

# Enhanced growth of ginger plants by an eco-friendly nitrogen-fixing *Pseudomonas protegens* inoculant in glasshouse fields

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## Abstract

**BACKGROUND:** Excessive nitrogen (N) fertilization in glasshouse fields greatly increases N loss and fossil-fuel energy consumption resulting in serious environmental risks. Microbial inoculants are strongly emerging as potential alternatives to agrochemicals and offer an eco-friendly fertilization strategy to reduce our dependence on synthetic chemical fertilizers. Effects of a N-fixing strain *Pseudomonas protegens* CHA0- $\Delta retS-nif$  on ginger plant growth, yield, and nutrient uptake, and on earthworm biomass and the microbial community were investigated in glasshouse fields in Shandong Province, northern China.

**RESULTS:** Application of CHA0- $\Delta retS-nif$  could promote ginger plant development, and significantly increased rhizome yields, by 12.93% and 7.09%, respectively, when compared to uninoculated plants and plants treated with the wild-type bacterial strain. Inoculation of CHA0- $\Delta retS-nif$  had little impact on plant phosphorus (P) acquisition, whereas it was associated with enhanced N and potassium (K) acquisition by ginger plants. Moreover, inoculation of CHA0- $\Delta retS-nif$  had positive effects on the bacteria population size and the number of earthworms in the rhizosphere. Similar enhanced performances were also found in CHA0- $\Delta retS-nif$ -inoculated ginger plants even when the N-fertilizer application rate was reduced by 15%. A chemical N input of 573.8 kg ha<sup>-1</sup> with a ginger rhizome yield of 1.31 × 10<sup>5</sup> kg ha<sup>-1</sup> was feasible.

**CONCLUSIONS:** The combined application of CHA0- $\Delta retS-nif$  and a reduced level of N-fertilizers can be employed in glasshouse ginger production for the purpose of achieving high yields while at the same time reducing the inorganic-N pollution from traditional farming practices.

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Supporting information may be found in the online version of this article.

**Keywords:** heavy nitrogen fertilization; biological nitrogen fixation; biofertilizer; ginger; glasshouse vegetable fields

## INTRODUCTION

China is now home to 1.411 billion people according to data issued by the National Bureau of Statistics.<sup>1,2</sup> As the most populous nation in the world, excessive input of fossil fuel-based fertilizers in soils, especially in glasshouse fields, remains the most convenient fertilization strategy to prevent future food scarcity.<sup>3</sup> However, excessive nitrogen (N) input from synthetic chemical fertilizers has exceeded crop demand, and only around 30–50% of the applied N-fertilizer can be absorbed by crops.<sup>4,5</sup> The total land area for glasshouse vegetable cultivation covered 6.3 million ha in 2019 in China, and more than 1000 kg N ha<sup>-1</sup> yr<sup>-1</sup> from chemical fertilizers was applied to Chinese vegetable production; however, the N use efficiency was only about 19.7%.<sup>6,7</sup> Long-term

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application of chemical N-fertilizers with low use efficiency of N is neither cost-effective nor eco-friendly.<sup>8</sup>

Inorganic-N losses from agricultural fields pose an unprecedented threat to the soil environment, aquatic ecosystems, and the atmosphere globally.<sup>9–12</sup> More than half of the N is lost through nitrate leaching, surface runoff and gas emissions [e.g. ammonia (NH<sub>3</sub>) and nitrous oxide (N<sub>2</sub>O)] in major Chinese croplands contributing to climate change, water pollution, and soil acidification.<sup>3,7,13,14</sup> Substantial environmental damages, in turn, lead to adverse effects on humans, as well as potential ecological risks to sustainable food production. Therefore, there is an urgent need to reform traditional farming practices and exploit eco-friendly strategies for reducing external fertilizer consumption.

Microbial inoculation offers an alternative approach to reducing our dependence on agrochemicals in agricultural practice. The application of plant growth-promoting rhizobacteria (PGPR) has gained increasing attention for its potential in establishing sustainable agricultural systems and for reducing the need for agrochemicals.<sup>15–17</sup> Widespread application of PGPR has now become feasible, and some organisms have been used as biofertilizers, which are less harmful to the environment and less expensive than chemical fertilizers.<sup>18</sup>

Several recent studies exploited genetically engineered *Pseudomonas* as inoculants to enhance plant productivity; for example, N-fixing *P. protegens* pf-5 mutants were employed for plant growth promotion in some pot experiments.<sup>19–21</sup> *Pseudomonas protegens* CHA0 is a well-characterized biocontrol agent capable of colonizing the rhizosphere, promoting plant growth, and controlling plant pathogens.<sup>22,23</sup> Complete genomic data of CHA0 revealed that it has a genome of 6.87 Mbp but no N-fixation genes, and furthermore, no nitrogenase activity was detected in this strain.<sup>24,25</sup> In a previous study, we successfully transferred a 49 kb *nif* gene island from *P. stutzeri* DSM 4166 to a *retS* deletion mutant of CHA0, resulting in a genetically engineered N-fixing strain named *P. protegens* CHA0- $\Delta$ *retS-nif*.<sup>26</sup> The *retS* deletion mutant was used as the basis for this N-fixing strain because the *retS* deletion can strongly enhance the expression of antimicrobial compounds in CHA0, thus providing additional useful properties.<sup>26</sup> CHA0- $\Delta$ *retS-nif* exhibited enhanced biocontrol activity and high levels of nitrogenase activity.<sup>26</sup>

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is one of the most widely used culinary spices worldwide and is a highly important commercial crop in China.<sup>27</sup> The annual ginger rhizome yield in China was estimated to be 540 million kilograms in 2017; however, soft rot disease causes ginger yield losses of up to 50 to 60%, and succession monocropping also resulted in significant yield reductions.<sup>28,29</sup> To improve economic benefits, excessive amounts of N-fertilizers have been applied by farmers to agricultural fields, especially in glasshouse fields (over two times higher than those in open fields).<sup>30</sup> Hence, sustainable farming practices need to be further explored for the production of ginger and other crops.

