

A METHOD FOR FUNGAL ENDOPHYTE-MEDIATED INDUCTION OF GROWTH AND RESILIENCE IN CROP PLANTS

FIELD OF THE INVENTION

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The present invention generally relates to the use of a plant growth-promoting composition comprising a fungal species, AR8, its progeny, hyphae and/or spores thereof, belonging to the genus *Tinctoporellus* to enhance the growth, yield and resilience in crop plants. More particularly, the said fungal isolate is *Tinctoporellus* species (AR8) having the NRRL Deposit
10 accession number 68276. The invention further relates to the direct and/or mixed and/or combinatorial (with AR8) use of beneficial metabolites belonging to the phenylpropanoid pathway, such as trans-Cinnamic acid, p-coumaric acid, caffeic acid, or ferulic acid, to increase the overall yield of farmed crops.

15 BACKGROUND OF THE INVENTION

The mycobiome, or mycobiota, is the fungal community associated with an organism. In general, plant roots interact with mycorrhizal fungi, as well as with various root-associated fungi. Importantly, since these soil fungi can drive multiple pivotal ecological functions and
20 confer beneficial effects on their host, plants can affect their performance and adaptation to the environment by changing this below-ground mycobiome. Given this scenario, understanding plant-fungus interactions and the members of symbiotic mycobiome in the rhizosphere is an imperative goal for plant biologists. The assembly of these fungi in the rhizosphere is affected by biotic and abiotic factors (Bais, H. P., *et al.*, (2006); Turlings, T., *et al.*, (2012); Dastogeer, K., *et al.*, (2020); Edwards, J. *et al.* (2015); Fitzpatrick, C. R. *et al.* (2018)). Host species and genotype, habitat conditions, and edaphic factors of the soil are important determinants of rhizosphere fungal communities. Mycorrhizal fungi are a classic
25 example of a symbiotic interaction, which has been intensively studied. Recently, fungal endophytes have attracted a great deal of attention due to their potential applications in agriculture to promote plant growth and/or health.
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There is a need for new methods of improving plant growth and/or health.

SUMMARY OF THE INVENTION

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The inventors isolated the mycobiome associated with the roots/rhizosphere of the model

- plant *Arabidopsis thaliana*, and then identified fungal isolates that could individually promote growth and biomass increase when applied to green leafy vegetables (Choy Sum and Kailan) in nutrient deficient soil. The inventors were particularly interested in identifying the endophytic species and were successful in isolating a fungus strain that belongs to
- 5 *Tinctoporellus* in preliminary analyses, designated AR8. Through rigorous testing, the inventors found that AR8 establishes itself as an endophyte in 10-14 days post-inoculation in the roots of the target host plants belonging to the Brassicaceae family, including the major vegetable crops Choy Sum (*Brassica rapa* var. *parachinensis*) and Kailan (*Brassica oleracea* var. *alboglabra*); the Asteraceae family including Lettuce (*Lactuca sativa*); and the Poaceae
- 10 family including Barley (*Hordeum vulgare*). A method of AR8 inoculation and usage was developed that was the most efficient in promoting robust growth in the aforementioned vegetable crops. AR8 has been deposited at the Agricultural Research Service Culture Collection (NRRL) under Deposit number 68276 on 17 April 2023.
- 15 Through detailed metabolomics analysis of the AR8-plant interaction, it was found that this endophytic fungus itself produces and secretes plant growth promoting phytohormones, and upregulates the host defense pathway components such as the phenylpropanoids during such rapid growth increase. In particular, the production of trans-Cinnamic acid was found to be one of the beneficial metabolites that was capable of inducing growth in vegetable crops
- 20 and the model plant species. Thus, AR8 serves a dual function in promoting growth (vegetative and reproductive) as well as resilience in the target hosts under nutrient limiting conditions. This invention unveils a beneficial endophyte and several beneficial metabolites capable of improved growth and metabolic resilience in urban farmed crops even under nutrient limiting conditions.
- 25 The AR8 seed/seedling inoculant improves vegetative growth and crop yield by increasing the ability of the host plant to respond to nutrient stress, and by enhancing the natural immunity.
- 30 The fungal isolate AR8 showed a robust growth promotion effect (up to 35% increase) in Choy Sum shoot and root system. Analysis of the AR8-Choy Sum root interaction revealed systemic spread of the fungus from the root surface, into specific intercellular regions, and biotrophic establishment as an endophyte into the intracellular region of Choy Sum root cortex. More importantly, the untargeted metabolomics analysis indicated the upregulation of
- 35 several primary metabolites with the significant perturbations in metabolic pathways, including phenylpropanoid biosynthesis, glyoxylate metabolism, and glucosinolate

biosynthesis in the AR8-inoculated Choy Sum plants. Exogenously provided metabolites belonging to the phenylpropanoid pathway, including trans-Cinnamic acid, were capable of independently inducing growth in Choy Sum and Arabidopsis. Preliminary analysis also provided a cross-kingdom transport of AR8-derived phosphate assimilating activity, and some growth-promoting phytohormones (mimics) as likely contributing mechanisms for such endophyte-based induction of growth in host plants. Taken together, the inventors report a novel fungal endophyte and associated beneficial metabolites that facilitate the growth of green leafy vegetables under greenhouse conditions; and uncover an important potential agricultural biofertilizer agent for sustainable yield increase in urban farmed crops.

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In a first aspect, the present invention provides a plant growth-promoting composition comprising a fungal species belonging to the genus *Tinctoporellus*, its progeny, mycelia/hyphae, spores, cell-free culture filtrate, exudate thereof, or any combination thereof.

15 In the context of this application, plant growth can refer to one or more of increased shoot biomass, leaf area and increased root branching and/or length. Shoot biomass and leaf area are of particular importance.

