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Comparative Evaluation of Microbially-Produced Biostimulants on Peanut Growth

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Abstract: Improper fertilization has become an essential factor limiting peanut yield and quality improvement. To improve peanut yield and quality, the effects of different fertilizer additives on peanut growth and yield were investigated. In this work, the effects of four fertilizer additives produced by microorganisms (CL, T6, T4, and P1) on peanut growth and yield were evaluated through pot and field trials. The results indicated that all fertilizer additives significantly increased the branch number and biomass of peanuts compared to the control. Additionally, T6 and CL treatments led to significantly higher peanut yields in the field. The aboveground nitrogen concentration of peanuts treated with CL and T6 was also significantly higher than that of the control, while T4 treatment did not show a significant difference. Overall, CL and T6 had the best positive effect on the growth of peanuts. The potential application values of CL and T6 in peanuts showed that fertilizer additives produced by microorganisms could be used as effective measures to achieve highly efficient production in agriculture.

Keywords: fertilizer additives; yield; *Pantoea alhagi*; chito-oligosaccharide; polyglutamic acid

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1. Introduction

The use of fertilizers significantly contributes to global food production; however, the consumption of fertilizer per cropland area in the world has reached a historical peak [1]. Global food security is becoming more and more of an issue as a result of the rising cost of agricultural resources. With the reform of agricultural production and the requirement of zero growth of chemical fertilizer use in China, the development and application of novel functional fertilizers and fertilizer additives to increase the efficiency of nutrients are essential measures to ensure food security [2]. Fertilizer additives, such as biochar, nanofertilizer, and biofertilizer, play a promising role in benefiting soil structure and fertility, increasing crop yield and quality, and promoting sustainable agriculture [3,4].

Algal biofertilizers with nanoscale applications hold significant prospects in agriculture [5]. Microbial fertilizers, consisting of living microorganisms that can enhance soil fertility and plant growth, have been reported to increase plant biomass, improve nutrient availability, increase plant root development, increase the resistance to biotic and abiotic stresses, and promote the growth of beneficial soil microorganisms [6,7]. This results in increased crop yields, improved plant health, and reduced dependence on synthetic fertilizers, which can have negative environmental impacts [8]. Previous studies have shown that biofertilizers produced with rhizobia and agrobacterium significantly increased crop yields [9–11]. Recently, cell-free formulations such as fermentation broth have gained attention. Since some plant-beneficial microorganisms exhibit multiple activities, their culture extracts contain various metabolic products such as antibiotics, siderophores, toxins,

lytic enzymes, and solubilized phosphate, which positively affect plant growth [12]. The symbiosis of bacteria (and archaea) in soil with some plant groups (especially legumes) is noteworthy, as it enables nitrogen fixation [13,14]. This symbiosis is utilized in the production of biofertilizers and substances containing living bacteria (or their persisting form, such as spores) which are applied to seeds, plant surfaces, or soil to promote crop growth [15,16]. *Pantoea alhagi* is a type of bacteria that has been studied for its potential to improve crop growth and yield through various mechanisms, including drought tolerance, nitrogen fixation, disease suppression, and salinity tolerance by secreting exopolysaccharides [17–20].

Microbial fertilizers offer an effective and environmentally sustainable option for improving crop yields and soil health. However, their efficacy can depend on factors such as soil type, crop type, and environmental conditions [5,8]. Based on microbial engineering technology, the industry could produce novel and economical chemicals that hold promise for application in agriculture as supplementary for synthetic fertilizers [21]. Previous studies have shown that the biosynthesis of byproducts from tryptophan and lysine could increase the harvest index of maize [22]. Oligosaccharides made from natural polysaccharides can boost plant growth and health, acting as signal molecules regulating plant development and defense [23]. However, the fertilizer additives produced using microorganisms are more commonly used in other crops.

Peanut (*Arachis hypogaea* L.) is an important oilseed crop that is widely cultivated in tropical and subtropical regions. In 2021, the global harvest areas and production of peanuts were estimated to be 32.7 million hectares and 53.9 million tonnes [1]. Although peanut can form N-fixation nodulation, it still needs a large amount of N during the whole growth period in China. The conventional amount of N application in Hubei and Henan province during peanut cultivation always reaches 900 kg/hectare, which needs tremendous reduction. In this study, we used peanut as a model crop to assess the effect of four kinds of fertilizer additives fermented with *Pantoea alhagi* through industrialized production. By comparing the effects of different microbial additives on the growth and development of peanuts, we aim to provide a scientific basis for the application and promotion of microbial fertilizer additives.

2. Materials and Methods

2.1. Experimental Set-Up

The Field experiments were carried out in Huanggang City, Hubei Province, China (30°44′ N 114°87′ E), from 23 May to 30 September 2021, using a randomized block design with four treatments. The soil nutrient conditions were as follows: 79.33 mg/kg of alkali hydrolyzable nitrogen, 17.59 mg/kg of Olsen available phosphorus, and 157.47 mg/kg of available potassium. All the plots, including the control group, were provided 135 kg N equivalent with urea, 90.0 kg P_2O_5 equivalent with KH_2PO_4, and 97.5 kg K_2O equivalent with KH_2PO_4 and KCl per hectare. The plot size was set as 6.0 × 2.0 m^2 with three biological replicates. The tested cultivar was peanut (*Arachis hypogaea* L. ZH16) and was sown using direct sowing cultivation. The planting density was 1.0 feet between rows and 0.6 feet between plants, resulting in 300,000 plants per hectare and two peanut seeds per hole.