The N-fixing bacterium *P. protegens* CHA0- $\Delta$ *retS-nif* has strong potential for eco-friendly and high-yield glasshouse vegetable production. This study firstly aimed to assess the effects of CHA0- $\Delta$ *retS-nif* inoculation on the growth, yield, and nutrient levels of ginger, as well as on earthworm biomass and the microbial community. We also evaluated whether CHA0- $\Delta$ *retS-nif* inoculation was effective at supporting plant N requirements in glasshouse fields with reduced N-fertilizer inputs. Understanding the benefits of applying N-fixing rhizobacteria for glasshouse vegetable cultivation in China is still lacking, and our study

should contribute to the further development of *Pseudomonas* inoculation as an improved method for glasshouse farming practices in China.

## MATERIALS AND METHODS

### Experimental area and plant species

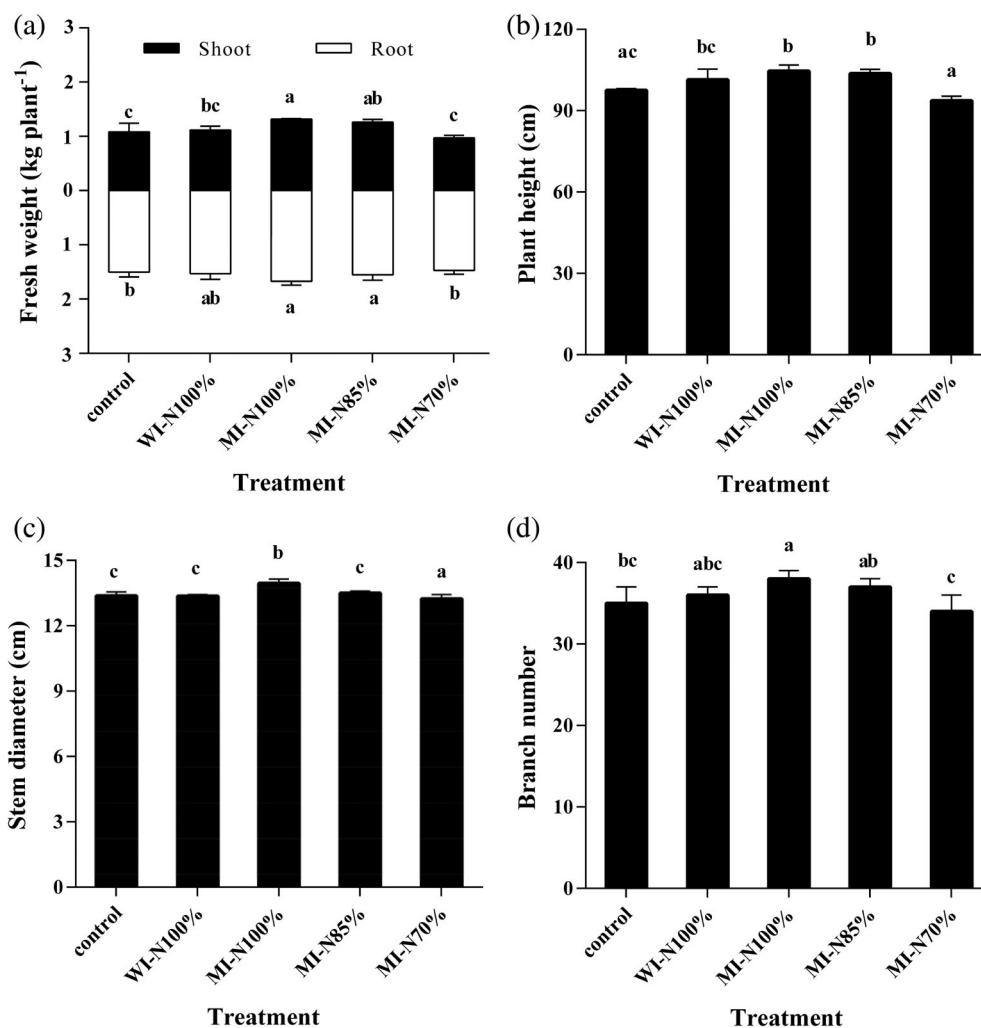
Plastic glasshouse trials were conducted from April 2, 2018 to October 15, 2019 in Beimeng County, Changyi City, Shandong Province, China [36°64' N, 119°54' E, 40 m above sea level (a.s.l)] (Supporting Information Fig. S1). The experimental location is characterized as a seasonal temperate semi-humid monsoon climate, and the mean annual temperature and precipitation are 12 °C and 650 mm, respectively. Precipitation mostly occurs from June to September. Chemical properties of the soil, primarily comprised of cinnamon soil, were measured prior to the initiation of the study, and are presented in Supporting Information Table S1. Ginger seeds utilized for experiments were Shandong mianjiang, a cultivar of ginger, reserved by the local farmers. Ginger has been grown continuously for many years in the experimental area.

