grow and develop properly.

As used herein, the terms "medium" or "plant growth medium" are used interchangeably and in the context of the present invention, refer to any substrate which may conventionally be used to cultivate plants and enables plant growth and development.

As used herein, the terms "plant growth-promoting", "plant growth-enhancing", "promoting and/or enhancing plant growth" and any other of equivalent grammatical meaning thereof may be used interchangeably and refer to the improvement of one or more characteristics of plant growth and / or development in a treated plant as compared to a control plant (for example, a plant treated with the composition of the present invention compared to a control untreated plant). Examples of characteristics of plant growth / development would be understood by those skilled in the art to include, but not limited to, plant height, number of leaves, leaf surface area, length of leaf, leaf mass, volume of leaf, rate of photosynthesis, rate of rhizogenesis, area of root, volume of root, surface area of root, length of root, root diameter, mass of root, number of root, number of shoot, number of tiller, diameter of shoot, length of shoot, mass of shoot, shoot diameter, shoot surface area, shoot volume, number of seed per plant, weight of seed, seed germination, seedling development, amount of phosphorus in root, amount of phosphorus in shoot, aluminium tolerance, amount of aluminium in root, amount of iron in root, amount of callose in root, amount of nitric oxide in root, amount of iron in root, etc.

As used herein, the term “reducing aluminium (Al) stress/toxicity” refers to a decrease in the development of symptoms of aluminium susceptibility and/or an increased tolerance to the presence of aluminium in the growth medium. Used in this context for example, a plant grown in the medium treated with the fungal strain and/or composition of the present invention may show a reduction in symptoms of aluminium toxicity (such as a reduction the aluminium-mediated deposition of callose in its roots), when compared to a control untreated plant grown under similar environmental conditions.

As used herein, the term "source of phosphorus" refers to a phosphate compound which, at least in the condition of the medium (such as soil) under consideration, does not make the element (phosphorus) fully available for plant uptake.

A description of exemplary, non-limiting embodiments of the invention follows.

The present invention is based, in part, on the identification of a novel strain of *Penicillium*, named *Penicillium olsonii* TLL1 (“POT1”), which has been found to have a high phosphate-solubilizing capability and has the ability to mitigate aluminium toxicity in plants. In this regard, the inventors have successfully determined that inoculating the medium in which the plant is grown in with the fungal strain of the present disclosure may positively affect the quality and internal milieu of said growth medium, thereby enhancing plant growth and development.

In one aspect, disclosed herein is an isolated fungal strain of species *Penicillium olsonii*, having the deposit accession number NRRL 68252, deposited with Agriculture Research Service Culture Collection (NRRL) on 18 January 2023.

Plant growth-promoting compositions

Disclosed herein in another aspect, is a plant growth-promoting composition comprising the isolated fungal strain of the present disclosure, its mycelia, hyphae, spores and/or cell-free culture filtrate thereof. In some embodiments, the composition comprises an inoculum of the isolated fungal strain disclosed herein.

The compositions disclosed herein have been designed to promote plant growth by changing the growth medium environment in which the plant is grown in. For example, the compositions disclosed herein are able to effectively solubilize insoluble or sparingly soluble sources of phosphorus which have low availability and poor mobility in a growth medium such as soil. Advantageously, the compositions disclosed herein may increase the

bioavailability of phosphorus in the growth medium and facilitate the nutrient uptake of usable phosphorus to promote plant growth and development.

It would be appreciated that aluminium is a known plant growth inhibitor and is considered toxic to most plants. Advantageously, the compositions disclosed herein may also promote aluminium tolerance by decreasing aluminium uptake and translocation into the plants.

Further, the compositions disclosed herein may also advantageously promote root elongation by inhibiting and reducing the deposition of iron, nitric oxide, and/or callose in the plant roots, which may result from phosphate deficiency and/or aluminium toxicity. Accordingly in some embodiments, the compositions disclosed herein may, amongst other things, (a) increase the availability of phosphorus for plant uptake in a medium used to grow the plant, (b) reduce aluminium stress/toxicity in a plant grown in a medium comprising aluminium, and / or (c) inhibit negative components of plant root growth such as accumulation of iron, nitric oxide, and/ or callose in the roots.

In view of the phosphate-solubilizing capability of the compositions disclosed herein, the compositions may further comprise an additional source of phosphorus to increase the bioavailability of phosphorus for plant uptake. In some embodiments, the source of phosphorus may be a soluble phosphorus compound, a sparingly soluble phosphorus compound, or an insoluble phosphorus compound. Preferably, the source of phosphorus is an insoluble or sparingly soluble phosphorus compound. Examples of insoluble or sparingly soluble phosphorus compounds may include but not limited to, calcium phosphate, dicalcium phosphate, tricalcium phosphate, tetra-calcium phosphate, iron phosphate, aluminium phosphate, hydroxyapatite, rock phosphate, and fluorapatite. In some embodiments, the source of phosphorus is calcium phosphate, tricalcium phosphate, iron phosphate, aluminium phosphate and/or hydroxyapatite.

It would be appreciated by a skilled artisan that any medium which is conventionally used for cultivating plants may be suitable for use in the present invention. Examples of a plant growth medium may include, but not limited to, soil (such as paddy soil or upland soil), a liquid medium such as water, artificial soil, agar, vermiculite, compost, a polysaccharide, peat moss, mulch, bark, wood fibre, log, clay, mold, sand, gravel, peat, straw, growstones, rice husks, brick shards, perlite, sheep wool, pumice, rockwool, polystyrene packing peanuts, substrates suitable for hydroculture such as expanded clay aggregate, any aqueous growing medium suitable for hydroponics, or a combination thereof. In some embodiments, the medium in which a plant is grown in may be an agar medium, a liquid medium, soil or vermiculite.

Advantageously, the compositions disclosed herein are also capable of improving the quality of the medium in which the plant is grown in. Accordingly, it may be useful to apply the compositions of the present disclosure to plant growth medium that may not be ideal / particularly conducive to support plant growth. For example, the medium in which the plant is grown in may be phosphorus deficient, may comprise low phosphorus content and/ or comprise insoluble or sparingly soluble phosphate compounds. In another example, the medium in which the plant is grown in may be acidic and / or may comprise aluminium.

The compositions disclosed herein may be formulated as a solid, liquid or powder form, as is known in the art. In some embodiments, the composition may be formulated as a granular form, a concentrated form, or a ready-to-use form. In some embodiments, the composition may also be formulated as a seed coating.

In some embodiments, the compositions may comprise a carrier. For example, the carrier may be an agriculturally acceptable carrier or a soil compatible carrier. In some embodiments, the carrier may include but not limited to, water, oil, a surfactant, an additive, a thickener, a dispersant, an anti-caking agent, a stabilizer, a coating, a preservative, a colouring agent, a polymer or a combination thereof. It would be appreciated that a skilled artisan can readily determine the appropriate carrier that may be used, taking into account factors such as the growth medium in which the composition is to be applied to, the type of plant, the formulation of the composition (e.g., either in solid, liquid or powder form), and the like.