In some embodiments, the fungal species is *Tinctoporellus* strain AR8, which has been deposited at the Agricultural Research Service Culture Collection (NRRL) under Deposit number 68276 on 17 April 2023.

20 In some embodiments, the composition comprises spores, mycelia/hyphae, and/or exudates of *Tinctoporellus*.

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In some embodiments, the fungal spore concentration in the composition is in the range of about 1×10^3 spores/ml to about 1×10^9 spores/ml. For example, in some embodiments the fungal spore concentration is about 1×10^6 spores/ml.

30 In some embodiments, the spores are conidia.

In some embodiments, the fungal species is an endophyte. For example, AR8 grown with Choy Sum is an endophyte. However, this fungus is not an endophyte in rice in which AR8 hyphae only attach to the root surface without colonizing the cortical regions of the roots.

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In some embodiments, the composition may further comprise at least one phenylpropanoid selected from the group consisting of trans-cinnamic acid, p-coumaric acid, caffeic acid and ferulic acid. It will be appreciated that other isomers of these phenylpropanoid may be used.

- 5 In some embodiments, the concentration of phenylpropanoid may be in the range of 1 to 10 μ M. In preferred embodiments, the concentration of trans-cinnamic acid may be in the range of 1 to 5 μ M.

- 10 In some embodiments, the fungal species may have a genome comprising a nuclear ribosomal internal transcribed spacer (ITS) sequence that is at least 97% identical to the polynucleotide sequence set forth in SEQ ID NO: 1. In some embodiments, the fungal species has a genome comprising a further 7 loci comprising a sequence that is at least 97% identical to one or more of the sequences set forth in the group comprising SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8. Each of SEQ ID NOs: 2 to 8 are predicted mRNA sequences based on whole genome assembly and annotation.

- In some embodiments, the plant is selected from the group comprising Brassica species, Arabidopsis species, Asteraceae species and Poaceae species. In some embodiments, the plant may be Choy Sum, kailan, barley, or lettuce.

In some embodiments, the Brassica species is selected from the group comprising *Brassica rapa* and *Brassica oleracea*.

- 25 In some embodiments, the Asteraceae species is *Lactuca sativa* and the Poaceae species is *Hordeum vulgare*.

- In a second aspect, the invention provides a method of increasing growth of a plant, said method comprising applying an efficacious amount of a growth-promoting composition according to the first aspect to the plant to increase the plant growth compared to an untreated plant.

- In some embodiments, the growth-promoting composition is applied to a propagative part of the plant.

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In some embodiments, the propagative part is a seed or a root.

In some embodiments, the plant is selected from the group comprising Brassica species, Arabidopsis species, Asteraceae species and Poaceae species.

- 5 In a third aspect, the present invention provides a plant growth-promoting composition comprising trans-cinnamic acid.

In some embodiments, the concentration of trans-cinnamic acid may be in the range of about 1 to about 10 μM , preferably in the range of 1 to 5 μM .

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In a fourth aspect, the present invention provides use of the plant growth-promoting composition according to the third aspect to increase the growth of Choy Sum plants, compared to untreated Choy Sum plants.

- 15 In a fifth aspect, the invention provides an isolated fungal species of a *Tinctoporellus* strain, designated AR8, which has been deposited at the Agricultural Research Service Culture Collection (NRRL) under Deposit number 68276 on 17 April 2023.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1. AR8 demonstrates the growth promotion effect on Choy Sum in soil. Shoot fresh weight of Choy Sum ($n=14$ plants each) incubated with or without AR8 conidia using different inoculated methods were grown in soil for 21 days. Asterisks represent significant differences compared to the mock control at $P < 0.05$ (*) and $P < 0.001$ (***) (Student's t-test). Numbers represent the fold change compared to mock control.

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Figure 2. AR8 demonstrates a growth promotion effect on Kailan in soil. Shoot fresh weight of Kailan ($n = 12-20$) in soil inoculated with water (mock) or AR8. Asterisks (*) represent significant differences compared to the mock control at $P < 0.05$ (Student's t-test).

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Figure 3. Fungal isolate AR8 promotes seed germination and seed-to-seedling growth in Choy Sum. **(A)** Germination efficiency of Choy Sum seeds inoculated with AR8 conidia or AR8 spent medium. Choy Sum seeds (40 seeds per experiment; three replicates of the experiment) inoculated with water (mock control), AR8 conidia, 1% Complete medium, or AR8 exudate/spent medium at 1% v/v (culture filtrate). Germination efficiency (failed germination, poor germination, or proper germination) was qualitatively and quantitatively

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assessed at 4 days post inoculation (dpi). **(B)-(D)** Seed-to-seedling growth from proper germinating Choy Sum seedlings shown in (A). Hypocotyl length, shoot fresh weight, and root length (20-40 plants per experiment) from each group were measured at 4 dpi. **(E)** Effect of seed inoculation on the early stage of Choy Sum growth. Choy Sum seeds were inoculated with water (mock control), AR8 conidia, 1% CM medium, or 1% AR8 exudate/spent medium (culture filtrate). Shoot fresh weight of proper germinating seedlings (50-60 plants per experiment; three replicates) was measured at 10 dpi. The boxes reveal the first quartile, median and third quartile; the whiskers indicate the min and max values. Asterisks represent significantly different means compared to the mock control at $*P < 0.05$, $***P < 0.001$ (Student's t-test).

Figure 4. Effect of phenylpropanoid (PP) compounds on the growth of Choy Sum. The measurements of PP compounds on Choy Sum ($n = 9$) in **(A)** shoot fresh weight and **(B)** hypocotyl length treated with *trans*-cinnamic acid (2.5 μM), *p*-coumaric acid (60 μM), ferulic acid (20 μM), and caffeic acid (20 μM) for 10 days.