### Bacterial strains and experimental setup

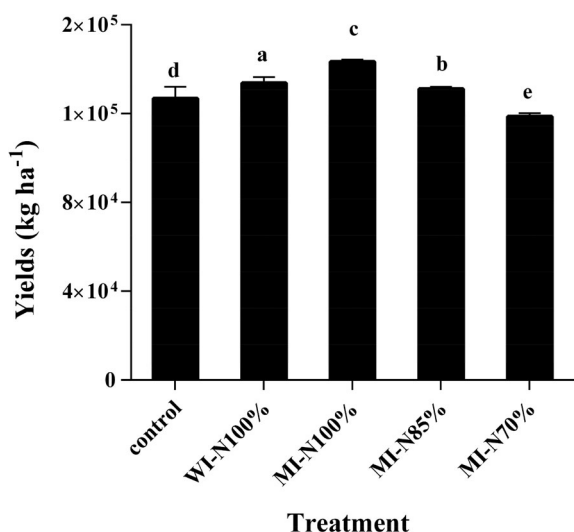
Bacterial strains used in this study were the wild-type *P. protegens* CHA0 strain and the genetically engineered N-fixing strain CHA0- $\Delta$ *retS-nif*.<sup>26</sup> Inoculant products were mass-produced in KB medium (pH 7.0; K<sub>2</sub>HPO<sub>4</sub>, 1.5 g L<sup>-1</sup>; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.5 g L<sup>-1</sup>; peptone, 20 g L<sup>-1</sup>; glycerin, 10 mL L<sup>-1</sup>) to obtain adequate quantities at 30 °C.<sup>26</sup> Groups were designed as follows: control, full dose of fertilizers without inoculation; WI-N100%, full dose of fertilizers with CHA0 inoculation; MI-N100%, full dose of fertilizers with CHA0- $\Delta$ *retS-nif* inoculation; MI-N85%, CHA0- $\Delta$ *retS-nif* inoculation but with the N fertilizer input reduced by 15%; and MI-N70%, CHA0- $\Delta$ *retS-nif* inoculation but with the N fertilizer input reduced by 30%. All groups were managed by the local farmers, and full doses of basal fertilizers were used based on the traditional tillage (Table S2), including 675 kg N ha<sup>-1</sup>, 375 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>, and 1186.5 kg K<sub>2</sub>O ha<sup>-1</sup>. All groups included full fertilization with phosphorus (P) and potassium (K). Pre-germinated ginger seeds were planted on April 2 at a depth of 25 cm with a spacing of 60 cm between plants. Liquid inoculants (5 × 10<sup>10</sup> CFU mL<sup>-1</sup> of viable bacteria) were applied on May 15, June 4, June 24 and July 24 with a volume of 60 L ha<sup>-1</sup> at every time point. Each block had an area of 45 m<sup>2</sup> (7.5 m × 6 m) with ten rows, and each group was set up in a completely randomized block with three replicates. Pesticides were applied based on conventional practices, and no pesticides were applied in the inoculant-treated groups. Irrigation and herbicide applications were conducted as need.

### Sampling and measurements

Samples aboveground or underground were collected by cutting plants at the soil surface, and roots were cleaned in sterile, distilled water to remove loosely adherent soil. Plant biomass (in g plant<sup>-1</sup>), plant height (in centimeters), stem diameter (in centimeters), and branch number were obtained on July 13, August 12, September 11, and October 15 from ten randomly collected plants per block. Total yields of ginger rhizome (in kg ha<sup>-1</sup>) were weighed after harvesting on October 15; and N, P, and K contents (g kg<sup>-1</sup> dry weight) of the plants were calculated based on the Kjeldahl method, molybdenum blue method, and atomic absorption spectrophotometry method, respectively.<sup>31,32</sup> Total protein content (in g kg<sup>-1</sup> dry weight) in the ginger rhizome was assayed based on the semi-micro Kjeldahl



**Figure 1.** Effects of different treatments on shoot and root weight (a), plant height (b), stem diameter (c) and branch number (d). Values are means with standard error bars ( $n = 3$ ). Means sharing the same letter are not significantly different between treatments ( $P < 0.05$ ).



**Figure 2.** Effects of different treatments on ginger rhizome yields. Values are means with standard error bars ( $n = 3$ ). Means sharing the same letter are not significantly different between treatments ( $P < 0.05$ ).

method.<sup>33</sup> Soluble sugar content and starch content (in g kg<sup>-1</sup> dry weight) in the ginger rhizome were assayed based on protocols GB 5009.9-2016 and NY/T 1278-2007, respectively, from the China National Standardization Management Committee. Polyphenol content and flavonoid content (in g kg<sup>-1</sup> dry weight) in the rhizome were assayed by measuring the absorbance using a spectrophotometer at 735 nm and 415 nm, respectively, according to a previous report.<sup>34</sup>

Earthworm surveys were conducted using cubes of soil (length, 1.0 m; width, 2.0 m; and height, 0.3 m) and excavated manually after the ginger rhizome harvest. All of the earthworms were counted, washed, blotted dry, and then their mass was determined.<sup>35</sup> Culturable bacteria, actinomycetes, and fungal strains, at depths of 0 to 20 cm and 20 to 40 cm, were determined by the dilution agar plating method on July 13.<sup>36-38</sup> Percent increases in ginger rhizome yield (%) associated with inoculation and different N-fertilizer levels were assayed and compared to the uninoculated control. Prices in the current study are expressed in US dollars based on the exchange rate of 6.534 Chinese Yuan per dollar. The local market price of ginger rhizome was \$0.62 kg<sup>-1</sup> in 2019. The price of pure N was an average of \$0.55 kg<sup>-1</sup>, in the last