The pot experiment was conducted in Wuhan City, Hubei Province, China (30°58′ N, 113° 41′ E). Every pot contained 12 kg of soil with an additional 10.54 g $Ca(NO_3)_2$, 7.91 g KH_2PO_4, 2.29 g KCl, 2.97 g $MgSO_4$, 0.24 g $CuSO_4$, and 0.26 g $ZnSO_4 \bullet 7H_2O$. The original soil nutrient conditions were 66.49 mg/kg of alkali hydrolyzable nitrogen, 4.72 mg/kg of Olsen available phosphorus, and 68.60 mg/kg of available potassium.; all the nutrients were supplied in soluble form. It was sown in pots with an upper diameter of 40 cm, a lower diameter of 30 cm, and a height of 23.5 cm; it was sown on 22 May 2021 and harvested on 10 September 2021, with six plants per pot. Each pot was watered with 500 mL of water per pot every seven days.

Four fertilizer additives produced by microorganisms were tested in this work, all provided by Hubei Sanning Chemical Industry Co., Ltd. (Yichang, China) (www.hb30.com

(accessed on 8 May 2020)). They were all fermented with *Pantoea alhagi* by Nanjing Shineking Biotech Co., Ltd. (Nanjing, China) (www.bioshineking.com (accessed on 8 May 2020)). The names and related patent numbers of the additives are listed below. Details of how they were produced are not listed due to commercial security concerns, but mainly components are provided. The methods for producing these components were described according to the original patent files.

1. CL (patent number: CN108893435A) mainly contains 1–10% *Pantoea alhagi* culture (1×10^9–9×10^{10} CFU/mL), 0.1–10% polyglutamic acid, 50–70% organic matter. The *Pantoea alhagi* was grown in LB liquid medium for 40 h under 30 °C (>1×10^{10} CFU/mL). The polyglutamic acid was produced by *Bacillus Subtilis* according to patent CN202010945386 and CN107881124A. The organic matter was a mixture of fulvic acid, sugars, and yeast extracts.
2. P1 (patent number: CN111903702A) mainly contains 0.02–5% chito-oligosaccharide and 0.003–0.5% brassinolide. The *Pantoea alhagi* was grown in LB liquid medium for 12 h under 37 °C, and then the *Pantoea alhagi* culture was transferred into the chito-oligosaccharide medium (35 g/L sucrose, 5.5 g/L peptone, 1 g/L $MgSO_4$, and 2 g/L K_2PO_4; pH 6.5). After 700 rpm, 24 h growth, the fermentation broth was heated to 121 °C for 20 min in an oil bath. Then, the chito-oligosaccharide supernatant was collected through a plate and frame filter. After distillation and rotary evaporation in 3-times volume ethanol, the chito-oligosaccharide precipitation was collected by centrifugation. P1 were the mixtures of 0.02–5% chito-oligosaccharide, 0.003–0.5% brassinolide, 6% lignosulfonate, 1.5% tween-60, 0.6% benzoic acid, 0.6% organic silicone, and 0.4% urea.
3. Both T4 (CN109824430A) and T6 (CN113412247A) are mixtures of P1 and polyglutamic acid. Although the proportions are different, the components of T4 and T6 are in the same range as a result of the flexible components in P1. According to the information of related patents, T4 and T6 mainly contain 5–35 g/L polyglutamic acid, 8–15 g/L chito-oligosaccharide, and 80–120 g/L organic matter. The polyglutamic acid was produced by *Bacillus Subtilis* (patent number: CN107881124A). The chito-oligosaccharide was made according to patent CN108865951B.

All the additives were applied in soil with fertilizer before sowing both in field and pot experiments. In the field experiments, 30 mL additives were sprayed on soils after diluting to 10 L. In the pot experiments, the same concentration (0.3%) of additives was watered 50 mL per pot. In the control groups (CK) of the field and pot experiments, the additives were replaced by distilled water with the same volume. Each treatment had four replicates.

2.2. Phenotypic Investigation

The agronomic traits of peanuts were measured during the harvest stage, including the main stem height (the length from the first pair of lateral branches bearing the body to the terminal leaf node) and the total number of branches (the total number of branches over 5 cm throughout the plant, excluding the main stem). The plants were then divided into shoots, roots, and pods. All samples were killed after 30 min at 105 °C in an oven and then dried at 80 °C to a constant weight to evaluate dry matter weight.