Figure 5. ITS rRNA repeats-based phylogenetic identification of AR8. Phylogenetic tree based on the Neighbor-Joining method. Strain AR8 is grouped together with other *Tinctoporellus* isolates. The respective genomic sequences (indicated Accession number NRRL 68276) for rDNA were obtained from NCBI database. Sequence comparison and alignment was performed using Megalign program via standard methodologies.

Figure 6. AR8 systematically colonizes Choy Sum roots in soil conditions. Laser scanning confocal microscopy of AR8 stained with WGA-Alexa488 (bright white; fungal cell wall) and Choy Sum roots stained with propidium iodide (grey; root cell wall). **(A)** Choy Sum roots were inoculated with water (mock control) and cultivated under sterilized soil conditions for 4 days and stained with the aforementioned reagents. No fungal conidia or mycelia are evident around the roots. Scale bar, 250 μm . **(B)** Choy Sum roots inoculated with AR8 conidia in sterilized soil for 4 days and stained with WGA-Alexa488, and propidium iodide. AR8 fungal hyphae attach to the root surface without penetrating the inner region of the root. Scale bar, 250 μm . **(C and D)** Enlargement of the section from projections is shown in (B). Scale bar, 25 μm . **(E)** Choy Sum root inoculated with water (mock control) for 7 days. No fungal attachment was found around the root. Scale bar, 250 μm . **(F)** Choy Sum root inoculated with AR8 conidia for 7 days. Fungal hyphae colonize both the external and internal roots. Scale bar, 250 μm . **(G)** The intercellular colonization of AR8 between root epidermal cells. Scale bar, 75 μm . **(H)** Enlargement of the section from projections and **(I)** orthogonal

- projection is shown in (G). Arrows indicate the intercellular hyphae between Choy Sum root epidermal cells. Scale bar, 75 μm (H) and 20 μm (I). (J) Choy Sum root was inoculated with water (mock control) for 14 days. No fungal attachment was found around the root. Scale bar, 250 μm . (K) Choy Sum root inoculated with AR8 conidia for 14 days. Extra-radical fungal hyphae enveloped Choy Sum root with the interstitial and intracellular hyphal colonization in the root epidermis. Scale bar, 250 μm . (L) AR8 shows endophytic growth and colonization in Choy Sum roots. The intracellular colonization of AR8 inside a root epidermal cell. Scale bar, 75 μm . (M) Enlargement of the section from projections shown in (L). Scale bar, 75 μm .
- 10 **Figure 7.** Fungus AR8 assimilates phosphate for the host plants, and promotes shoot growth under phosphate-limiting conditions. 7 day-old of Col-0 and *phr1* mutant arabidopsis seedlings ($n = 12-16$) were planted in the phosphate (Pi)-rich (1250 μM) and Pi-poor (100 μM) medium with or without for 10 days to measure (A) shoot fresh weight and (B) root length.
- 15 **Figure 8.** Time course analysis of Choy Sum shoot and root fresh weight in soil inoculated with water (mock control) or AR8 conidia. Four day old Choy Sum seedlings were inoculated with or without AR8 conidia and grown in soil. Shoot and root fresh weight (12-20 plants per experiment; three replicates of the experiment) were measured at 7, 14, and 21 dpi. Data presented (mean $\pm$ S.D) was derived from 3 independent replicates of the experiment. Asterisks (***) represent significant differences compared to the mock control at $P < 0.001$ (Student's t-test).
- 20 **Figure 9.** AR8 promotes robust growth in *Arabidopsis thaliana* Col-0 under soil conditions. Shoot fresh weight of *Arabidopsis thaliana* Col-0 inoculated with AR8 conidia in soil conditions. The boxes reveal the first quartile, median and third quartile; the whiskers indicate the min and max values. Asterisks (***) represent significantly different means compared to the mock control at $P < 0.001$ (Student's t-test).
- 25 **Figure 10.** Silique number and shoot branching rate of Choy Sum inoculated with AR8 in soil. The boxes reveal the first quartile, median and third quartile; the whiskers indicate the min and max values. Asterisks (*) represent significantly different means compared to the mock control at $P < 0.05$ (Student's t-test).
- 30 **Figure 11.** The increased accumulation of phosphorous (P) in AR8-inoculated Choy Sum plants. The concentration of (A) nitrogen (N), (B) phosphorus (P), and (C) potassium (K) in

Choy Sum shoots inoculated with water (mock control) or AR8 conidia for 21 days. The nutrient concentration was calculated in mg/kg based on the shoot dry weight. The boxes reveal the first quartile, median and third quartile; the whiskers indicate the min and max values. Asterisk (**) represents significantly different means compared to the mock control at $P < 0.01$ (Student's t-test).

Figure 12. The growth of Choy Sum seedlings shows growth promotion upon treatment with or without AR8 conidia and trans-cinnamic acid (tCA). Shoot fresh weight of Choy Sum (n=8 plants each) in soil inoculated with AR8 and tCA. Asterisks represent significant differences compared to the mock control at * $P < 0.05$, *** $P < 0.001$ (Student's t-test).

Figure 13. t-CA displays growth-promoting activity in Choy Sum in a dose-dependent manner. **(A)** Representative images of Choy Sum seedlings treated with t-CA in a dose-dependent manner. Four day-old Choy Sum seedlings were transferred into MS medium supplemented with t-CA (0, 1, 2.5, 5, 10, and 50 μM) for 7 days. Scale bar, 5 cm. **(B)** Shoot fresh weight, **(C)** hypocotyl length, and **(D)** lateral root density of Choy Sum seedlings grown in MS medium with t-CA in different concentrations. 4 day-old Choy Sum seedlings (10 plants per experiment; three replicates of the experiment) were grown in MS medium and t-CA and shoot fresh weight, hypocotyl length, and lateral root density were measured at 7 dpi. Data presented (mean $\pm$ S.E) was derived from 3 independent replicates of the experiment. Different letters are significantly different means between treatments at $P < 0.05$ as calculated using the Tukey's HSD test. Letters 'a' to 'd' indicate the significant differences compared to the treatments. Data points with no significant difference are labelled with the same letter.