**Table 1.** Effects of different treatments on economic income

Treatment	Nitrogen application (kg ha <sup>-1</sup> )	Plant nitrogen content (kg ha <sup>-1</sup> )	Yield (×10 <sup>5</sup> kg ha <sup>-1</sup> )	Increase in yield (%)	Nitrogen production efficiency (kg kg <sup>-1</sup> )	Economic income (\$ ha <sup>-1</sup> )
Control	675.0	137.51 ± 8.54 b	1.27 ± 0.05 d	—	188.04	78 696.6 d
WI-N100%	675.0	145.14 ± 3.91 b	1.34 ± 0.02 a	5.45	198.30	82 987.0 a
MI-N100%	675.0	177.46 ± 10.52 a	1.43 ± 0.01 c	12.93	212.36	88 870.8 c
MI-N85%	573.8	162.50 ± 12.22 a	1.31 ± 0.01 b	3.33	228.58	81 319.2 b
MI-N70%	472.5	141.02 ± 3.60 b	1.19 ± 0.02 e	-6.48	251.22	73 594.0 e

Values presented are mean ± standard error (*n* = 3). Means sharing the same letter are not significantly different between treatments (*P* < 0.05). Plant nitrogen (N) content refers to the whole plant N acquisition measured on October 15.

**Table 2.** Effects of different treatments on plant acquisition of nitrogen (N), phosphorus (P) and potassium (K)

Treatment	Control (kg ha <sup>-1</sup> )	WI-N100% (kg ha <sup>-1</sup> )	MI-N100% (kg ha <sup>-1</sup> )	MI-N85% (kg ha <sup>-1</sup> )	MI-N70% (kg ha <sup>-1</sup> )
<i>Nitrogen</i>					
July 13	22.08 ± 0.44bC	17.74 ± 0.99cC	21.65 ± 2.07bC	25.79 ± 1.45aC	13.21 ± 2.32dC
August 12	49.66 ± 2.27dB	64.10 ± 3.26bB	69.08 ± 1.74aB	66.34 ± 0.33abB	59.62 ± 1.54cB
September 11	130.43 ± 16.36bA	132.02 ± 5.69bA	169.37 ± 12.01aA	157.18 ± 14.84aA	114.64 ± 9.40bA
October 15	137.51 ± 8.54bA	145.14 ± 3.91bD	177.46 ± 10.52aA	162.50 ± 12.22aA	141.02 ± 3.60bD
<i>Phosphorus</i>					
July 13	4.20 ± 1.11aC	15.22 ± 1.34bC	5.42 ± 1.25aC	4.25 ± 0.36aC	8.98 ± 1.63cC
August 12	13.98 ± 0.51bB	18.17 ± 2.75aAC	18.92 ± 1.05aB	17.42 ± 0.44aB	16.89 ± 0.93aB
September 11	23.60 ± 1.77aA	28.93 ± 0.41bA	25.39 ± 2.33aA	27.22 ± 1.08aA	27.98 ± 1.34bA
October 15	34.20 ± 1.87aD	26.87 ± 1.93bB	35.36 ± 1.57aD	35.00 ± 1.69aD	26.16 ± 1.81bD
<i>Potassium</i>					
July 13	68.96 ± 6.52dC	196.31 ± 13.06bcC	236.40 ± 8.98abC	275.16 ± 33.16aC	157.88 ± 36.05cC
August 12	240.96 ± 11.53cB	424.25 ± 22.64abB	467.06 ± 32.64abB	476.03 ± 35.61aB	415.11 ± 42.93bB
September 11	505.54 ± 44.37dA	608.91 ± 10.41cA	759.04 ± 61.02aA	723.20 ± 75.91abA	637.98 ± 24.90bcA
October 15	837.37 ± 51.98dD	900.74 ± 45.83cdD	1031.52 ± 27.75abD	1072.19 ± 31.59aD	948.80 ± 71.80bcD

All values presented are mean ± standard error (*n* = 3). Means sharing the same lowercase letter are not significantly different between treatments (*P* < 0.05). The capital letters indicate significant differences for time effects (*P* < 0.05).

few years in China, according to the National Bureau of Statistics of China. Nitrogen production efficiency (in kg kg<sup>-1</sup>) was estimated as the ginger yield (in kg ha<sup>-1</sup>) divided by the N input (in kg ha<sup>-1</sup>) and then multiplied by 1000.

### Data analysis

GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA) was used to generate the figures. Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA) was used for data processing. Statistical analyses of the data were carried out using SPSS 25 software (SPSS Inc., Chicago, IL, USA). The differences between mean values (*n* = 3) were assessed by one-way analysis of variance (ANOVA) according to Duncan's test at a *P* < 0.05 level of significance.

## RESULTS

### CHA0-ΔretS-nif inoculation enhanced ginger plant growth

Data collected on the harvest date (October 15) are shown in Fig. 1. Results showed that plants that were both inoculated with CHA0-ΔretS-nif and fertilized with full inorganic-N (group MI-N100%) achieved the highest fresh weight of whole plants compared to all other groups (Fig. 1(a)). The fresh weight of shoots of plants in group MI-N100% was significantly increased by

21.79% and 17.98% compared with plants in the control and WI-N100% groups, respectively, and root fresh weight increased by 11.33% and 8.93%. Moreover, the fresh weight of whole plants decreased with decreased levels of N-fertilizer. Shoot and root fresh weights in group MI-N85% reduced by 4.25% and 7.06%, respectively, when compared to group MI-N100%. However, a higher fresh weight of whole plants was observed in group MI-N85% compared to group WI-N100%, with an increase of 12.97% in shoot weight and 1.24% in root weight. Among all groups, the lowest fresh weight of whole plants was observed in group MI-N70%. With regard to plant height, stem diameter and branch number, group MI-N100% was superior to all other groups, and group MI-N70% had lower growth than groups received with abundant N (Fig. 1(b-d)). Variations in plant growth parameters in samples collected on July 13, August 12, September 11, and October 15 are shown in Fig. S2.