After drying the peanut pods, 500 g of peanut pods were randomly selected to determine and calculate peanut yield and yield components, including seed kernel weight, hundred fruit weight, hundred-kernel weight, kernel yield, pod fullness, single plant yield, and yield per hectare:

$$\text{Full fruit weight rate (\%)} = (\text{full fruit weight}/\text{total fruit weight}) \times 100\%;$$

$$\text{Kernel yield (\%)} = (\text{seed kernel weight}/\text{pod weight}) \times 100\%;$$

$$\text{Full kernel weight rate (\%)} = (\text{full kernel weight}/\text{seed kernel weight}) \times 100\%;$$

$$\text{Pod fullness} = \text{kernel emergence rate} \times \text{kernel weight rate}.$$

2.3. Determination of Nitrogen Concentration of Each Tissue

The dried tissue samples were ground into powder. Samples were digested by a graphite digestion instrument (Hanon, SH220F, Chongqing, China) in 50 mL digestion tubes with 5 mL sulfuric acid at 340 °C. Then, the digested solutions were used to determine N concentration by a Kjeldahl nitrogen analyzer (Shanghai, China).

2.4. Statistical Analysis

Data were analyzed and plotted using Excel 2016 and Graph Pad Prism 8. One-way analysis of variance (ANOVA) was performed using SPSS 26 for the significance of differences between data, where the differences were significant ($p < 0.05$) and highly significant ($p < 0.01$).

3. Result

3.1. Effect of Different Microbial Additives on the Phenotype of Peanut Plants

Compared with the CK, the number of peanut meristems treated with fertilizer additives CL, T6, T4, and P1 significantly increased, with the effect of CL and T4 being the most significant. CL had no significant effect on peanut plant height compared to the CK. In contrast, peanut plant height in T4 treatments was significantly lower than the control. As shown in Figure 1, the number of peanut branches treated with fertilizer additives T4 and CL in the field trials was significantly increased (Figure 1a). In addition, CL and T4 had a significant effect on the number of peanut branches. On the other hand, P1, T6, and CL treatments had no significant effect on peanut plant height in the field trials (Figure 1b), while peanut plant height in the T4 treatment was significantly lower than that in the CK. In the pot trials, peanut plant height was higher than the control in all treatments, but there was no significant difference (Figure 1c).

3.2. Effect of Different Fertilizer Additives on Plant Biomass and Yield of Peanut

Differences in the dry weight of each peanut tissue at the pegging stage are shown in Figure 2. Compared with the control, the shoot biomass of peanuts treated with T6, T4, and CL was significantly reduced at the pegging stage (Figure 2a), while there was no significant difference in root biomass (Figure 2b). There was no significant difference in shoot, root, and pod biomass between the treatments and control group at the podding stage (Figure 2c–e). At the maturity stage, the CL treatment increased the total biomass and pod biomass of the peanuts, while T4, T6, and P1 showed no significant difference with the CK (Figure 2f,g). At the same time, T6 and CL treatment significantly increased the yield of the peanuts (Table 1). As shown in Table 1, CL, T6, T4, and P1 treatments increased the hundred-fruit weight by 6.3%, 14.5%, 5.9%, and 4.1%, respectively, compared to the CK. The effects on hundred-kernel weight were consistent with those on hundred-fruit weight. Overall, all four microbial fertilizer additives had positive effects on peanut yield in the field, and the hundred-fruit weight, hundred-kernel weight, kernel yield, and pod fullness were all higher than those of the CK. Among them, the yield of peanut in the field treated with T6 and CL was significantly higher than that of the control, by 35.7% and 28.2%, respectively, reaching a significant level. Moreover, T4 and P1 yielded slightly higher than the CK, by 26.0% and 6%, respectively, indicating that fertilizer additive T6 had the best effect on the yield of peanuts.