Figure 14. AR8 promotes Choy Sum growth in urban farm conditions. Shoot fresh weight of Choy Sum inoculated with or without 1×10^6 spores/ml AR8 in the urban farm. Choy Sum (n=25-30 plants) were randomly selected and shoot fresh weight measured at 28 dpi. The boxes reveal the first quartile, median and third quartile; whereas the whiskers indicate the minimum and maximum values. Asterisks (***) represent significantly different means compared to the mock control at $P < 0.001$ (Student's t-test).

Figure 15. AR8 functions in different monocot and dicot crop species, and significantly promotes the shoot growth. **(A)** Representative images of Kailan (dicot), barley (monocot), and lettuce (dicot) in soil treated with either water (mock control) or AR8 conidia (1×10^6 conidia/ml). Shoot fresh weight of **(B)** Kailan, **(C)** barley, and **(D)** lettuce inoculated with AR8

conidia in soil conditions. Three replicates of the experiment were performed, using 16-24 plants per experiment. The boxes reveal the first quartile, median and third quartile; the whiskers indicate the minimum and maximum values. Asterisks (***) represent significantly different means compared to the mock control at $P < 0.001$ (Student's *t*-test).

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DETAILED DESCRIPTION OF THE INVENTION

Certain terms employed in the specification, examples and appended claims are collected here for convenience. Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each of the appended claims.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

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 "inoculant composition" and "inoculant" refer to a composition comprising microbial cells and/or spores, said cells/spores being capable of propagating/germinating on or in a suitable growth medium or substrate when conditions (e.g., temperature, moisture, nutrient availability, pH, etc.) are favorable for germination and/or microbial growth.

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As used herein, the term "spores" includes sexual spores and asexual spores of the fungus AR8, the asexual spores also known as "conidia". In this specification, the unit "spores/mL" may therefore be used to include the presence of one or both types of spores. The term "conidia/mL" may also be used.

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As used herein, the term "cell-free culture filtrate" or "culture filtrate" refers to the cell-free spent medium after fungal growth or biomass is removed, and the term "exudate" refers to all extra-cellular secretions from the fungal mycelia and/or conidia that collects in the surrounding growth medium. In this specification, the terms "exudate" and "cell-free culture
10 filtrate" may be used interchangeably, since it is difficult to rid of all secretions from the fungus while harvesting mycelia and/or conidia.

The present invention is described by reference to the following examples, which is offered by way of illustration and is not intended to limit the invention in any manner. A person skilled
15 in the art will appreciate that the present invention may be practiced without undue experimentation according to the methods given herein. The methods, techniques and chemicals are as described in the references given or from protocols in standard biotechnology and molecular biology textbooks. Standard molecular biology techniques known in the art and not specifically described were generally followed as described in
20 Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (2001).

EXAMPLES

25 Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana Col-0, the mutant *phr1* and Choy Sum (*Brassica rapa* var. *parachinensis*) was used and cultivated based on the methods provided by Tan, et al (2020).

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Seeds were surfaced-sterilized with 70% ethanol and placed on Murashige and Skoog basal salts (Sigma-Aldrich; no. M5524) with 1% sucrose and 1% agar (MS-agar) for germination at 22°C and relative humidity 60% with 16/8 (light/dark) photoperiod at a 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ Photosynthetic Photon Flux Density (PPFD), or 8880 Lux For fungal co-culture experiments,
35 seedlings were transferred to either sucrose-free MS-agar medium or a tray containing autoclaved (121 °C, 1 h) peat/perlite mix (BVB Substrates, Netherlands) after fungal

inoculation. Plants in the tray were watered with deionized water (1000 ml/day) and no additional fertilizers were added during the experimental period.

Example 1: Isolation and identification of fungal isolate AR8 from *Arabidopsis thaliana*

Fungal isolation from the roots of Arabidopsis thaliana

Roots from 14-day-old *Arabidopsis thaliana* Col-0 plants were collected and ground in 1x phosphate-buffered saline (PBS) to acquire the rhizosphere fungal microbiome. The 100x diluted root homogenate was plated on Prune agar (PA) 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) supplemented with Tetracycline (5 µg/ml), Streptomycin (100 µg/ml), Carbenicillin (50 µg/ml), and Kanamycin (50 µg/ml) and incubated at 28°C for 2-3 days. The individual fungal colonies were selected and subcultured on PA medium with 50 mm filter-paper discs twice for purification, and storage.

Fungal growth conditions and DNA extraction

Prune agar or Potato dextrose agar medium was used for fungal culture. For conidiation, fungal isolates grown on PA medium in the dark for 2-3 days were transferred and cultivated under constant illumination at room temperature for 5-7 days. The conidia were collected by scraping with inoculation loops on the surface of fungal colonies in the presence of water. The suspension was then filtered through two layers of Miracloth (Calbiochem, San Diego, CA, USA) and diluted to 1×10^6 spores/ml for the following inoculations.

For DNA extraction, conidia were inoculated in the liquid Complete medium (CM) (6 g/L yeast extract, 6 g/L casein hydrolysate, 10 g/L sucrose) and incubated at 28°C, 180 rpm on an orbital shaker for 5 days. Subsequently, AR8 fungal mycelia were collected and ground to a powder in liquid nitrogen for the following DNA extraction procedure.

Genomic DNA was extracted from mycelia by using the MasterPure Yeast DNA Purification kit (Lucigen). Ten nanograms of fungal DNA was used for PCR amplification using specific primers that target the internal transcribed spacer (ITS) region, and the nuclear ribosomal large subunit (LSU) and small subunit (SSU) rRNA genes. Oligonucleotide primers used for identification of fungal genera are given in Table 1.