### CHA0-ΔretS-nif inoculation enhanced ginger yield

Total yields of fresh ginger rhizomes for each of the groups were ranked as follows: group MI-N100% > WI-N100% > MI-N85% > control > MI-N70%, as shown in Fig. 2. The yield for group MI-N100% was significantly increased, by 12.93% and 7.09%,

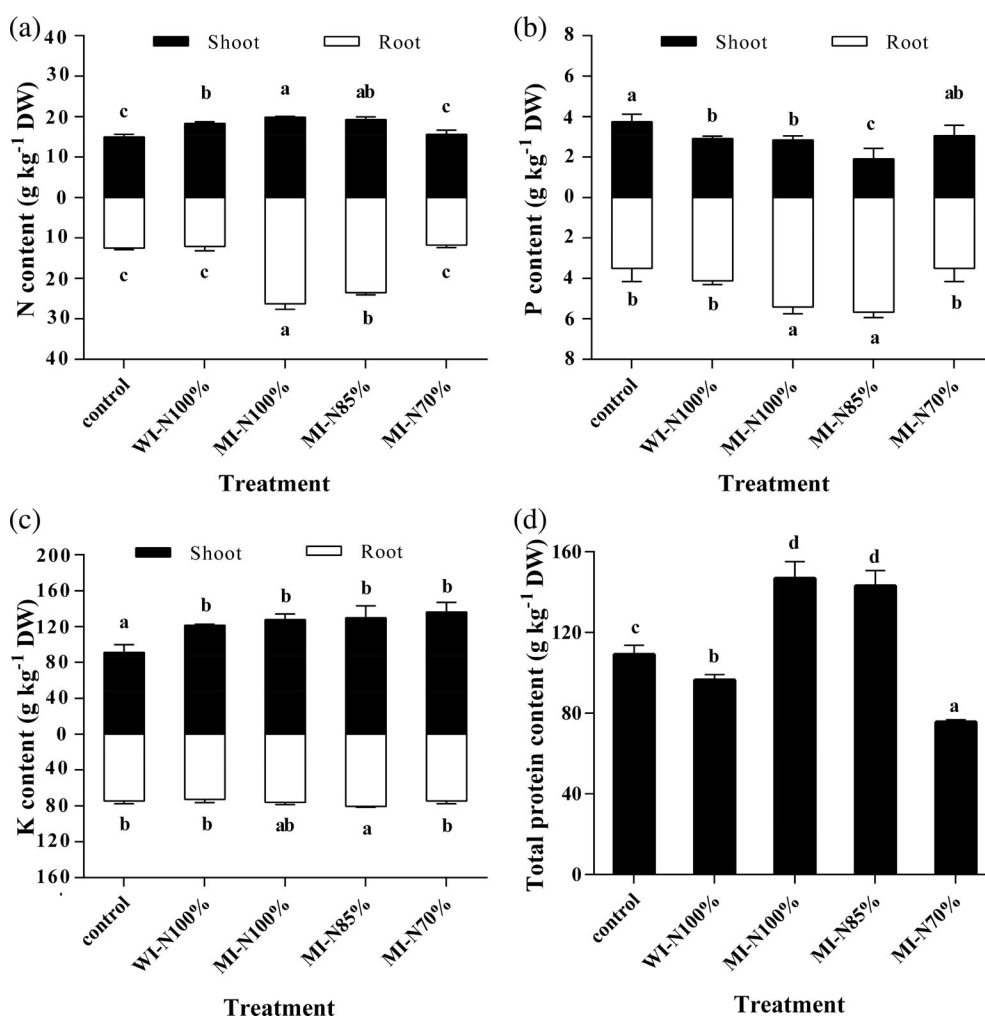
respectively, when compared to yields for the control and WI-N100% groups. Group MI-N85% also had increased yield, by 3.33%, in comparison to the control. The yield for group MI-N85% was similar to that of group WI-N100%, although the input of N-fertilizer in the MI-N85% group was reduced by 15%. However, the yield for group MI-N70% was decreased by 6.48% when compared to the control. The total economic income for group MI-N100% reached \$88 870.8 ha<sup>-1</sup>, more than 10 000 dollars above the income from the control (\$78 696.6 ha<sup>-1</sup>) (Table 1). Groups WI-N100% and MI-N85% gave similar increments of 5.45% and 3.33% of total income in comparison to the control. The lowest total economic benefit were obtained in group MI-N70%, although this group had the highest N production efficiency due to a reduction of the chemical N supply by 30%, followed by the MI-N85% and MI-N100% groups.

### Contribution of *CHA0-ΔretS-nif* inoculation to ginger plant nutrition

Plants inoculated with *CHA0-ΔretS-nif* had a higher N acquisition when compared to the uninoculated control and the wild-type *CHA0*-treated plants (Table 2). Plant N accumulation in group MI-N100% was significantly increased by 22.04%, 7.77%, 28.29%, and 22.27% for July 13, August 12, September 11, and October

15, respectively, when compared to group WI-N100%. Group MI-N85% also had a markedly higher level of N accumulation, with increases of 45.38%, 3.49%, 19.06% and 11.96% when compared to group WI-N100% for July 13, August 12, September 11, and October 15, respectively. Groups WI-N100%, MI-N100% and MI-N85% showed significant increases in plant N accumulation in the August 12, September 11 and October 15 samples, compared to those of the control. However, a higher level of N accumulation was not observed in group MI-N70% compared to the other groups.