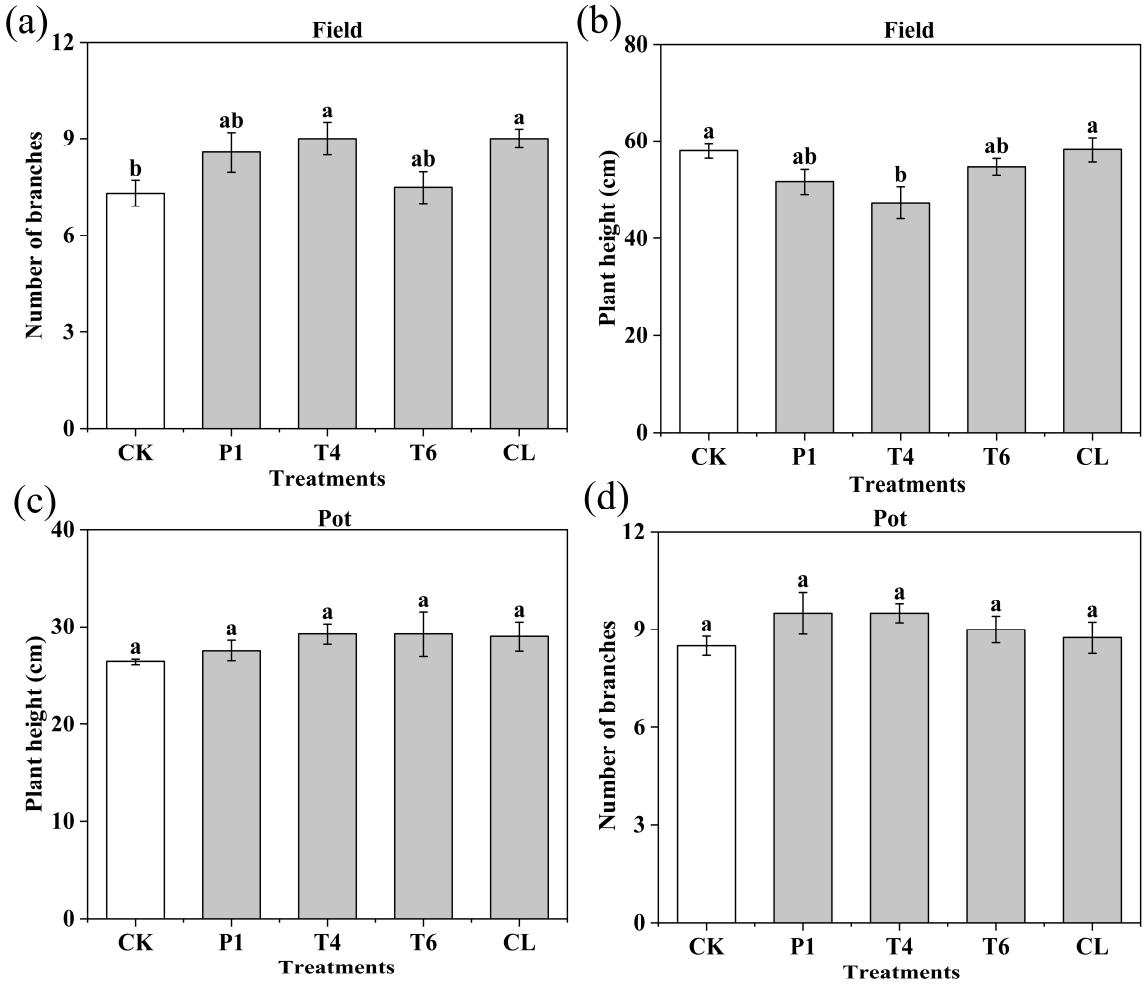

Figure 1. Effect of different fertilizer additives on branch number and plant height of peanut at the maturity stage. (**a**,**d**) The number of branches of peanut plants. (**b**,**c**) The height of the plant. Different letters indicate significant differences at $p < 0.05$ (Tukey's test). CK, control group; P1, the mixtures of chito-oligosaccharide and brassinolide; T4, the mixtures of *Pantoea alhagi*, polyglutamic acid and organic matter; T4, the mixtures of P1 and polyglutamic acid; T6, the mixtures of P1 and polyglutamic acid; CL, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter.

Table 1. Effect of bioavailability enhancers on peanut yield in the field.

Treatment	Hundred Pods Weight (g)	Hundred Seeds Weight (g)	Kernel Yield (%)	Pod Fullness	Yield (kg acres^{-1})	Full Fruit Weight Rate (%)	Full Kernel Weight Rate (%)
CK	145.90 ^{b}	59.40 ^{a}	64.29 ^{a}	0.43 ^{a}	156.16 ^{c}	63.54 ^{b}	67.47 ^{a}
P1	151.85 ^{ab}	59.35 ^{a}	65.48 ^{a}	0.48 ^{a}	165.55 ^{abc}	68.7 ^{ab}	72.58 ^{a}
T4	154.45 ^{ab}	61.25 ^{a}	67.04 ^{a}	0.47 ^{a}	196.62 ^{bc}	67.64 ^{ab}	70.55 ^{a}
T6	167.80 ^{a}	61.50 ^{a}	67.32 ^{a}	0.48 ^{a}	211.92 ^{a}	72.03 ^{a}	70.82 ^{a}
CL	155.10 ^{ab}	63.55 ^{a}	66.88 ^{a}	0.46 ^{a}	200.20 ^{ab}	71.47 ^{a}	69.29 ^{a}

Kernel yield indicates the ratio of seed kernel weight to pod weight; Full fruit weight rate indicates the ratio of full fruit weight to total fruit weight; Full kernel weight rate indicates the ratio of full kernel weight to total seed kernel weight; Pod fullness equals the product of kernel yield and full kernel weight rate. Different letters in superscript indicate significant differences at $p < 0.05$ (Tukey's test).