Table 1: Oligonucleotide primers used for fungal identification and taxonomy.

Primers	Primer Sequences	SEQ ID NO
¹ ITS1	TCCGTAGGTGAACCTGCGG	9
¹ ITS4	TCCTCCGCTTATTGATATGC	10
² LSU-F	ACCCGCTGAACTTAAGC	11
² LSU-R	TCCTGAGGGAAACTTCG	12
² SSU-F	GTAGTCATATGCTTGTCTC	13
² SSU-R	CTTCCGTCAATTCCTTAAG	14

¹White et al., 1990; ²Schoch et al., 2012

5 PCR products were sequenced and DNA barcoding performed on the NCBI BLAST database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), assigning the species identity of our fungal isolates by utilizing the highest identity/similarity score. An ITS-based phylogenetic analysis by Neighbor-Joining method placed AR8 as being highly similar to the other *Tinctoporellus* species (Fig. 5). *Tinctoporellus* is a genus of fungi in the family Polyporaceae.

10 The genome of AR8 was sequenced using whole-genome sequencing by Illumina NovaSeq 6000 at Axil Scientific Pte Ltd, Singapore. The sequencing of the AR8 genome produced an assembly of 1658 contigs and an estimated genome size of 44 Mb. Notably, the genome comparison of randomly selected annotated ORFs provided high similarities with the
15 orthologous sequences of *Tinctoporellus* in the NCBI database, supporting the relevance of AR8 to *Tinctoporellus* species. The ITS nucleotide loci sequence from the AR8 genome that showed significant sequence similarity to the orthologous sequence in the published *Tinctoporellus epimiltinus* genome, is set forth in SEQ ID NO: 1, whereas SEQ ID NOs: 2 to
20 8 represent annotated nucleotide sequences of 7 selected genes (ORFs) selected from the whole genome sequence of AR8 isolate.

Example 2: AR8 demonstrates growth promotion effect on Choy Sum and Kailan

25 To quantify plant growth promotion mediated by fungus AR8 on green leafy vegetables Choy Sum and Kailan, two different conidial inoculation methods were used (conidia refers to the asexual spores of the fungus). Surface-sterilized seeds were germinated on Murashige & Skoog (MS) agarose medium. In the first method, AR8 conidia (1 x 10⁶ spores/ml) obtained from the AR8 fungal colony grown on PA medium were mixed with autoclaved soil in a 1:10

(v/v) ratio and 4 day-old plant seedlings were then transferred to the soil-conidia mixture. In the second method, AR8 conidia (1×10^6 spores/ml) were directly inoculated to the seedling roots and their surrounding soil. Heat-killed spores or sterile distilled water was used as a mock control in these two methods with the same settings. Shoot fresh weight was measured at 21 days post-inoculation (dpi) (Fig. 1 and Fig. 2).

The results show that AR8 has a growth promotion effect on Choy Sum and Kailan in soil. Direct root inoculation was selected to use in the following examples, unless otherwise stated, given the higher efficiency for Choy Sum growth promotion. For Kailan, shoot biomass of AR8-inoculated plants was increased by 18.94% ($P < 0.05$) at 21 dpi.

Example 3: AR8 promotes growth of Choy Sum during both vegetative and reproductive stages

The beneficial effect of AR8 were further investigated. 4 day-old Choy Sum seedlings were inoculated with or without 10^6 /ml AR8 conidia and grown in soil. Shoot and root fresh weight (12-20 plants per experiment; three replicates of the experiment) were measured at 7, 14, and 21 dpi.

As shown by Figure 8, the improved Choy Sum growth provided by AR8 under soil conditions was at 14 days post-inoculation (dpi). The AR8-inoculated plants exhibited a significantly higher shoot biomass increase of 22.01% ($P < 0.001$) at 14 dpi, and 37.07% at 21 dpi ($P < 0.001$).

To address the long-term impact of AR8 colonization, the reproductive growth of Choy Sum was studied as an index of fertility. Four-day-old Choy Sum (8 seedlings per experiment; three replicates of the experiment) were inoculated with water (mock control) or 10^6 /ml AR8 spores. Silique number and shoot branching were measured at 49 dpi. It was observed that Choy Sum inoculated with AR8 displayed an earlier floral transition and provided more siliques (Figs. 10A and 10B), thus indicating that the beneficial fungus AR8 promotes the growth and development during both vegetative and reproductive stages of the host plants.

Example 4: AR8 promotes increased shoot biomass in *Arabidopsis*

The impact of AR8 on model Brassica species *Arabidopsis thaliana* accession Col-0 was tested. Seven-day-old *Arabidopsis thaliana* Col-0 seedlings (12 plants per experiment; three replicates of the experiment) were inoculated with water (mock control) and 10^6 /ml AR8.

Shoot fresh weight was measured at 14 dpi. Remarkably, shoot biomass of *Arabidopsis* demonstrated a significant improvement with an average increase of 53.61% ($P < 0.001$) upon AR8 inoculation under soil conditions (Fig. 9).

5 **Example 5: AR8 promotes Choy Sum seed germination and improves seed-to-seedling performance in soil**

Surface-sterilized Choy Sum seeds were placed and inoculated with either 1 ml axenic AR8 culture filtrate or AR8 conidia (1×10^6 spores/ml). For the production of axenic AR8 culture
10 filtrate, AR8 conidia were inoculated in the CM liquid medium with a concentration of 1×10^5 spores/ml at 28°C, 180 rpm for 5 days on an orbital shaker. The AR8 medium was filtered through two layers of sterilized Miracloth and a syringe filter (0.2 µm hydrophilic cellulose acetate membrane, Minisart®). The inoculated seeds were then lightly covered with soil and cultivated for 4 days in indoor conditions (16 h light, 8 h darkness, 22°C, 60% relative
15 humidity, $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, or 8880 Lux). Seed germination rate and seed-to-seedling performance including hypocotyl length, seedling fresh weight and root length were measured in comparison to the mock control (Fig. 3).