The compositions disclosed herein may also comprise other ingredients that may facilitate the growth of the fungal strain in the medium. For example, the composition may further comprise inorganic salts, nutrients such as sugars (e.g., glucose and sucrose), and/or other organic materials that can serve as a substrate for the fungus of the present disclosure.

The amount of the composition for use in the present disclosure is not limited in any particular respect. The amount of the composition to be used may depend on a variety of factors such as the type of plant species being treated, the type of growth medium and the volume of the growth medium in which the composition is to be applied to, the formulation of the composition, climate conditions and the like. For any given case, an effective amount of the composition disclosed herein should be applied and may be readily determined by a person ordinarily skilled in the art by simple routine experimentation. In this regard, an effective amount is the amount required to establish sufficient fungal growth such that the plant growth is improved in the presence of the composition. As would be appreciated, a positive, observable effect on plant growth will be obtained if an effective amount is used.

Likewise, the positive effect may not be observed if an insufficient amount is applied. By way of example only, the amount of composition to be applied may comprise from about 0.001 – 1.0 Kg fungal spores and mycelium (fresh weight) per hectare, or about 1.0×10^2 colony forming units (CFU)/mL to about 5.0×10^{12} CFU/mL per acre. In some embodiments, the composition may comprise at least 0.1 g of mycelia. In some embodiments, the composition may comprise at least 0.5 g of mycelia. In some embodiments, the composition to be applied may comprise at least 0.5 g of mycelia for each pot of plant.

In another aspect, there is provided a plant seed coated with the isolated fungal strain of the present disclosure or the composition as disclosed herein. In some embodiments, the plant seed is further coated with a soil compatible carrier. By way of example only, the amount of composition to be applied may comprise about 1.0×10^2 – 1.0×10^7 CFU per seed. Methods of preparing a formulation for seed coating is known in the art.

In another aspect, there is provided the composition of the present disclosure for use as a fertilizer. In another aspect, there is also provided a use of the composition as disclosed herein as a fertilizer. In some embodiments, the fertilizer may be formulated as a liquid, powder, or a granular formulation. In some embodiments, the fertilizer may be formulated as a soluble or a slow release formulation. In some embodiments, the fertilizer may be formulated as a dry biomass (such as dust or granules) or a suspension (such as water-based or emulsion). It would be appreciated that methods of formulating fertilizers are known in the art.

Methods of promoting plant growth

Also disclosed herein are methods of promoting plant growth. In one aspect, there is provided a method of promoting plant growth, said method comprising applying an efficacious amount of a growth-promoting composition of the present disclosure to a medium used to grow the plant, wherein the application of the composition promotes plant growth compared to a plant grown in an untreated medium.

Advantageously, the methods disclosed herein may be promote plant growth by positively changing the growth medium environment and improving the medium quality in which the plant is grown in. In some embodiments, the application of the composition in the method disclosed herein may (a) increase the availability of phosphorus for plant uptake in the medium used to grow the plant, (b) reduce aluminium (Al) stress/toxicity in the plant grown in a medium comprising aluminium, and / or (c) inhibit negative components of root growth such as accumulation of iron, nitric oxide, and/ or callose in the roots.

In some embodiments, the method disclosed herein may be applied to a medium comprising at least one of the following: low phosphorus content, insoluble phosphate, or aluminium. In some embodiments, the medium may be an agar medium, a liquid medium, soil or vermiculite. In some embodiments, the soil may be paddy soil or upland soil.

For purposes of the compositions and methods of the present disclosure, the target plant(s) are not particularly limited. For example, the plant may be a dicotyledon or a monocotyledon. Examples of plants may include but not limited to, Gramineae plants (for example, barley, maize, wheat, Sorghum bicolor, sugar cane, and rice species such as *Oryza sativa* or *Oryza sativa* var. Indica (Temasek™ rice)), Leguminosae plants (for example, soybean, horse bean, and garden pea); Brassicaceae plants (for example, broccoli, cauliflower, cabbage, Chinese cabbage, Japanese radish, turnip, Arabidopsis species such as *Arabidopsis arenosa, Arabidopsis lyrata*, *Arabidopsis thaliana,* wild-type *Arabidopsis thaliana* (Col-0), Brassica species such as *Brassica chinensis komatsuna, Brassica campestris, Brassica rapa* var. *nipposinica, Brassica juncea*, and *Brassica rapa* var. *chinensis* (Bok Choy)), Solanaceae plants (for example, tobacco, eggplant, tomato, pimento, and capsicum), Chenopodiaceae plants (for example, beet and spinach), and Compositae plants (for example, lettuce and butterhead lettuce). In some embodiments, the plant may be a Brassica species, Arabidopsis species or Oryza species. In some embodiments, the plant may be Bok Choy, wild-type *Arabidopsis thaliana* (Col-0) or *Oryza sativa* var. Indica (Temasek™ rice).

Unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in various embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. “About” in reference to a numerical value generally refers to a range of values that fall within ±10%, in some embodiments ±5%, in some embodiments ±1%, in some embodiments ±0.5% of the value unless otherwise stated or otherwise evident from the context. In any embodiment in which a numerical value is prefaced by “about”, an embodiment in which the exact value is recited is provided. Where an embodiment in which a numerical value is not prefaced by “about” is provided, an embodiment in which the value is prefaced by “about” is also provided. Where a range is preceded by “about”, embodiments are provided in which “about” applies to the lower limit and to the upper limit of the range or to either the lower or the upper limit, unless the context clearly dictates otherwise. Where a phrase such as “at least”, “up to”, “no more than”, or similar phrases, precedes a series of numbers, it is to be understood that the phrase applies to each number in the list in various embodiments (it being understood

that, depending on the context, 100% of a value, e.g., a value expressed as a percentage, may be an upper limit), unless the context clearly dictates otherwise. For example, “at least 1, 2, or 3” should be understood to mean “at least 1, at least 2, or at least 3” in various embodiments. It will also be understood that any and all reasonable lower limits and upper limits are expressly contemplated.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Example 1: Plant growth promotion and improved phosphate use efficiency by phosphate-solubilizing fungus (PSF), *Penicillium olsonii TLL1*

Methods and Materials

Identification and Genotyping of PSF Strain

A PSF was isolated by inoculating environmental samples on Pikovskaya (“PVK”) agar containing 0.5% tricalcium phosphate (CaP), pH 6.8-7.2, and incubated at 25ºC for 7 days. A clear zone of dissolved phosphorous around fungal colonies indicated recalcitrant phosphate solubilization. The ratio of colony diameter and a circular band of dissolved phosphorus was calculated to find the phosphate-solubilizing rate.

Total genomic DNA was extracted from the PSF strain using the CTAB method. One hundred nanograms of genomic DNA of the PSF strain were used to amplify seven genetic loci such as Internal transcribed spacer (*ITS*), β-tubulin (*benA*), translation elongation factor-1-α (*TEF1α*), calmodulin (CaM), 28S large ribosomal subunit (*LSU*), 18S small ribosomal subunit (*SSU*) and mini-chromosome maintenance protein (*MCM7*) using high-fidelity Phusion™ DNA polymerase. The genotyping was analyzed with primers shown in Table 1. The PCR reaction profile consisted of initial denaturation at 98ºC for 3 minutes followed by 35 cycles of 98ºC for 10 seconds, 55-68ºC for 30 seconds, and 72ºC for 1 minute and final extension of 72ºC for 5 minutes. The sequences of PCR amplicons were determined using Sanger’s Sequencing, and the contigs were assembled using Bioedit tool Software and were analysed by BLAST searches at NCBI.