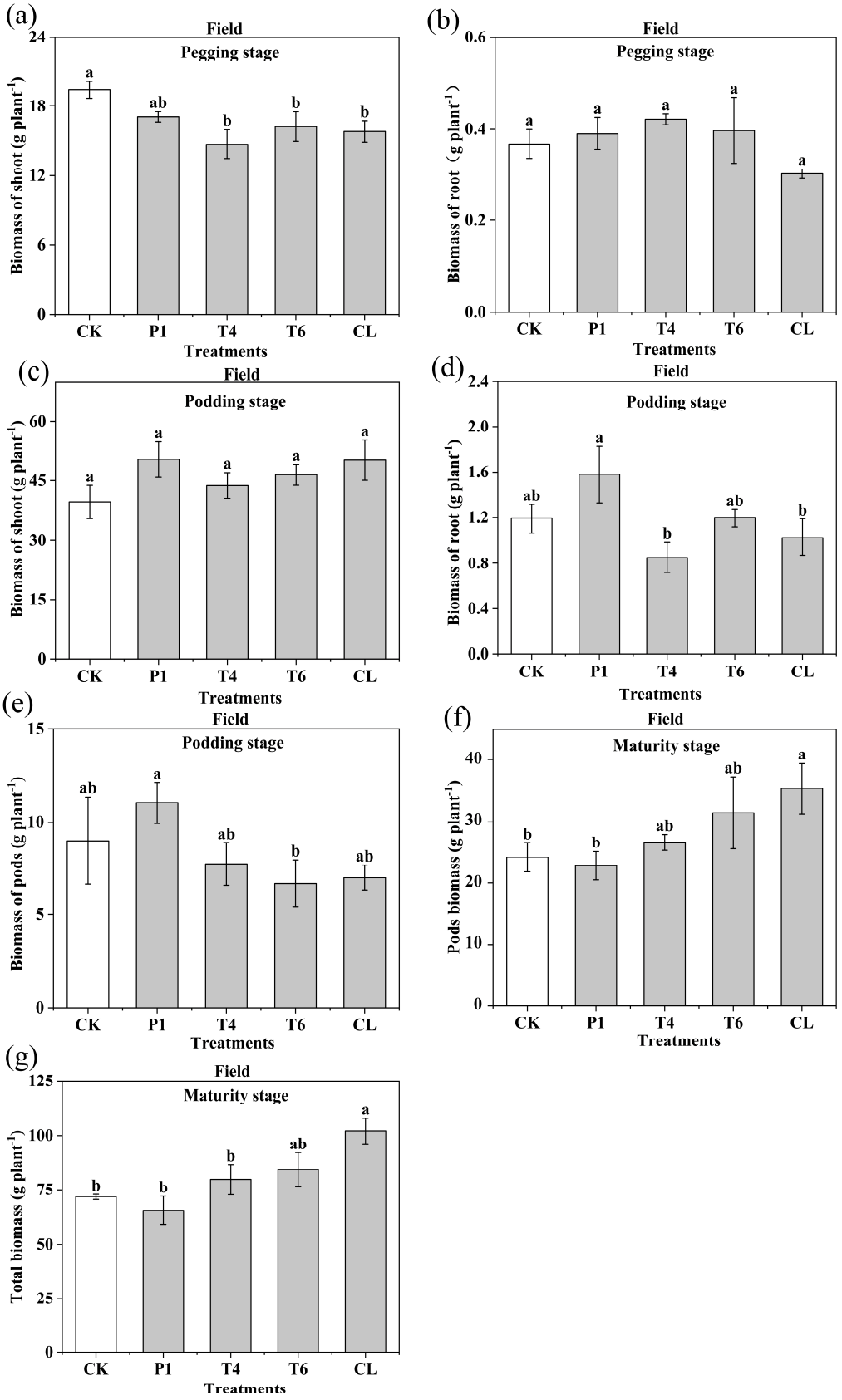

Figure 2. Effect of different fertilizer additives on tissue biomass of peanut at the pegging stage and podding stage in the field. (**a**,**b**) The shoot and root biomass in the pegging stage. (**c**,**d**) The shoot and

root biomass during the podding stage. (**c**–**e**) The shoot and root biomass and pod biomass at the podding stage, respectively. (**f**,**g**) The single plant biomass and pod biomass at maturity, respectively. Different letters indicate significant differences at $p < 0.05$ (Tukey's test). CK, control group; P1, the mixtures of chito-oligosaccharide and brassinolide; T4, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter; T4, the mixtures of P1 and polyglutamic acid; T6, the mixtures of P1 and polyglutamic acid; CL, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter.

The shoot and root biomass of peanut was higher in CL, T6, T4, and P1 treatments than in the control, but the differences were not significant (Figure 3a,b). Application of fertilizer auxiliaries CL, T6, T4, and P1 in the pot trials had no significant effect on the biomass of peanut pods at maturity compared to the control (Figure 3c). The biomass of peanut was higher than the control in the treatments with fertilizer auxiliaries CL, T6, T4, and P1, but did not reach significant differences (Figure 3d).