Seeds inoculated with either AR8 exudates or AR8 conidia showed a significantly higher rate
20 of germination (Fig. 3A). Moreover, the hypocotyl length and shoot fresh weight were significantly increased at the seedling stage in the presence of AR8 exudates and in AR8 conidia-inoculated groups (Figs. 3B and 3C). The promotion effect on seed-to-seedling performance was on the aerial part of the plants (AR8 conidia: 31.65% increase in hypocotyl length and 30.95% increase in shoot fresh weight; AR8 medium: 16.55% increase in
25 hypocotyl length).

The axenic AR8 culture filtrate were analyzed by LC-MS. The axenic AR8 culture filtrate was filtered and vacuum-dried by CentriVap concentrator. The samples were reconstituted with 80% methanol (v/v) before analysis. The samples were spiked with 500 ppb of standards to
30 reconfirm the identity of sample peaks if needed (retention time shifted). The analysis was performed in an Agilent 1290 Infinity II coupled to an Agilent 6495 Series Triple Quadrupole LCMS (Agilent, USA) with a Jet Stream ESI ion source. The separation of compounds was achieved on a Kinetex C18 column (100 mm length, 2.1 mm inner diameter, 1.7 µm particle size, 100 Å, Phenomenex). The autosampler and column were maintained at 4 °C and 35 °C,
35 respectively. For analysis, plant samples and fungal samples were injected with 2 µL and 10 µL extraction solvent (80% v/v methanol) respectively. Separation was performed at a

constant flow rate of 0.3 ml/min, in a gradient of solvent A (water acidified with 0.1% formic acid) and B (acetonitrile acidified with 0.1% formic acid). The gradient program was: 5% solvent B (0-1 min); 5-100% solvent B (1-10.5 min); 100% solvent B (10.5-13.4 min); re-equilibration from 100-5% solvent B (13.4-13.5 min); and reconditioned with 5 % solvent B (13.5-16.5 min). Electrospray ionization was performed in both positive and negative ion mode with the following source parameters: drying gas (N₂) temperature of 250 °C with a flow rate of 12 L/min, nebulizer gas pressure of 35 psi, sheath gas temperature of 350 °C with a flow rate of 11 L/min and capillary voltage of 4000 V. Mass spectra were acquired in the multiple reaction monitoring (MRM) mode.

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The LC-MS analysis confirmed the production of gibberellic acid (GA₄) from AR8. The LC-MS analysis also confirmed that AR8 secretes GA₄ and abscisic acid into the culture filtrate.

Example 6: Chemical complementation assay for phenylpropanoids

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The following phenylpropanoids/chemicals were used for chemical complementation assays: trans-cinnamic acid (t-CA; Sigma), p-coumaric acid (Sigma), Caffeic acid (ChromaDex), and Ferulic acid (ChromaDex). The chemicals were prepared at 1 mM as stock solutions in methanol, which was in parallel used as the solvent/mock control in treatments. To quantify the effect of phenylpropanoid compounds in plant growth promotion, 4 day-old Choy Sum seedlings (n = 10) germinated on MS agar were transferred to the medium containing 2.5 μM t-CA, 60 μM p-coumaric acid, 20 μM caffeic acid, or 20 μM Ferulic acid in growth chamber (16 h light/8 h dark; 21°C) for 10 days. The shoot fresh weight and hypocotyl length of Choy Sum were measured as parameters to examine the growth promotion effect of phenylpropanoid compounds.

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As shown in Figure 4, shoot biomass of t-CA treated plants was increased by 28.86% (P < 0.01). Hypocotyl length of t-CA treated plants was increased by 21.81% (P < 0.05). Hypocotyl length of p-coumaric acid treated plants was increased by 36.36% (P < 0.05). Hypocotyl length of ferulic acid treated plants was increased by 41.81% (P < 0.05). In summary, t-CA has a general growth-promoting effect on Choy Sum.

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Example 7: Effect of t-CA concentration on plant growth promotion in Choy Sum

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To further understand the function of t-CA on plant growth promotion, the effect of different concentrations of t-CA (0, 1, 2.5, 5, 10, 50 μ M) on Choy Sum seedling shoot fresh weight, hypocotyl length, and lateral root density therein were tested (Fig. 13A). Four day-old Choy Sum seedlings were transferred into MS medium supplemented with t-CA (0, 1, 2.5, 5, 10, and 50 μ M) for 7 days.

In contrast to the untreated control plants, a clear trend towards growth promotion was observed in t-CA-treated Choy Sum. The shoot biomass increased by 22.93%, 38.01%, and 24.69% while hypocotyl length was induced by 6.25%, 13.54%, and 15.62% for seedlings treated with low concentrations (1, 2.5, and 5 μ M) of t-CA, respectively (Figs. 13B and 13C). Moreover, the growth-promoting effect of t-CA on Choy Sum was not restricted to the aerial parts of the seedlings. The roots of Choy Sum treated with low concentrations (1, 2.5, and 5 μ M) of t-CA triggered the formation of lateral roots without inhibiting primary root growth, leading to higher lateral root density (64.14%, 73.26%, and 87.81%, respectively) (Fig. 13D). In contrast, high concentrations of t-CA caused growth inhibition in Choy Sum. Both shoot biomass and hypocotyl length were greatly reduced in the Choy Sum seedlings treated with 10 or 50 μ M of t-CA compared to the mock control (Figs. 13B-13C).