Table 1: Oligonucleotide primers for genotyping.

Locus	Primer ID	Oligonucleotides (5' - 3')	Annealing temp. (°C)	Reference
Internal transcribed spacer region of rDNA (ITS)	ITS1	TCCGTAGGTGA CCTGCGG (SEQ ID NO. 1)	55	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC (SEQ ID NO. 2)		
Translation Elongation Factor 1-alpha (TEF1α)	EF1–1018F	GAYTTCATCAAGAACATGAT (SEQ ID NO. 3)	57	Stielowet al. 2015
	EF1–1620R	GACGTTGAADCCRACRTTGTC (SEQ ID NO. 4)		
Small subunit rDNA (SSU)	NS1	GTAGTCATATGCTTGTCTC (SEQ ID NO. 5)	55	White et al. 1990
	NS4	CTTCCGTCAATTCCTTTAAG (SEQ ID NO. 6)		
Beta-tubulin (BenA)	Bt2a	GGTAACCAAATCGGTGCTGCTTTC (SEQ ID NO. 7)	68	Glass and Donaldson 1995
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC (SEQ ID NO. 8)		
Calmodulin (CaM)	CF1	GCCGACTCTTTGACYGARGAR (SEQ ID NO. 9)	60	Peterson et al. 2005
	CF4	TTTYTGCATCATRAGYTGGAC (SEQ ID NO. 10)		
Mini chromosome maintenance protein (MCM7)	MCM7-709for	ACNMGNGTNTCVGAYGTHAARCC (SEQ ID NO. 11)	55	Schmitt et al. 2009; Raja et al. 2011
	MCM7-1348rev	GATTTDGCIACICCIGGRTCWCCCAT (SEQ ID NO. 12)		
Large ribosomal subunit (LSU)	LR6R-F	CGCCAGTTCTGCTTACC (SEQ ID NO. 13)	58	Raja et al. 2017
	LROR-F	ACCCGCTGAACTTAAGC (SEQ ID NO. 14)		

A phylogenetic tree was constructed using MEGA v 7.0 Software separately for each genetic locus using maximum-likelihood (ML) algorithm using Tamura Nei model with 1000 bootstrap replications. The reference sequences of the genes from the genus *Penicillium* were also used for alignment. The final tree was presented to show a bootstrap cut off level above 65% (Visagie et al., 2014). The identified PSF strain was designated *Penicillium olsonii TLL1 (POT1).*

Plant materials and growth conditions

For seedling growth, Arabidopsis WT (Col-0), Bok Choy (*Brassica rapa chinensis*) and Rice (Temasek™ rice) seeds were germinated on media containing Murashige and Skoog salts, 0.25 mM MES, 10 g/l sucrose and 0.8% agar.

In vitro growth test of Arabidopsis ecotype, Col-0 were assessed in modified Hoagland's solution under full, low P and -P conditions as 0.50 mM, 0.01 mM and 0 mM KH_2PO_4, respectively, at pH 5.6. Four-day-old Arabidopsis seedlings were transferred to the test media pre-cultured with POT1 inoculum. The root length of the seedlings was estimated after 10 days using Image J software (https:imagej.nih.gov/ij/download).

For soil test, vermiculite was used as nutrient-free soil for studying plant growth under insoluble phosphate conditions. It was washed three times by deionized water in order to remove any soluble P and air-dried at room temperature. The plants were grown at 22ºC (Arabidopsis) and 25ºC (Bok Choy) under 75% relative humidity.

The plants were irrigated weekly twice with Hoagland's that contained full P (0.5 mM KH_2PO_4), low P (0.01 mM KH_2PO_4, -P (0 mM KH_2PO_4), 0.5 mM tricalcium phosphate (0.5 mM CaP), 0.5 mM aluminium phosphate (0.5 mM AlP), 0.5 mM iron phosphate (0.5 mM FeP), and 0.5 mM Hydroxy apatite (0.5 mM HA).

To test plant growth by P solubilizing activity, POT1 mycelia (0.5 g) were drenched in vermiculite for 7 days prior to the transfer of the seedlings. 7-day-old seedlings of Arabidopsis and Bok Choy were transferred to the vermiculite and subjected to different phosphate sources.

Tests were conducted in vermiculite soil medium to compare CaP solubilization ability of POT1 with other *Penicillium* strains such as *P. bilaiae* (ATCC20851), *P.chrysogenum* (NBRC 4626), *P. janthinellum* (NBRC31133) and *P. simplicissimum* (NBRC106922). The fungal mycelia (0.5 g) were drenched in vermiculite for 7 days prior to the transfer of the Arabidopsis seedlings and the plants were irrigated weekly twice with Hoagland's solution that contained tricalcium phosphate as sole source of phosphorus.

Analysis of insoluble phosphate-solubilizing activity of POT1

POT1 was cultured on Prune agar medium (40 ml/L prune juice, 1 g/L yeast extract, 2.5 g/L lactose, 2.5 g/L sucrose, 20 g/L agar, pH 6.5) two days in dark and three days in light for sporulation. The PSF conidial spore suspension (10^8 spores/ml) was inoculated in 100 ml

modified Pikovskaya broth containing 1 g/L sparingly soluble inorganic phosphates such as tricalcium phosphate (pH 7.0), iron phosphate (pH 2.5), aluminium phosphate (pH 4.0) and hydroxyapatite (pH 7.0) at 25ºC, 120 rpm for 14 days. Uninoculated media with different P sources were also kept as control. The cultures were centrifuged at 5000 rpm for 15 minutes, and the supernatant was filtered with 0.22 µm syringe filter. The soluble phosphorus content was determined using phosphomolybdenum spectrophotometry (Ames, 1966). The insoluble phosphate solubilizing activity of POT1 was also compared to the commercial biofertilizer strain, *P. bilaiae* (ATCC20851) to solubilise tricalcium phosphate, iron phosphate, aluminium phosphate and hydroxyapatite. The phosphate solubilizing activity of POT1 was also compared with other Penicillium strains such as *P.chrysogenum* (NBRC 4626), *P. janthinellum* (NBRC31133) and *P. simplicissimum* (NBRC106922) in CaP amended modified Pikovskaya broth. The phosphate solubilising rate was calculated using the following formula: ([soluble phosphorus content of inoculation group-soluble phosphorus content of control group]/inorganic phosphorus content of each experimental group*100) described by Qiao et al., 2019.

Effect of POT1 under local soil conditions

The growth test of Bok Choy and Temasek rice was conducted in soil collected from Lim Chu Kang, Singapore (N 1° 26' 7.3284", E 103° 42' 48.456") under in greenhouse conditions. POT1 mycelia (0.5 g per pot) including heat killed POT1 were drenched in Lim Chu Kang soil seven days before transferring the Bok Choy and Rice seedlings.