No significant differences in plant P accumulation were detected among the groups over time (Table 2). Plant K accumulation in group MI-N100% increased compared to levels in the control and group WI-N100% (Table 2). Plant K accumulation in group MI-N100% increased by 20.42%, 10.09%, 24.66% and 14.52% for July 13, August 12, September 11 and October 15, respectively, when compared to group WI-N100%. In October, the highest level of K accumulation was observed in group MI-N85%, with increases of 28.04%, 19.03%, and 3.94%, respectively, compared to the control group, WI-N100%, and MI-N100%. Interestingly, plants in group MI-N70% had a higher K accumulation in the October samples compared with those of the control group and group WI-N100%.



**Figure 3.** Effects of different treatments on plant contents of N (a), P (b) and K (c), and total protein (d) in the ginger rhizome. Values are means with standard error bars ( $n = 3$ ). Means sharing the same letter are not significantly different between treatments ( $P < 0.05$ ). DW, dry weight.

CHA0- $\Delta retS-nif$ -inoculated plants in group MI-N100% had significantly higher N content in both shoots and roots when compared to the control and group WI-N100% (Fig. 3(a)). On October 15, the N content of plants in group MI-N100% was increased by 8.62% and 116.17% in shoots and roots, respectively, compared to levels in group WI-N100%. Similar increments in plant N content were observed in group MI-N85%, especially in plant roots, with a significant increase of 93.65% compared to the level in group WI-N100%. However, these beneficial increments were not observed in group MI-N70%. Significant increments in P content were only observed in plant root (Fig. 3(b)). In the October 15 samples, groups MI-N100% and MI-N85% had increases of 31.75% and 38.10%, respectively, in plant root P content, when compared to group WI-N100%. CHA0- $\Delta retS-nif$  inoculation had no apparent effect on shoot P content.

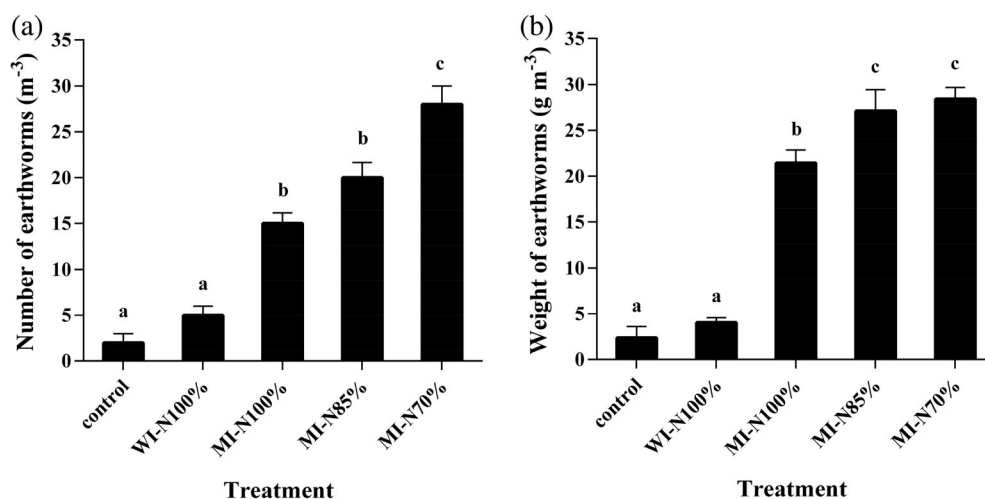
In contrast to the results for N and P contents, group MI-N70% gave the highest K content in shoots on October 15, with an increase of 49.49% and 12.23% compared to the control and WI-N100% groups, respectively, followed by group MI-N85%, which had increases of 42.53% and 7.00% over those two groups (Fig. 3(c)). Group MI-N85% had the highest K content in root, with levels 7.91% and 10.51% higher than those in the control and group WI-N100%, respectively, followed by group MI-N100%, which had increases of 1.81% and 4.26% over those two groups.

The total protein contents in ginger rhizomes in groups MI-N100% and MI-N85% were significantly increased, by 52.38% and 48.55%, respectively, when compared to levels in group WI-N100% (Fig. 3(d)). However, similar positive effects were not noted in group MI-N70%. The impacts of inoculation with CHA0- $\Delta retS-nif$  on the contents of polyphenol, flavonoid, starch,

**Table 3.** Effects of different treatments on microbial populations in ginger rhizosphere

Treatment	Microbial biomass ( $\times 10^6$ CFU g $^{-1}$ )	Bacteria ( $\times 10^6$ CFU g $^{-1}$ )	Actinomycetes ( $\times 10^5$ CFU g $^{-1}$ )	Fungi ( $\times 10^4$ CFU g $^{-1}$ )	Percentage (%)			Bacteria/ Fungi
					Bacteria	Actinomycetes	Fungi	
<i>0–20 cm</i>								
Control	2.23 $\pm$ 0.05d	2.03 $\pm$ 0.05d	1.83 $\pm$ 0.13b	1.60 $\pm$ 0.20a	91.07	8.21	0.72	126.49
WI-N100%	2.13 $\pm$ 0.05d	1.83 $\pm$ 0.06d	2.83 $\pm$ 0.22a	1.60 $\pm$ 0.13a	85.96	13.29	0.75	114.61
MI-N100%	2.57 $\pm$ 0.21c	2.43 $\pm$ 0.21c	1.30 $\pm$ 0.07c	0.53 $\pm$ 0.06b	94.73	5.06	0.21	451.10
MI-N85%	3.67 $\pm$ 0.28a	3.57 $\pm$ 0.29a	0.93 $\pm$ 0.07d	0.63 $\pm$ 0.11c	97.28	2.55	0.17	572.24
MI-N70%	3.09 $\pm$ 0.26b	3.03 $\pm$ 0.17b	0.50 $\pm$ 0.05e	1.10 $\pm$ 0.14c	98.03	1.62	0.36	272.31
<i>20–40 cm</i>								
Control	0.53 $\pm$ 0.01d	0.43 $\pm$ 0.02d	0.80 $\pm$ 0.16c	1.47 $\pm$ 0.18b	82.07	15.15	2.78	29.52
WI-N100%	0.78 $\pm$ 0.07c	0.63 $\pm$ 0.07c	1.37 $\pm$ 0.07a	1.13 $\pm$ 0.08c	80.77	17.56	1.45	55.70
MI-N100%	1.89 $\pm$ 0.20a	1.77 $\pm$ 0.21a	1.17 $\pm$ 0.08b	0.53 $\pm$ 0.04d	93.54	6.18	0.28	334.07
MI-N85%	1.06 $\pm$ 0.13b	1.03 $\pm$ 0.13b	0.23 $\pm$ 0.06d	0.60 $\pm$ 0.08d	97.24	2.20	0.56	173.64
MI-N70%	1.10 $\pm$ 0.02b	0.97 $\pm$ 0.09b	1.10 $\pm$ 0.03b	2.03 $\pm$ 0.07a	88.12	10.03	1.85	47.63