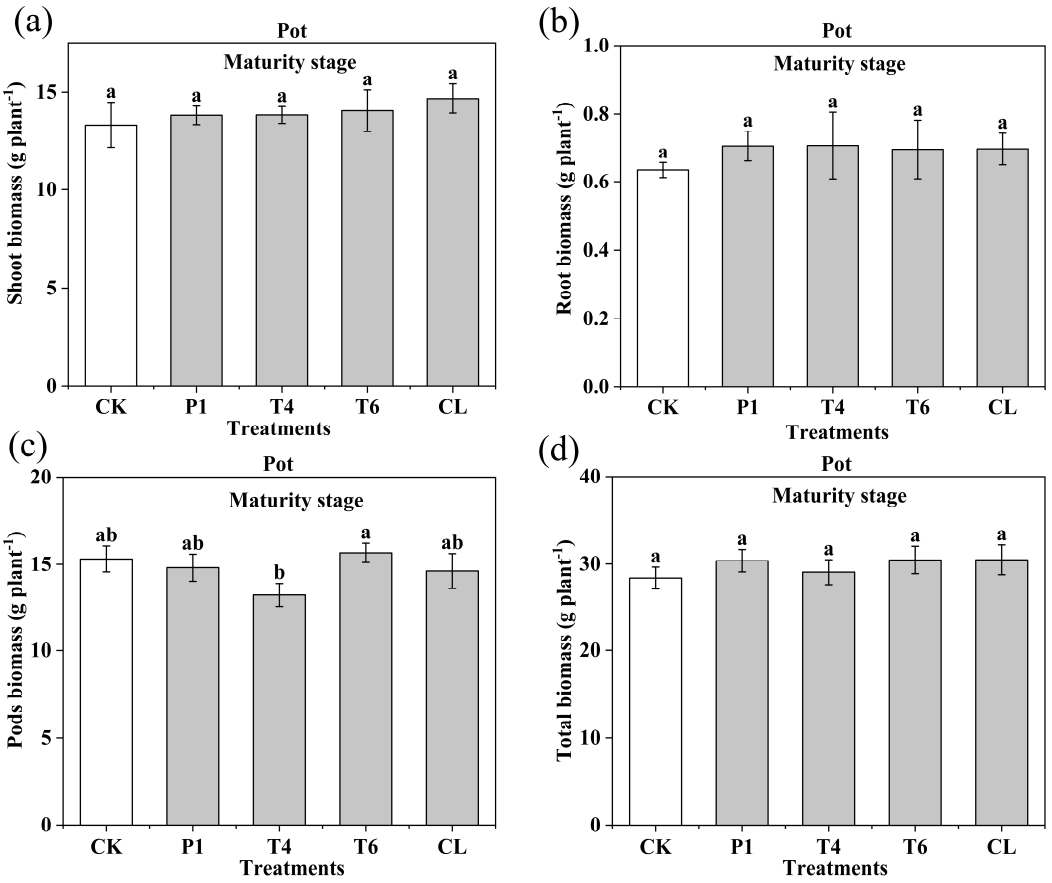

Figure 3. Effect of different fertilizer additives on plant biomass and pods biomass at maturity in pots. (**a**) Shoot biomass; (**b**) root biomass; (**c**) pod biomass; (**d**) total biomass. Different letters indicate significant differences at $p < 0.05$ (Tukey's test). CK, control group; P1, the mixtures of chito-oligosaccharide and brassinolide; T4, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter; T4, the mixtures of P1 and polyglutamic acid; T6, the mixtures of P1 and polyglutamic acid; CL, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter.

3.3. Effect of Different Fertilizer Additives on the Nitrogen Concentration of Peanut Tissues

It shows that neither the shoot nor root N concentration of peanuts at the pegging stage significantly differed compared to the control (Figure 4a,b). When compared to the control, there were no appreciable differences in the concentration of N in the shoots, roots, shells, and seeds of peanut pods when compared to the control, except for peanut plants treated with P1, which accumulated much more N at the podding stage (Figure 4c–g). The

nitrogen concentration of peanut seed kernels in the field trials of fertilizer additive CL, T6, T4, and P1 treatments at harvest was not significantly different from the control (Figure 5b), while the nitrogen concentration in the aboveground parts of peanut treated with P1 was significantly lower than that of the control (Figure 5a). The aboveground nitrogen concentration of peanuts treated with T4 was not significantly different from that of the control (Figure 5c). CL, T6, and T4 treatments were significantly higher than the control, with the CL and T6 treatments having the best effect. The nitrogen concentration of peanut pod pericarp treated with fertilizer additives CL, T6, T4, and P1 was slightly lower than the control, although there was no significant difference between them. N accumulation in peanut plants at maturity in CL, T6, T4, and P1 treatments was significantly higher than in the control (Figure 5d).