Analysis of total phosphorus content

Roots and shoots of Arabidopsis plants were collected after growing in different phosphorus sources. The total phosphorus content was measured by digesting 45 mg of the leaf or root tissue in 1 mL 3:1 HNO_3/HCl in a microwave oven at 240^{o}C for 15 mins and, after digestion, it was diluted by ten times with water. The phosphorus content was determined using inductively coupled plasma–atomic emission spectroscopy (ICP-OES).

Callose staining

The Arabidopsis plant roots grown under full P and low P conditions were incubated for 2 hours in 150 mM K_2HPO_4 and 0.01% aniline blue under dark. Callose depositions were quantified in a fluorescent microscope using DAPI filter (excitation 370 nm and emission 509 nm, Olympus FV 3000 (20X objective)).

NO staining

The root was excised from Arabidopsis plants grown under full P and low P media for 10 days. The roots were treated with 10 µM DAF-FM DA (3-amino, 4-aminomethyl-2′,7′-Difluorescein diacetate) for 15 minutes and samples were washed with HEPES buffer (pH 4.0) for 10 minutes. The fluorescence was observed with a fluorescence microscope (excitation 495 nm and emission 515, Olympus FV 3000 (20X objective).

Anthocyanin measurement

The anthocyanin content was determined in leaves of Arabidopsis plants following the protocol by Laby et al., 2000. The leaves of 14-day old Arabidopsis plants co-cultivated with and without POT1 were divided into three groups (leaves 1-4, 5-8, 9-12). The leaves were extracted with 99:1 methanol:HCl (v/v) overnight at 4^{o}C. The anthocyanin contents were determined by acquiring OD at 530 and 657 nm and was calculated using the formula: OD_{530}-(0.25*OD_{657}) * extraction volume (ml)* 1/ weight of the tissue.

Analysis of total macro/micro-nutrient content in Lim Chu Kang soil

A soil nutrient analysis was conducted to determine the available nutrients in the Lim Chu Kang soil and commercial soil used for the cultivation of Bok Choy and rice. 1 kg commercial soil and local soil collected from Lim Chu Kang, Singapore were analyzed for nitrogen, phosphorus, potassium, calcium, magnesium, sodium, copper, manganese, zinc and boron using Mehlich 3 extraction (Mehlich, 1984) followed by ICP-OES analysis at National Parks Board, Singapore. 10 g of soil was mixed with 20 ml of sterile distilled water and stirred with magnetic stirrer for 1 hour. The pH of the soil mixture was recorded using a pH meter.

Siderophore assay

A modified CAS-agar universal assay was used to analyse the secretion of siderophores by POT1 (Milagres et al., 1999) by adding CAS-blue agar on one half of the Petri plate and malt extract agar on the other half. A POT1 conidial spore suspension (10^8 spores/ml) was inoculated in the centre of the media and incubated at 25^{o}C for 14 days to observe siderophore production. The positive reaction results in a colour change of reagent (from blue to orange, purple or purplish red).

Results

Identification and genotyping of POT1

The phylogenetic relationships between POT1 and the representative fungal strains in maximum-likelihood trees were constructed using seven barcode markers such as ITS, benA, TEF1α, SSU, LSU, CaM and MCM7. All the sequences of the DNA barcode markers

of POT1 strain clustered with *P. olsonii* sequences and the strain was ascertained as *Penicillium olsonii* (FIG. 1A). Figure 1B showed the morphology of POT1 in MEA and PDA.

Plant root growth promotion by POT1 under P sufficient and deficient conditions

Arabidopsis growth promotion mediated by *P. olsonii* TLL1 (POT1) was tested under P sufficient and P deficient media conditions (FIG. 1C and H). FIG. 11 showed whole plates of Arabidopsis growth test of FIG. 1C and FIG. 1H. Under P sufficient condition, Arabidopsis plants showed 1.2-fold longer primary roots with POT1 inoculation (FIGS. 1C and D) but their shoot, root fresh weight and shoot P content were not significantly increased, compared with uninoculated plants (FIG. 1E, F and G). Under P deficient condition, POT1-inoculated plants showed 2-fold longer primary root length (FIG. 1) and their shoot and root fresh weight were significantly increased by 1.5- and 2-fold (FIGS. 1J and K), compared with uninoculated plants. Arabidopsis growth by POT1 inoculation under no P condition was also tested (FIG. 12A). Despite no phosphate source in the medium, the co-cultivated plants with POT1 showed 2-fold longer primary root and 2-fold increased root fresh weight (FIGS. 12B and D), whereas there was no difference in shoot fresh weight between inoculated and non-inoculated plants (FIG. 12C). The shoot phosphate content in plants inoculated with POT1 under -P conditions increased 1.5-fold compared to the uninoculated plants (FIG. 12E).

Inhibition of callose deposition and NO accumulation by POT1

To confirm that POT1 inhibits negative components of root growth, we investigated callose deposition and nitric oxide (NO) accumulation under phosphate sufficient or deficient condition and the effect of POT1 inoculation (FIG. 2). Under phosphate deficiency, massive callose formation occurred predominantly in the root apical meristem, whereas no extra callose deposition was observed by POT1 inoculation, similar to phosphate sufficient condition with/without POT1 (FIG. 2A). In addition, as NO is a downstream signalling molecule in root development under phosphorus deficiency, we tested the capability of POT1 to inhibit endogenous NO accumulation in roots using the fluorescent probe DAF-FM DA. The green fluorescence was clearly observed under phosphate-deficient condition, indicating phosphate-dependent NO production. In contrast, after inoculating POT1, the NO level was decreased in root, suggesting the involvement of POT1 in root development under phosphate deficiency (FIG. 2B).

Quantitative assay for phosphate-solubilizing activity of POT1

When POT1 was grown in PVK and NBRIP media amended with $Ca_3(PO_4)_2$ for 7 days at 25 °C, the strain generated visible halo zones. We measured the diameter of the colony (d) and a circular band of dissolved phosphorus (D) and calculated the phosphate solubility index from the d/D ratio of PSF isolated from the environmental sample. When cultured on Pikovskaya and NBRIP media, the PSF strain had a d/D ratio of 1.161 and 1.212, respectively, (FIGS. 3A, B).

The POT1 solubilized tricalcium phosphate [$Ca_3(PO_4)_2$], hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4.4H_2O$) were accompanied by a decrease in pH of the supernatants in modified PVK media (FIG. 3). POT1 had the highest phosphate-solubilizing activity in media containing $Ca_3(PO_4)_2$, followed by hydroxyapatite, aluminium phosphate, and iron phosphate (FIG. 3C). The soluble phosphorous concentration reached 2.95 mg/ml in CaP and 1.574 mg/ml in hydroxyapatite. POT1 could also solubilise $AlPO_4$ and $FePO_4$ to release soluble P at 0.924 mg/ml and 0.436 mg/ml, respectively, after 14 days post-inoculation (FIG. 3D). The phosphate-solubilizing rate was 57% for CaP, 31% for hydroxyapatite, 20% for AlP and 3% for FeP.

Siderophore production by POT1

Siderophores are iron chelating ligands produced by plant growth-promoting rhizobacteria (PGPR) and fungi to help the solubilization and transport of iron through the formation of soluble Fe^{3+}. To investigate the possibility of inhibiting iron accumulation in root apical meristem by siderophore production by POT1, POT1 was grown on modified Cas-agar-MEA media and observed for colour change. FIG. 24 shows that secreted siderophores were confirmed by the formation of a purplish red colour in the medium.