Example 8: Systemic colonization of AR8 in Choy Sum roots

To visualize in planta colonization process of AR8 by live-cell confocal imaging, 10^6 /ml AR8 conidia were inoculated on Choy Sum roots and stained with WGA-Alexa488 and propidium iodide (PI) to visualize fungal hyphae and root cell walls, respectively, under soil conditions. Tracing the WGA-Alexa488 signal, there was a lack of fungal hyphae/structures in the roots of control samples during in planta colonization analysis (Figs. 6A, 6E, and 6J). By contrast, the WGA signal in AR8-inoculated plants indicated that AR8 hyphae are attached to the root surface at 4 dpi (Figs. 6B-6D). However, at this stage, none of the fungal hyphae penetrated the epidermal region or colonized the endosphere. Around 7 dpi, AR8 showed a stable interaction with host roots, with fungal hyphae in the rhizosphere gradually enveloping the roots (Fig. 6F). More importantly, AR8 entered the roots interstitially, producing intercellular hyphae that advanced between PI-labeled epidermal cells (Figs. 6H-6I). Based on this data, it was hypothesized that AR8 establishes a biotrophic interaction with plant roots (causing no damage/death, and keeping the host alive). Physically intact host root cells clearly outlined by the intercellular hyphae, suggested the viability of host and fungal cells during such AR8 colonization. Following the entry into the root epidermis, AR8 further colonized the root cortex with both inter- and intracellular hyphae at 14 dpi (Figs. 6L and 6M). The root cortical

cells colonized by intracellular hyphae remained unharmed and inferred to have established a stable biotrophic and symbiotic interaction established within the root system. At this stage, an extensive network of extra-radical hyphae formed and enveloped the host roots (Fig. 6K).

- 5 Overall, the data indicates that AR8 is a beneficial fungal endophyte, which colonizes the intra- and intercellular compartments of the host roots and provides significant beneficial effects to the plants without causing any apparent damage or detrimental symptoms.

Example 9: Macronutrient assimilation in Choy Sum

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The inventors measured the 3 major macronutrients, phosphorus (P), nitrogen (N), and potassium (K), in Choy Sum by using Inductively Coupled Plasma mass spectrometry (ICP-MS) (Fig. 11). Choy Sum roots were inoculated with water (mock control) or (10^6 /ml) AR8 conidia for 21 days. Choy Sum shoots (16 plants per experiment; three replicates of the
15 experiment) from each group were pooled to measure shoot N, P, and K content by Inductively-coupled plasma mass spectrometry (8 samples per experiment). The nutrient concentration was calculated in mg/kg based on the shoot dry weight. The samples of Choy Sum shoot were dried for 5 days at 65°C. For pre-digestion, 100 mg of dry homogenized plant powder was performed by 2.5 ml of HNO₃ (66% v/v) and 0.5 ml of H₂O₂ (30% v/v)
20 overnight. High Performance Microwave Digestion System was followed to digest samples for 3 h. Final solution was diluted by 1:10 dilution with deionized water and stored at 4°C before analysis. The determination of nitrogen (N), phosphorus (P), and potassium (K) levels was performed with an Agilent 7700 ICP-MS (Agilent) following the manufacturer's instructions.

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The results show that AR8 fungal inoculation significantly increased (by about 17%) the Phosphate levels in the shoots whereas the concentration of N and K remained unperturbed therein.

30 Example 10: Molecular mechanism of enhanced phosphate transport

The inventors tested hyphal transport in the bi-compartment system with wild-type and the *phr1* mutant (defective in Phosphate Starvation Response 1) Arabidopsis plants (Fig. 7A). In this system, AR8 mycelium plugs were inoculated in the inner compartment (hyphal
35 compartment; HC) while plants were cultivated in the external compartment (root and hyphal compartment; RHC). These two compartments were separated by a plastic barrier, which

only allowed the crossing of fungal hyphae. By adjusting the inorganic phosphate (Pi) concentration in RHC, the inventors aimed to understand whether Pi accumulation in host plants is due to the AR8 hyphal transport. Remarkably, in the low-Pi conditions (100 μ M), shoot biomass demonstrated a significant improvement only in the wild-type plants inoculated with AR8. By contrast, the *phr1* mutant which is defective in phosphate starvation response was impaired in AR8-mediated plant growth promotion. The shoot fresh weight of *phr1* mutant plants was comparable between mock and AR8 inoculation. (Col-0: 1.52-fold; $P < 0.001$ versus *phr1*: 1.23-fold; $P = 0.179$). Similarly, in the high-Pi conditions (1250 μ M), the promotion of shoot fresh weight in the AR8 inoculated plants demonstrated a significant improvement in wild-type plants compared to *phr1* mutant plants (Col-0: 1.44-fold; $P < 0.001$ versus *phr1*: 1.29-fold; $P < 0.05$) (Fig. 7A).

In the bi-compartment system, the colonization of AR8 reflects an expanded capacity of host roots to access Pi by the long-distance transport of/via fungal hyphae. The transfer of Pi from AR8 hyphae to host plants supports the shoot growth under both low- and high-Pi conditions. PHR1 is the regulatory factor of the phosphate starvation response (PSR) in *Arabidopsis thaliana* which controls the transcriptional activation of a large set of PSR genes during Pi deficiency. The defect of growth promotion in *phr1* mutant plants in the bi-compartment system reinforces the idea of Pi transport by AR8 to growth promotion in Choy Sum. The reduced capacity for shoot growth indicates the functional engagement of AR8 hyphae in Pi transport. Taken together, the inventors uncovered a novel role for the beneficial fungus AR8 in expanding the range of nutrient absorption for and transport into the host plants. The colonization of AR8 forming the extraradical hyphal networks likely increased the nutrient absorptive area of the root system. Furthermore, the extended soil exploration by AR8 might be conducive to plant growth and development in natural soils, under low as well as high bioavailable Pi conditions, and thus in improving crop productivity.