Values presented are mean  $\pm$  standard error ( $n = 3$ ). Means sharing the same lowercase letter are not significantly different between treatments ( $P < 0.05$ ).



**Figure 4.** Effects of different treatments on earthworm number (a) and weight (b) per m $^3$ . Values are means with standard error bars ( $n = 3$ ). Means sharing the same letter are not significantly different between treatments ( $P < 0.05$ ).

and soluble sugars in rhizomes are shown in Fig. S3, with no significant differences observed among the different groups.

### CHA0- $\Delta retS-nif$ inoculation altered soil microbial diversity in rhizosphere

As shown in Table 3, the total microbial numbers at a depth of 0 to 20 cm were higher in all the CHA0- $\Delta retS-nif$ -treated groups when compared to the uninoculated control and the wild-type-strain-treated group. The maximum bacterial population size observed in group MI-N85% was significantly increased by 75.86% and 95.08% in comparison to those of the control and group WI-N100%, respectively, and the ratio of bacteria to fungi was increased by more than four times when compared to levels with the other two groups. The total numbers of microbes and bacteria were also increased in group MI-N70% when compared to levels in the control, WI-N100%, and MI-N100% groups. The proportions of actinomycetes and fungi with the CHA0- $\Delta retS-nif$ -treated groups exhibited decreases. The total numbers of microbes and bacteria were decreased at a depth of 20 to 40 cm in the ginger rhizosphere in all groups, compared with those found at a depth of 0 to 20 cm. Plants inoculated with the wild-type strain CHA0 gave the lowest bacterial population in soils at a depth of 0 to 20 cm when compared with all groups; however, the bacterial population size for group WI-N100% at a depth of 20 to 40 cm was a little higher than that of the control. Group MI-N100% had the maximum bacterial population size at a depth of 20 to 40 cm, followed by groups MI-N85% and MI-N70%, with increases of 180.95%, 63.49%, and 53.96%, respectively, when compared with group WI-N100%.

An increase in the population density of earthworms was found in plots inoculated with the wild-type strain CHA0, when compared to the control, but the increment was markedly lower than that of the groups with CHA0- $\Delta retS-nif$  inoculation, in which the number of earthworms ranged from 15 to 28 per m<sup>3</sup> (Fig. 4). The greatest earthworm biomass was noted in group MI-N70%, which had N-fertilizer input reduced by 30%.

## DISCUSSION

It has been previously reported that *Pseudomonas* inoculation resulted in significant increases in plant growth parameters, including root length, stem diameter, leaf area, and consequently, the total yield of the plants.<sup>39,40</sup> Several recent studies exploited genetically modified *Pseudomonas* as inoculants to enhance plant biomass production for example, N-fixing *P. protegens* pf-5 mutants were applied for plant growth promotion in pot experiments.<sup>19-21</sup>

Results in this study showed that N-fixing CHA0- $\Delta retS-nif$  inoculation could significantly enhance the growth and production of ginger plants when compared to the uninoculated control and the wild-type strain-treated plants, which suggested that inoculating with CHA0- $\Delta retS-nif$  was more effective than inoculating with wild-type CHA0 for plant growth promotion. Moreover, these enhancements were maintained under low N-fertilizer input conditions (approximately 85% of full N supply), indicating that the capacity of the mutant strain to fix biological N can partially compensate for the need for N-fertilizers. This finding is in agreement with earlier investigations showing that reasonable use of captured N<sub>2</sub>-fixation ability can largely improve N uptake in maize, wheat, and tomato plants.<sup>19,41</sup>

The novelty of our study is that proposed an improved N-fertilizer management strategy that can be applied to enhancing

glasshouse vegetable production in northern China. Most previous studies applied microbial inoculants to benefit major cereal crop yields and to reduce N fertilization.<sup>15,34,40,42</sup> However, limited information has been available on the impact of inoculation on the glasshouse vegetable yields. Fast-growing vegetables produced in glasshouses demand larger amounts of fertilizers and pesticides compared to cereal production.<sup>3</sup> Increased risk of N loss in vegetable fields is one of the major barriers to environmental protection and synthetic chemical N inputs can be reduced in many agricultural systems.<sup>43,44</sup> Results in this study demonstrated that a smaller reduction of N-fertilization levels (by approximately 15%) might be more suitable for reducing the use of chemical fertilizer without compromising ginger yield. When the amount of chemical N-fertilizers was reduced by 30%, ginger plant growth promotion was considerably suppressed despite inoculation with CHA0- $\Delta retS-nif$ , in contrast to the findings of Ju *et al.* in which a 40% reduction in chemical N application rate had no significant effect on glasshouse vegetable production.<sup>6</sup>