Growth test by POT1 under insoluble phosphate condition in Arabidopsis and leafy vegetable

To investigate the phosphate-solubilizing capability of POT1 on Arabidopsis growth under P sufficient and deficient conditions including insoluble P sources, Arabidopsis plants were co-cultivated in vermiculite which is a nutrient free soil (FIG. 4). Arabidopsis plants grown in low P and insoluble P sources such as tricalcium phosphate (CaP), aluminium phosphate (AlP), iron phosphate (FeP) and Hydroxyapatite (HA) conditions are stunted without POT1 inoculation (FIG. 4A). Consistent with Arabidopsis growth condition, shoot fresh weight, leaf index, number of leaves and shoot phosphate content were higher than the plants not inoculated by POT1 (FIGS. 4B, C, D, E and FIG. 13). The anthocyanin accumulation, which is a typical phenotype of plants grown under phosphate deficiency, was higher in non-

inoculated POT1, whereas that of POT1-inoculated plants were lower that of non-inoculated plants (FIGS. 14A-C).

Under -P condition (P deficient) using vermiculite soil, shoot fresh weight, number of leaves and leaf area index of Arabidopsis plants were increased significantly, compared with non-inoculated plants (FIGS. 15B-D). However, plants grown under -P condition still showed growth defects when compared with phosphate-sufficient plants. POT1 colonization was also examined as to whether it can also promote growth of leafy vegetables, such as Bok Choy (*B. rapa chinensis*), by growing in vermiculite under different phosphate sources (FIG. 5A). After four weeks, Bok Choy plants grown in low P, tricalcium phosphate (CaP), aluminium phosphate (AlP), iron phosphate (FeP) and hydroxyapatite (HA) conditions without POT1 appeared to have stunted growth, whereas the biomass of POT1-inoculated plants were increased as indicated by fresh shoot weight, number of leaves and leaf area index (FIGS. 5B-D and FIG. 16). In addition, total phosphate content was higher in all the inoculated plants in insoluble phosphate condition as well as low phosphate condition, compared with non-inoculated Bok Choy (FIG. 5E).

When -P condition was simulated in vermiculite soil, shoot fresh weight, number of leaves and leaf area index were increased in plants treated with POT1 compared to non-inoculated plants (FIG. 16).

Growth test by PSF under local soil conditions

It was investigated whether POT1 could function in local soil at Lim Chu Kang in Singapore to improve Bok Choy and Rice growth. Firstly, the pH and macro/micro-nutrient content of local soil were analyzed. As a result, the pH of Lim Chu Kang soil was 6.5, whereas that of a commercial soil was 5.7 which is in the range of highest phosphate availability (FIGS. 6A and C). FIG. 6B shows the phosphate content in commercial and Lim Chu Kang soil. The phosphate content of commercial soil was 4-fold higher than that of Lim Chua Kang soil. However, a 50 mg/kg phosphate content in Lim Chu Kang soil meant there was still sufficient condition for plant growth in soil (Philip et al., 2021). In addition, other macro/micro-nutrients such as nitrogen, potassium, calcium, magnesium, iron, sodium, copper, manganese zinc and boron were also analyzed in commercial and Lim Chu Kang soil (FIG.18A). Notably, the content of copper, manganese and zinc in Lim Chu Kang soil was higher than that of the commercial soil (FIG. 18B).

For four weeks, Bok Choy plants were grown in Lim Chu Kang soil with/without POT1 (FIG. 19). FIG. 7 shows the growth condition of Bok Choy plants was improved by POT1 inoculation because the shoot fresh weight, number of leaves and leaf index were higher

than those without POT1 (FIGS. 7A-D). Consistent with Bok Choy growth index, total P content in the leaves was higher than non-inoculation POT1 plants (FIG. 7E). Moreover, it was confirmed that POT1 had the phosphate-solubilizing activity in rice as a monocot plant in paddy soil (FIG. 8, FIG. 20). The co-cultivated rice plants with POT1 were improved on plant height (FIGS. 8A, B), number of tillers (FIG. 8C), and shoot P content (FIG. 8D) when compared with plants grown without POT1 inoculation. Consistent with the increased tiller number, the seed number (FIG. 8E) and seed dry weight (FIG. 8F) were increased by POT1 inoculation.

In addition, the effect on plant growth by heat-killed POT1 was tested as a negative control in Bok Choy and rice grown under Lim Chu Kang soil condition (FIG. 21, FIG. 22). It was found that shoot fresh weight, number of leaves and leaf index were decreased in the inoculation of Bok Choy with heat-killed POT1 compared to a live POT1 inoculation (FIGS. 21A-D). Similarly, in the rice growth test, heat-killed POT1 was non-functional indicated by a shorter plant height, less tiller numbers, and reduced seed weight and numbers compared with live POT1 (FIGS. 22A-E). These results suggest that the phosphate-solubilizing capability of POT1 helps plants to absorb phosphate sources from soils containing insoluble phosphate.

Comparison of five Penicillium strains on phosphate solubilizing activity and Arabidopsis growth

The tricalcium phosphate solubilization capability of POT1 was compared to other *Penicillium* strains such as *P. chrysogenum*, *P. janthinellum* and *P. simplicissimum* (FIG. 23). The soluble phosphate concentration in the culture supernatants of *P.chrysogenum*, *P. janthinellum* and *P. simplicissimum* in CaP were 1.69 mg/ml, 1.84 mg/ml and 1.97 mg/ml, respectively. The highest CaP solubilization was obtained for *P. bilaiae* (3.246 mg/ml) followed by POT1 (2.96 mg/ml).

The growth-promotion activity of POT1 was also compared with that of other *Penicillium* strains such as *P. bilaiae*, *P. chrysogenum*, *P. janthinellum* and *P. simplicissimum* under P-deficient conditions (FIG. 9). All strains showed longer primary root length than that of control which was without any fungal inoculation. However, when it was compared among 5 *Penicillium* strains, the primary root length inoculated by POT1 was longest (FIG. 9B). Similarly, shoot fresh weight in POT1 inoculated plants (2- fold) was the highest level (FIG. 9C). Unexpectedly, there was no difference in shoot fresh weight between plants inoculated with *P. simplicissimum* and control plants. Moreover, in the case of plants co-cultivated with *P. janthinellum*, a 2.56- fold decrease in shoot fresh weight was observed (FIG. 9C). The

root fresh weight inoculated by all fungi strains was higher that of non-inoculated plants and the highest root fresh weight was shown in *P. janthinellum* strain (FIG. 9D).

To investigate the plant growth by phosphate solubilizing activity of each fungus, Arabidopsis growth in vermiculite was tested, using the insoluble phosphate source calcium phosphate (CaP) (FIG. 10A). FIG. 10B shows that shoot fresh weight inoculated by POT1 was higher that other strains under insoluble phosphate condition. Furthermore, anthocyanin accumulation, which is a typical phosphate deficiency response in plants, was also measured. It showed similarly less anthocyanin accumulation in plants inoculated with POT1 and *P. simplicissimum* but POT1 produced a higher shoot fresh weight than *P. simplicissimum* (FIG. 10C).