Example 11: Co-culture of Choy Sum seedlings with AR8 conidia and trans-cinnamic acid (tCA) improves growth

4 day-old Choy Sum seedlings were inoculated with (1×10^8 /ml) AR8 conidia and tCA in MS-agar medium for 6 days and transferred to the soil for 14 days. As shown by the results in Figure 12, the growth-promoting effect on Choy Sum was further enhanced significantly if co-cultured with AR8 and t-CA.

Example 12: Field trial at an Urban Farm

Field trials were performed at a local urban farm in Singapore. Choy Sum seeds were sown on coco peat bungs and inoculated with the conidial suspension of AR8. Specifically, about 3-4 (unsterilized) seeds were sown on the surface of coco peat (114 coco peat bungs per group in total) and inoculated with AR8 fungal suspension (1×10^6 spores/ml). The irrigation system was kept on for 24 h to keep water flowing through the base of the coco peat bungs placed in the perforated plastic pots. Seed germination was checked at 7 dpi. Additional seedlings (per pot; if any) were removed to retain and maintain only one properly germinated seed/seedling in each coco peat to prevent the shade avoidance syndrome. Shoot fresh weight of Choy Sum plants was measured at 28 dpi.

The size of Choy Sum plants inoculated with AR8 was more uniform with larger leaf area and greater plant size. More importantly, the shoot fresh weight showed a significant increase by about 31.13% ($P < 0.001$) at 28 dpi (Fig. 14). As a result, the AR8 functional study in the less favorable field conditions suggests the increase of Choy Sum yield and highlights the potential use of AR8 as a biofertilizer for urban farming.

Example 13: AR8 functions in different crop species, and significantly promotes shoot growth

Trials were performed with Kailan, barley, and lettuce (16-24 plants per experiment; three replicates of the experiment) in soil treated with either water (mock control) or 1×10^6 spores/ml AR8 conidia. Figure 15 shows that AR8 promoted an increase in shoot fresh weight of Kailan (B), barley (C), and lettuce (D).

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CLAIMS

1. A plant growth-promoting composition comprising a fungal species belonging to the genus *Tinctoporellus*, its progeny, mycelia/hyphae, spores, cell-free culture filtrate, exudate
5 thereof, or any combination thereof.
2. The plant growth-promoting composition according to claim 1, wherein the fungal species is *Tinctoporellus* strain AR8, which has been deposited at NRRL under Accession number 68276 on 17 April 2023.
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3. The plant growth-promoting composition according to claim 1 or 2, wherein the composition comprises spores, mycelia/hyphae, and/or exudates of *Tinctoporellus*.
4. The plant growth-promoting composition according to any one of claims 1 to 3,
15 wherein fungal spore concentration in the composition is in the range of 1×10^3 spores/ml to 1×10^9 spores/ml.
5. The plant growth-promoting composition according to claim 4, wherein the fungal spore concentration is about 1×10^6 spores/ml.
20
6. The plant growth-promoting composition according to any one of claims 1 to 5, wherein the spores are conidia.
7. The plant growth-promoting composition according to any one of claims 1 to 6,
25 wherein the fungal species is an endophyte.
8. The plant growth-promoting composition according to any one of claims 1 to 7, further comprising at least one phenylpropanoid selected from the group consisting of trans-cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid or other isomers thereof.
30
9. The plant growth-promoting composition according to claim 8, wherein the concentration of phenylpropanoid is in the range of about 1 μ M to about 10 μ M.
10. The plant growth-promoting composition according to claim 8 or 9, wherein the
35 concentration of trans-cinnamic acid is in the range of 1 μ M to 5 μ M.

11. The plant growth-promoting composition according to any one of claims 1 to 10, wherein the fungal species has a genome comprising a nuclear ribosomal internal transcribed spacer (ITS) sequence that is at least 97% identical to the polynucleotide sequence set forth in SEQ ID NO: 1.
- 5
12. The plant growth-promoting composition according to claim 11, wherein the fungal species genome further comprises polynucleotide sequences that have at least 97% identity to one or more of the polynucleotide sequences set forth in the group comprising SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and
- 10 SEQ ID NO: 8.
13. The plant growth-promoting composition according to any one of claims 1 to 12, wherein the plant is selected from the group comprising Brassica species, Arabidopsis species, Asteraceae species and Poaceae species.
- 15
14. The plant growth-promoting composition according to claim 13, wherein the Brassica species is selected from the group *Brassica rapa* and *Brassica oleracea*.
15. A method of increasing growth of a plant, said method comprising applying an
- 20 efficacious amount of a growth-promoting composition according to any one of claims 1 to 14 to the plant to increase the plant growth compared to an untreated plant.
16. The method according to claim 15, wherein the growth-promoting composition is applied to a propagative part of the plant.
- 25
17. The method according to any one of claims 15 to 16, wherein the propagative part is a seed or a root.
18. The method according to any one of claims 15 to 17, wherein the plant is selected
- 30 from the group comprising Brassica species, Arabidopsis species, Asteraceae species and Poaceae species.
19. A plant growth-promoting composition comprising trans-cinnamic acid.
- 35
20. The plant growth-promoting composition according to claim 19, wherein the concentration of trans-cinnamic acid is in the range of about 1 μ M to about 10 μ M.

21. The plant growth-promoting composition according to claim 19 or 20, wherein the concentration of trans-cinnamic acid is in the range of 1 to 5 μ M.
- 5 22. Use of the plant growth-promoting composition according to any one of claims 19 to 21 to increase the growth of Choy Sum, kailan, barley or lettuce plants, compared to untreated Choy Sum, kailan, barley or lettuce plants.
- 10 23. An isolated fungal species of a *Tinctoporellus* strain, designated AR8, which has been deposited at the Agricultural Research Service Culture Collection (NRRL) under Deposit number 68276 on 17 April 2023.