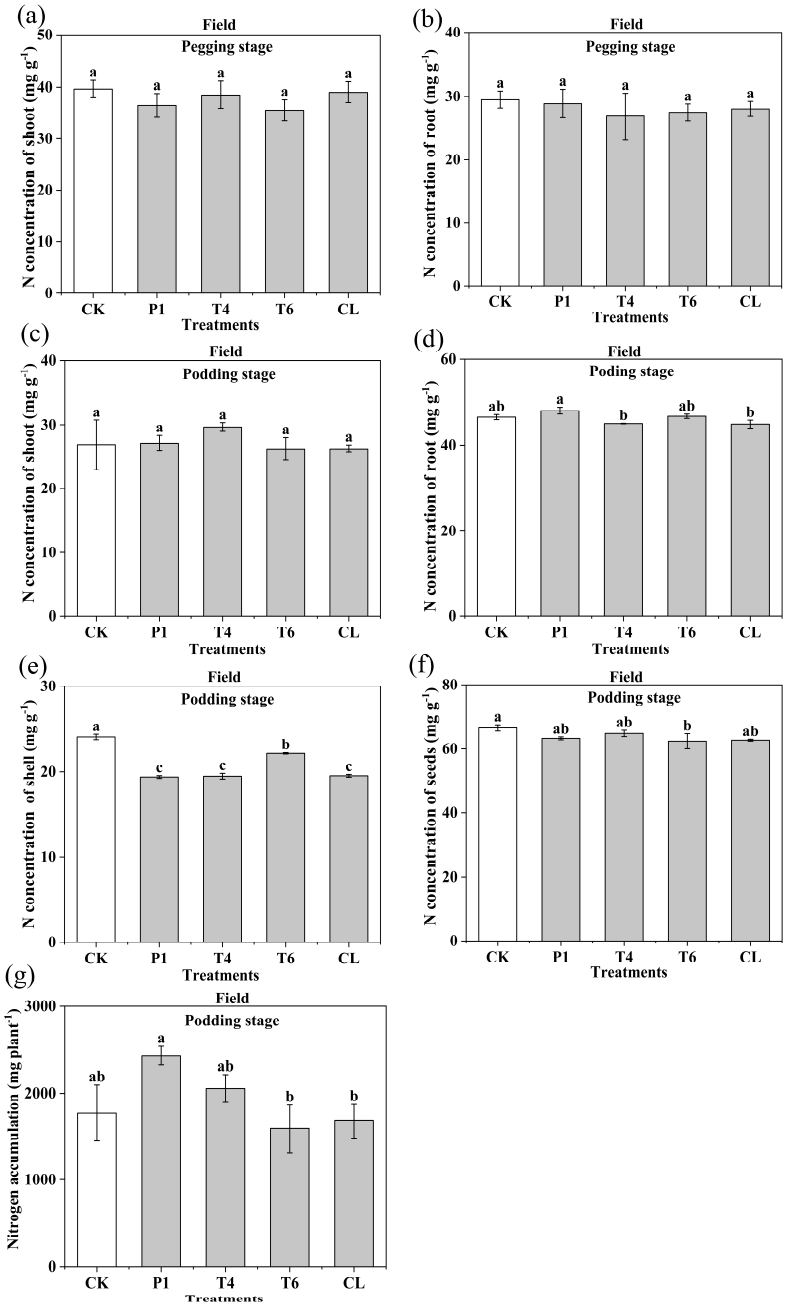

Figure 4. The N concentration and N accumulation in various tissues of peanut at different stages. The N concentration of (**a**) shoots and (**b**) roots in the pegging stage. The N concentration of (**c**) shoots,

(**d**) roots, (**e**) shells, and (**f**) seeds at the podding stage, respectively. (**g**) The total N accumulation amounts in peanut. Different letters indicate significant differences at $p < 0.05$ (Tukey's test). CK, control group; P1, the mixtures of chito-oligosaccharide and brassinolide; T4, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter; T4, the mixtures of P1 and polyglutamic acid; T6, the mixtures of P1 and polyglutamic acid; CL, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter.