Summary

The present invention relates to a newly isolated fungal strain, *P. olsonii TLL1* (POT1) which has high phosphate-solubilizing ability and promotes root elongation in inoculated plants (FIG. 1). Even if the phosphate source is completely omitted from the growth media, the primary root length is longer with POT1 inoculation (FIG. 12B).

Callose (β-(1,3)-D-glucan) deposition in root apical meristem due to phosphate deficiency obstructs cell to cell communication which are necessary for root elongation. Moreover, the accumulation of nitric oxide (NO) acts as an inhibition factor of primary root elongation. It was confirmed that POT1 inhibited callose and NO accumulation in root cells (FIG. 2). Despite being grown under low phosphate condition, Arabidopsis roots elongate normally due to inhibition of callose and NO accumulation with root colonization of POT1 (FIG. 1H, FIG. 12A).

In addition, accumulation of iron in primary root tips is responsible for inhibition of primary root elongation of Arabidopsis under phosphate-limiting condition. Siderophores are iron-chelating agents produced by bacteria and fungi to scavenge iron from the environment. Evidence herein shows that POT1 is able to secrete siderophores (FIG. 24), suggesting that the secreted siderophores from POT1 scavenge iron and reduce iron accumulation in the plant roots grown under phosphate deficiency.

Root-associated endophytic fungi are the major contributors of phosphate solubilization in the rhizosphere of Arabidopsis plants. Phosphate solubilising fungi (PSF) thrive in the rhizosphere and establish symbiotic associations with root systems of plants. The active species of PSF involved in phosphate cycling are endophytic fungi such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Piriformospora*, and *Curvularia*. Phosphate-solubilizing fungi could be widely employed as a potential solution to improve phosphate uptake and use efficiency.

Insolubility by the phosphate fixation in soil depends on edaphic factors such as pH, organic matter content, presence of exchangeable cations such as Fe, Al, Ca, Mg and clay content in the soil. Iron and aluminium participate in phosphate precipitation reactions at acidic pH (below 5.5), whereas calcium ions become operative in alkaline pH (pH 6-8) and magnesium ions dominate at pH 7.5. The lowest fixation of P occurs between pH 6 to 7. The phosphate added as agro-fertilizer to the soil reacts with cation ions such as Al, Ca and Fe, and becomes fixed in the soil, which is inaccessible for uptake by the plant roots (Penn and Camberato, 2019). The phosphate-solubilizing activity of POT1 was confirmed in insoluble phosphate complexes such as tricalcium phosphate, aluminium phosphate, iron phosphate and hydroxyapatite (FIG. 3B). POT1 in culture media containing four different phosphate sources has shown high solubilizing capability for tricalcium phosphate.

Here, it was confirmed that plant growth condition in Arabidopsis, Bok Choy and Rice is improved due to soluble phosphate source increased by POT1 in vermiculite and Singapore local soil (FIG. 4A, FIG. 7, FIG. 8, FIG. 18, FIG. 19). In addition, comparing the effect of five *Penicillium* strains in plants, it was shown that POT1 was remarkably effective on root growth promotion and phosphate solubilizing activity under phosphate deficient medium and insoluble phosphate-contained soil (FIG. 9, FIG. 10). Thus, it is suggested that POT1 can be used as potent phosphate-solubilizing microorganism for both of monocot and dicot crop growth in fields containing insoluble phosphate as well as it can be applied not only upland soil but paddy soil, showing better activity than other *Penicillium* strains.

The application of fungal species as biofertilizer to arable land to improve soil quality is emerging as an eco-friendly approach in modern agriculture. There are several practices followed for the application of PSF inoculants such as seed coating, drenching into the soil, applying on the plant surfaces or root dipping etc. (Kumar et al., 2018).

The biofertilizers have been commercialised as dry biomass (dusts or granules) and as suspension (water based and emulsions) (Odoh et al., 2020). Among the *Penicillium* genus, *P. bilaji, P. italicum, P. albidum, P. frequentans, P. simplicissimum, P. rubrum, P. expansum, P. oxalicum, P. citrinum* have been commercially used as biofertilizers for the mobilization of P, Mn, Zn, Fe, Co, Cu, and Mo and biotic and abiotic stress tolerance (Odoh et al., 2020). There are a few commercialised biofertilizers formulated with *Penicillium spp* (*Penicillium bilaiae*) which increased P uptake and improved yield of canola (*Brassica napus*) (Chang and Yang, 2009). The other *Penicillium* species such as *Penicillium radicum* and *P. italicum* have also used to increase yield in the case of wheat and soyabean (Sneha et al., 2018). *P. bilaiae* was widely used as biofertilizer and several studies conducted in growth chambers and in the field increased the biomass, phosphate uptake and grain yield in wheat (*Triticum*

aestivum L.) (Kucey, 1988), canola (*Brassica napus L.*)(Kucey and Leggett, 1989) , bean, pea (Vessey and Heisinger, 2001) and lentil (Wakelin et al., 2007).

The optimal usage of chemical fertilizers is necessary to maintain ecosystem function and develop sustainable agriculture. Thus, application, research and methodology developments of biofertilizers need to be emphasized on improving effective, stable, nontoxic microbiome strains for promoting plant growth. The present invention provides the combined action of solubilization of insoluble phosphate that plant roots are unable to uptake and removal of inhibition factors of primary root elongation under phosphorus-limiting conditions.

Example 2: *Penicillium olsonii TLL1* strain (POT1), alleviates aluminium stress in plants.

Materials and Methods

Fungus isolation and identification

Penicillium olsonii TLL1 (POT1) was isolated and identified according to Example 1.

Plant materials and germination

Seeds of wild-type *Arabidopsis thaliana* (Col-0) and Bok Choy (*Brassica rapa chinensis*) were grown on 1.2% (w/v) and 0.8% (w/v) agar media containing Murashige and Skoog (MS) basal salts, 1% (w/v) sucrose and 2.5 mM MES, pH 5.6, respectively. After imbibition at 4ºC in the dark for 2 days, seedlings were germinated in a tissue culture chamber at 22ºC with 60% relative humidity under 16 h light/8 h dark condition (100 μmol $m^{-2}s^{-1}$ light intensity).

Arabidopsis growth test by POT1 inoculation under high Al condition

5-day-old Arabidopsis seedlings were transferred onto pH 4 and pH 5.6 modified Hoagland's nutrient medium containing 1.5% (w/v) phyto agar with the following treatments: (1) Control, (2) Al (100 μM $AlCl_3$), and (3) Al + POT1 (POT1 was inoculated on a Hoagland's medium plate containing 100 μM $AlCl_3$ and grown for 1 week). The modified Hoagland's nutrient contained 2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1 mM $MgSO_4$, 0.75 μM NaFeEDTA, 0.25 mM KH_2PO_4, 50 μM H_3BO_3, 0.075 μM $NH_4Mo_7O_{24}$, 2 μM $ZnSO_4$I, 10 μM $MnCl_2$, 1.5 μM $CuSO_4$, 0.2 μM $CoCl_2$ and 0.5 mM MES. All chemicals were obtained from Sigma-Aldrich. Transferred seedlings were grown vertically for 10 days. Four other *Penicillium* species, *P. bilaiae* (ATCC 20851), *P. chrysogenum* (NBRC 4626), *P. janthinellum* (NBRC 31133), and *P. simplicissimum* (106922) were used for comparison studies.