Nitrogen availability positively correlates with ginger plant development. In contrast to the significant increase in N content within CHA0- $\Delta retS-nif$ -treated ginger plants in full N supply, little effect was found on plants treated with the wild-type strain, indicating that the significant increase in N uptake associated with the mutant was primarily due to the exogenous N fixed by this strain. Similar beneficial effects from inoculation on plant N uptake and food production have also been found in other commercial crops.<sup>14,15</sup> Enormous amounts of atmospheric N becomes available to plants annually through the process of biological N fixation. Biologically fixed N has been confirmed as the most suitable nitrogenous source for resolving the conflict between over-production and environmental pollution.<sup>45</sup> Nitrogen-fixing CHA0- $\Delta retS-nif$  has high potential for use as an inoculant to increase plant N uptake even under low N-fertilizer input conditions (Fig. 3(a)). The combined application of CHA0- $\Delta retS-nif$  and suitable levels of N-fertilizer could be a desirable and promising strategy to reduce over-fertilization in vegetable systems, even though glasshouse vegetable planting generally demands more chemical fertilizers than do agricultural practices in open fields.<sup>46</sup> However, a higher level of N acquisition was not observed in group MI-N70% compared to the other groups, indicating that CHA0- $\Delta retS-nif$  inoculation cannot completely replace chemical fertilizer in terms of promoting N acquisition. Moreover, inoculation with CHA0- $\Delta retS-nif$  enhanced the uptake and efficiency of K-fertilizers. The enhanced K levels in CHA0- $\Delta retS-nif$ -treated plants compared to those of the uninoculated control can be attributed to multiple factors, such as the K solubilizing ability and the higher root uptake surface stimulated by inoculants.<sup>47</sup>

Application of reasonable amounts of synthetic chemical N-fertilizer would be an effective way to achieve both yield stability and environmental protection.<sup>48</sup> Heavy N input from chemical fertilizers has exceeded crop demand, and more than half of the N is lost, particularly through gas emission and nitrate leaching, in Chinese croplands resulting in serious environmental risks.<sup>7,14</sup> Poor farming practices with high input of synthetic chemical fertilizers, in particular N-fertilizer, has led to high fossil-fuel energy consumption and resulted in a significant increase in carbon emission.<sup>49</sup> China has made a commitment to a peak of carbon emission by 2030 under the Paris Agreement,<sup>50</sup> and hence, there is an urgent need to reform traditional management practices. The results of our work showed that CHA0- $\Delta retS-nif$  inoculation can reduce the input of chemical nitrogenous fertilizers, and consequently control the ever-increasing adverse effects of synthetic

chemical N loss on ecosystems. Considering both environmental effects and yield, group MI-N85% was optimal for glasshouse ginger production with the recommended N application rate in glasshouse fields of less than 600 kg ha<sup>-1</sup> (Table 1), significantly lower than the average annual N-fertilizer inputs for some vegetable glasshouses of northern China (about 1358 kg ha<sup>-1</sup> according to Ti *et al.*<sup>7</sup>).

A healthy rhizosphere microbial diversity is necessary for maintaining sustainable agricultural practices.<sup>51</sup> *Pseudomonas protegens* and its genetically modified derivatives have the ability to colonize a wide variety of crop plants and exert substantial effects on the microbial community structure in the rhizosphere.<sup>36,52–54</sup> Our results indicated that microbial community composition in the ginger rhizosphere was immensely affected by the inoculants and that CHA0- $\Delta retS-nif$  inoculation strongly increased the density of the bacteria population over that of fungi and actinomycetes; these findings may be attributable to the capacity of CHA0- $\Delta retS-nif$  to colonize plant roots and outcompete resident soil microflora and to enhance the biocontrol activities of this strain against the total fungal spectrum in the rhizosphere. Compared to levels in the soil at a depth of 0 to 20 cm, the total amount of microbial biomass decreased as soil depth increased, suggesting that soil depth had some effects on bacterial population size, as found in a previous report.<sup>46</sup> Significantly, group MI-N85% harbored the maximum amount of microbial biomass at a depth of 0 to 20 cm, indicating that some reduction of chemical fertilizers could enhance the survival of microorganisms, which is in agreement with a similar study showing that high N fertilization could negatively impact the diversity of native rhizobacteria.<sup>55</sup>

Likewise, agronomic practices can also affect the population of earthworms, since earthworms are capable of distinguishing and avoiding polluted environments.<sup>35,56,57</sup> Therefore, the earthworm population has been used as a biological indicator to assess soil health. Observations in this study demonstrated that CHA0- $\Delta retS-nif$  inoculation combined with reduction of N-fertilizer use is much more efficient than conventional cropping methods at increasing earthworm biomass. Microbial inoculants provide an additional food source for earthworms, and earthworm movements in turn contribute to improved soil nutrients and porosity, and therefore result in longer-term persistence of the microbial products.<sup>58</sup> Overall, earthworms prefer soils with low contents of chemicals.

## CONCLUSIONS

*Pseudomonas protegens* CHA0- $\Delta retS-nif$  inoculation positively supported ginger plant growth, yields, and nutrient uptake, significantly increased the population size of rhizosphere bacteria and earthworms, and partially reduced inorganic-N application in glasshouse fields. Overall, the application of CHA0- $\Delta retS-nif$  can mitigate environmental risks caused by the massive use of N-fertilizers without compromising yield. The effects of the genetically engineered N-fixing bacterium on various non-leguminous crops and in different soil and climate conditions will be a part of future investigations, as the performance and efficacy of bacterial inoculants may be affected by plant cultivars, indigenous microbes, soil types, climate, and other environmental factors.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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