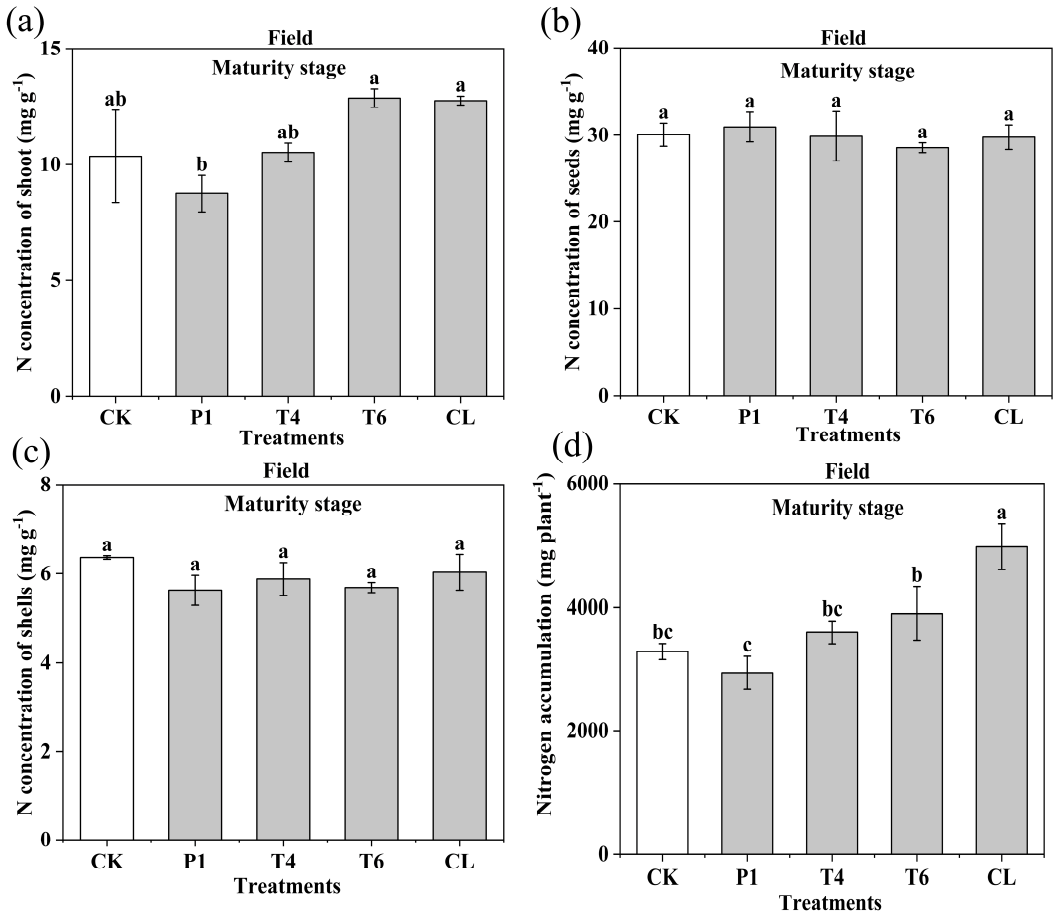

Figure 5. The N concentration and N accumulation in each tissue of peanut at maturity in field trials. The N concentration of (**a**) seeds, (**b**) shoots, and (**c**) shells at the maturity stage, respectively. (**d**) The total N accumulation amounts in peanut. Different letters indicate significant differences at $p < 0.05$ (Tukey's test). CK, control group; P1, the mixtures of chito-oligosaccharide and brassinolide; T4, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter; T4, the mixtures of P1 and polyglutamic acid; T6, the mixtures of P1 and polyglutamic acid; CL, the mixtures of *Pantoea alhagi*, polyglutamic acid, and organic matter.

4. Discussion

Fertilizer additives produced by microorganisms can have a positive impact on the growth of crops by improving nutrient availability, increasing soil fertility, and promoting plant growth. These additives can improve soil fertility, enhance nutrient availability, and promote plant growth through various mechanisms [4,7]. The application of biofertilizer was proven to make peanut plants short and robust with increased branching [24]. In our work, the application of fertilizer additives generated similar trends on peanut. The number of peanut branches was significantly increased in the CL, T6, T4, and P1 treatments compared to the CK (Figure 1). Although there was no significant difference in plant dry weight among treatments, aboveground dry weight and pod yield per plant were also significantly increased compared to the control (Figure 2). Potted peanut plants treated with

CL, T6, T4, and P1 were all slightly taller than the CK (Figure 3). Reasonable nutritional growth of peanut plants is the basis for obtaining higher yields [25] and is a prerequisite for yield formation; studies on rice, wheat, corn, soybean, and other crops have confirmed that the growth status of the aboveground is closely related to economic yield, so ensuring healthy growth of peanut plants is essential for stable and increased yields [26]. The use of peanut rhizobacteria plus peanut rhizobacteria promoter had a positive effect of promoting the growth and development of peanut, which had different degrees of improvement on the aboveground biological and economic yield of peanut [27]. T6 and CL treatments increased the biomass of peanut plants in the field (Figure 2), which was related to the significant increase in peanut hundred-fruit weight, yield, and full fruit weight rate compared to the CK (Table 1).

Previous work has shown that the application of seaweed fertilizer was superior to common fertilizers in increasing peanut hundred-kernel weight and yield, indicating that the active organic substances inside seaweed fertilizer can improve fertilizer utilization [28]. The results of this study showed that the nitrogen concentration of aboveground peanuts in pots and fields treated with a fertilizer additive was slightly higher than that of the CK (Figures 4 and 5). In comparison, the nitrogen concentration of peanut pod shells in fields treated with CL, T6, T4, and P1 was lower than that of the control (Figure 4e). However, it did not reach a significant difference, indicating that CL, T6, T4, and P1 may facilitate more N accumulation aboveground and regulate peanut plant growth and development in an appropriate direction. The increase in yield was related to the enhanced N nutrition at the maturity stage by stimulating with fertilizer additives. It is worth noting that poly(gamma-glutamic acid) (gamma-PGA) affects the nitrogen metabolism of Chinese cabbage [29]; PGA is also a component of CL, T4, and T6, which might be associated with enhanced N accumulation in peanuts (Figures 4g and 5e). Other reports have also revealed that the application of gamma-PGA facilitated the growth and tolerance to stress of crops [30–32]; thus, the better effects might be due to PGA rather than P1, CL, and T6. Previous works have proven the application of chito-oligosaccharide can help legumes establish symbiosis with *arbuscular mycorrhizal* fungi and nodule-inducing rhizobia in roots [33–35]. Although the application of P1 did not show a significant effect on peanut yields (Table 1), the increase in nitrogen accumulation suggests its potential use in facilitating N uptake (Figures 4g and 5e). However, further research is needed to understand the optimal soil types and application amounts for P1.

By improving nitrogen uptake, fertilizer additives produced by microorganisms can reduce the need for fertilizers with higher crop production. Based on microbial engineering technology, they can be cost-effective compared to synthetic fertilizers, especially in regions with limited access to external inputs. In all, they have the potential to play an increasingly important role in sustainable agriculture. However, further research is needed to fully understand their effectiveness in different agricultural systems and to develop best practices for their use.

5. Conclusions

By the comprehensive evaluation of pot and field trials, the additives used, and especially CL, have shown positive effects on peanut growth and development, as indicated by increased biomass, pod yield, and nitrogen concentration. However, more research is needed to fully understand the effectiveness of these additives in different agricultural systems and to develop best practices for their use. Overall, the use of fertilizer additives produced by microorganisms has the potential to play an important role in sustainable agriculture, promoting both economic and environmental sustainability.

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