Plant growth test by POT1 inoculation in Al containing soil

2-week-old Arabidopsis and 1-week-old Bok Choy seedlings were transplanted from germinating medium to soil. For POT1 induction treatments, 0.5 g of freshly grown and rinsed POT1 mycelia were inoculated to each soil pot one week before seedling transplant. Plants were grown for four weeks in a growth room at 23ºC with ~60% relative humidity under 16 h light/8 h dark condition (100 µmol $m^{-2}s^{-1}$ light intensity). All plants were watered twice per week. While control plants were watered with tap water, Al-treated Arabidopsis and Bok Choy plants were watered with tap water containing 50 mM and 20 mM $AlCl_3$, respectively.

Measurement of total anthocyanin and chlorophyll content

Anthocyanin levels were measured as previously described by Rabino and Mancinelli, 1986. 50 mg of Arabidopsis shoots were ground in liquid N_2 and anthocyanin was extracted overnight at 4°C with 500 µl of methanol-HCl (99:1). Anthocyanin levels (mg/g FW) were calculated using the equation: ($Absorbance_{(530\ nm)}$ - (0.25 x $Absorbance_{(657\ nm)}$)) x extraction volume (ml) x (1/sample weight (g)).

Chlorophyll levels were measured as previously described by Porra et al., 1989. Chlorophyll extraction was similar to anthocyanin except the use of 5 ml of 80% acetone as extraction buffer. Total chlorophyll levels (mg/g FW) were calculated using the equation: ((7.34 x Absorbance $_{(663\ nm)}$) + (17.76 x Absorbance $_{(646\ nm)}$))/10.

Al staining

Root tips of Arabidopsis seedlings grown under Control, Al, and Al + POT1 conditions were excise, washed in deionized water for 10 min and stained with 100 µM morin in 10 mM MES buffer, pH 5.5 for 30 min. Then, they were washed twice with the same MES buffer and deionized water for 5 min each. Stained root tips were mounted in 15% glycerol and viewed with eGFP epifluorescence microscopy (ZEISS AXIOPLAN 2).

Measurement of Al content

For the measurement of total Al content, Arabidopsis shoot and roots were dried at 60°C and ground into fine powder. To 100 mg of grounded powder, 5 mL of HNO_3 (68%) and 1 mL of HCl (37%) were added and incubated at 220 °C for 2 h using graphite digestion equipment (DigestLinc ST60D). Digested samples were then diluted to 10 ml with deionized water and the Al contents were analysed using Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) (Agilent 720) with a detection limit of 0.02 ppm.

NO detection

In situ measuring of nitric oxide (NO) was performed as described by Park et al., 2011. Root tips were stained with 5 µM DAF-FM DA (3-amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate, a fluorescent NO indicator dye) in 20 mM HEPES buffer (pH 7.4) for 15 min. The stained root tips were then washed with 20 mM HEPES buffer (pH 7.4) for 10 min and fluorescence was observed under Confocal Laser Scanning Microscope (CLSM) (excitation 495 nm, emission 515 nm; Olympus FV3000).

Callose staining

Callose staining was performed as described by Clay et al., 2009, with modifications. Root tips were washed in 150 mM K_2HPO_4 for 10 min and stained with 150 mM K_2HPO_4 and 0.01% (w/v) aniline blue for 1 hour under darkness. Callose depositions were visualised with CLSM (excitation 370 nm, emission 509 nm; Olympus FV3000).

Results

POT1 reduces Al stress of Arabidopsis

In order to assess plant-growth-promoting effects of the Al-tolerant POT1, 5-day-old Arabidopsis seedlings were transferred to agar plates of pH 4 and pH 5.6 containing 100 µM Al and 100 µM Al + POT1 and grown for 7 days. As known from previous studies, seedlings grown on pH 4 - $AlCl_3$ plates exhibited severe inhibition of primary root growth compared to untreated, control plants (FIGS. 25a, b). However, when the seedlings were grown on agar plates containing 100 µM Al + POT1, primary root length was significantly higher than that of Al-treated plants. At pH 5.6, Al treatment did not have a significant effect on primary root growth, as compared to pH 4. It is important to note that at both pH 4 and pH 5.6, primary root length of POT1-treated seedlings was higher than that of control and Al-treated seedlings, indicating that POT1 not only overcomes root growth inhibition caused by Al treatment but it also has inherent root growth promoting effects (FIG. 25a, b). Fresh weight of primary roots was in correlation with that of the primary root length (FIG. 25c). In addition, plants treated with POT1 also had higher shoot fresh weight and leaf area (FIG. 25d-f). FIG. 31 shows the effect of POT1 under two different experimental conditions in Arabidopsis. In the first experiment, Arabidopsis seedlings were transferred to a solid medium plate in which POT1 had been growing for a week whereas for the second experiment, POT1 and Arabidopsis seedlings were added to the solid media plates back-to-back on the same day. Using these two experimental conditions, we not only re-confirmed that plants inoculated with POT1 had longer primary roots than that of non-inoculated plants, but we also realised that pre-treatment of growth medium with POT1 is not essential to alleviate Al stress in plants.

POT1 colonizes plant roots and alleviates accumulation of Al, NO, and callose at the root apex.

To understand the influence of POT1 on the accumulation of Al, NO, and callose, the roots of the Arabidopsis plants cultivated with 100 µM Al and 100 µM Al + POT1 were observed under confocal microscope and brightfield microscope after different staining methods (FIG. 26). In the control, Al, NO, and callose were either undetectable or observed only as weak, diffused signals whereas high levels of Al-, NO-, and callose- specific signals could be observed in the roots exposed to aluminium. Interestingly, a dramatic decrease in the levels of Al, NO, and callose could be detected in Al+POT1-treated plants. When the Al content was measured using Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES), the Al content of Al+POT1-treated plants was indeed much less than in Al-treated plants (FIG. 32a). These results indicated that POT1 successfully alleviated the accumulation of Al, NO, and callose in the root elongation zone and/or root tips and thereby prevented the primary root growth inhibition.

Effects of POT1 on Arabidopsis and Bok Choy grown in high Al-containing soil

To test the effects of aluminium toxicity in Arabidopsis and Bok Choy, 1-week-old seedlings were transferred to soil and treated with different concentrations (0, 10, 20, 30, 40, 50 and 100 mM) of $AlCl_3$ solution once a week. As the concentration of Al increased in soil, Arabidopsis plants became smaller and smaller but at 100 mM Al, the plants were significantly growth retarded and appeared purple, indicating the accumulation of anthocyanins (FIG. 27a). Increasing concentrations of Al had similar effect on the Bok Choy plants (FIG. 28a). From these results, 50 mM and 20 mM $AlCl_3$ were chosen as optimum concentrations to study the effect of POT1 in relation to Al toxicity in Arabidopsis and Bok Choy plants, respectively. Although the Arabidopsis plants treated with 50 mM Al + POT1 were smaller than the non-treated control plants, they were significantly larger than the Al only treated plants (FIG. 27b). Not only were the Al-treated plants much shorter than the Al+POT1 inoculated plants, but they also senesced earlier too (FIG. 27c). The shoot fresh weight was nearly 4-fold lower in Al-treated plants than in control plants (FIG. 38d). Even though the shoot fresh weight of Al + POT1-treated plants were slightly lower than the control plants, they were 3-fold higher than that of Al-treated plants (FIG. 27d), indicating much less Al stress in POT1 inoculated plants (FIG. 27e). Not so surprisingly, POT1 inoculated plants showed less stress phenotype with less anthocyanin than that of non-

inoculated plants under 50 mM Al treatments (FIG. 27b). Consistent with the leaf phenotype, total anthocyanin and chlorophyll content were 2-fold less and 2-fold more, respectively, in POT1 inoculated plants than Al-treated plants (FIG. 27f, g). Similar to Arabidopsis, Bok Choy plants treated with Al+POT1 showed better growth condition such as higher shoot fresh weight and reduced shoot Al content, compared to Al-treated plants (FIG. 28b-d).

Comparison of other Penicillium species with POT1

To compare the capability of POT1 in alleviating Al stress with other *Penicillium* strains, Arabidopsis were grown under high Al conditions in the presence/absence of the following 5 *Penicillium* strains; *P. bilaiae*, *P. chrysogenum*, *P. janthinellum*, *P. simplicissimum,* and POT1 (Figure 3a). The results show that the plants inoculated with fungal strains had longer primary roots, higher root fresh weight and shoot fresh weight compared to the non-inoculated control plants (FIG. 29b-d). Interestingly, root fresh weight and shoot fresh weight were not only the highest in POT1 inoculated plants but also, they were significantly higher than those of the plants inoculated with other *Penicillium* strains (FIG. 29c, d). When Arabidopsis were grown under high Al conditions on soil that were not inoculated or inoculated with one of these 5 Penicillium strains, we observed the least anthocyanin accumulation and highest chlorophyll content in POT1 inoculated plants compared to the plants inoculated with the other 4 *Penicillium* strains (FIG. 30).

Summary

The plant growth promoting potential of POT1 was tested in an assay with Arabidopsis seedlings growing on agar plates with 100 μM Al. POT1 was able to clearly promote plant growth in the presence of Al. Increased primary root length, root fresh weight, shoot fresh weight and leaf areas coincided with the inoculation of POT1. It is important to note that POT1-inoculated Arabidopsis seedlings had better primary root growth, root fresh weight and shoot fresh weight than control plants. The growth improvement of POT1 added plants could have resulted from the phosphate solubilizing property of POT1 (see Example 1) and increased nutrient uptake as transport of nutrients through the hyphae from distant points beyond the root depletion zones has been documented (George, 2000). Similar to plants growing on agar, POT1 offered Al resistance to Arabidopsis and Bok Choy grown on soil under Al stress.

Arabidopsis plants have been shown to accumulate high levels of Al, Fe, NO, and callose in the root tips upon sensing Al stress (Zhang et al., 2019). Our microscopy analysis showed that Al + POT1 treatment significantly reduced the accumulation of Al, NO, and callose in the Arabidopsis roots when compared to Al-only treated plants. This result suggests that Al +

POT1-treated Arabidopsis plants do not experience as much Al toxicity as the Al-only treated plants, which might be contributed by POT1-dependent detoxification of Al.

The results demonstrate that POT1, an Al-tolerant fungus isolated from an indoor environment, showed plant-growth-promoting traits in the presence of Al. The results suggest that the inclusion of POT1 with organic fertilizer may be a possible strategy to alleviate Al toxicity and promote plant biomass production.

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CLAIMS

1. An isolated fungal strain of species *Penicilium olsonii*, having the deposit accession number NRRL 68252, deposited with Agriculture Research Service Culture Collection (NRRL) on 18 January 2023.

2. A plant growth-promoting composition comprising the isolated fungal strain of claim 1, its mycelia, hyphae, spores and/or cell-free culture filtrate thereof.

3. The plant growth-promoting composition according to claim 2, wherein the composition comprises the culture filtrate or the mycelia of the isolated fungal strain of claim 1.

4. The plant growth-promoting composition according to claims 2 or 3, wherein the composition

(a) increases the availability of phosphorus for plant uptake in a medium used to grow the plant,

(b) reduces aluminium (Al) stress/toxicity in a plant grown in a medium comprising aluminium, and / or

(c) inhibits negative components of plant root growth such as accumulation of iron, nitric oxide, and/ or callose in the roots.

5. The plant growth-promoting composition according to any one of claims 2 to 4, wherein the composition comprises at least 0.1 g mycelia.

6. The plant growth-promoting composition according to claim 5, wherein the composition comprises at least 0.5 g mycelia.

7. The plant growth-promoting composition according to any one of claims 2 to 6, wherein the composition comprises at least one source of phosphorus.

8. The plant growth-promoting composition according to claim 7, wherein the at least one source of phosphorus is selected from the group consisting of: calcium phosphate, tricalcium phosphate, iron phosphate, aluminium phosphate and hydroxyapatite.

9. The plant growth-promoting composition according to any one of claims 4 to 8, wherein the medium is an agar medium, a liquid medium, soil or vermiculite.

10. The plant growth-promoting composition according to any one of claims 4 to 9, wherein the medium comprises at least one of the following: low phosphorus content, insoluble phosphate, or aluminium.

11. The plant growth-promoting composition according to any one of claims 2 to 10 for use as a fertilizer.

12. Use of the growth-promoting composition according to any one of claims 2 to 10 as a fertilizer.

13. The use according to claim 12, wherein the fertilizer is formulated as a dry biomass, preferably dust or granules, or a suspension, preferably water-based or emulsion.

14. A method of promoting plant growth, said method comprising applying an efficacious amount of a growth-promoting composition according to any one of claims 2 to 10 to a medium used to grow the plant, wherein the application of the composition promotes plant growth compared to a plant grown in an untreated medium.

15. The method according to claim 14, wherein the composition

(a) increases the availability of phosphorus for plant uptake in the medium used to grow the plant,

(b) reduces aluminium (Al) stress/toxicity in the plant grown in a medium comprising aluminium, and / or

(c) inhibits negative components of root growth such as accumulation of iron, nitric oxide, and/ or callose in the roots.

16. The method according to claim 14, wherein the medium comprises at least one condition selected from the group comprising low phosphorus content, insoluble phosphate and aluminium.

17. The method according to claim 14, wherein the medium is an agar medium, a liquid medium, soil or vermiculite.

18. The method according to claim 17, wherein the soil is paddy soil or upland soil.

19. The method according to any one of claims 15 to 18, wherein the plant is a Brassica species, Arabidopsis species or Oryza species.

20. The method according to any one of claims 15 to 19, wherein the plant is Bok Choy, wild-type *Arabidopsis thaliana* (Col-0) or *Oryza sativa* var. Indica.

21. A plant seed coated with the isolated *Penicillium olsonii* strain of claim 1 or the composition according to any one of claims 2 to 10.

22. The plant seed of claim 19, further coated with a soil compatible carrier.