Improve Plant Photosynthesis by a New Slow-Release Carbon Dioxide Gas Fertilizer

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ABSTRACT: In the natural state, the concentration of carbon dioxide in the atmosphere is about 300 μmol mol−1. Plants need a suitable balance of CO2 to achieve optimal growth. The optimum CO2 content corresponding to a high photosynthesis rate is between 0.1 and 1.0% by volume. However, air has only a CO2 content of 0.03% by volume, so plants cannot use all of their growth potential. The use of fertilizer to assist in the supply of CO2 increases the rate of photosynthesis. In this work, a slow-release CO2 gas fertilizer inspired by polyphenol chemistry was prepared to provide sustainable CO2 that could improve plant photosynthetic capacity and get a higher crop yield. The core–shell structure was designed to confer gas fertilizers slow-release property. Micron-sized calcium carbonate particles with uniform particle size and regularity morphology, as carbon sources for plant photosynthesis, was a core, and tannic acid was coated on it as a shell via oxidative oligomerization and cross-linked by polyetherimide. The structure and morphology of fertilizers were characterized by scanning electron microscopy, X-ray energy dispersive spectroscopy, transmission electron microscopy, X-ray photoelectron spectroscopy, and thermogravimetric analysis. In vitro experiments, the prepared fertilizers were proved to have slow-release properties. And then through net photosynthesis rate, chlorophyll fluorescence parameters, chlorophyll content, leaf area, leaf mass per area, and dry matter to study the effects of slow-release CO2 gas fertilizers on plant physiology of Brassica chinensis. The results revealed that the slow-release CO2 gas fertilizers not only had good slow-release properties but also can well improve plant photosynthesis.

INTRODUCTION

To meet the future food requirement caused by the ever-increasing population, it is urgent to solve the problem of present crop productivity.1 High photosynthesis rates can guarantee a high grain yield. In the total output of crops (including root, stem, leaf, seed etc.), only 5–10% of the nutrients is from the soil by roots, and 90–95% is from photosynthesis.2,3 Carbon dioxide (CO2) is the primary limiting factor for photosynthesis because of its low concentration not only in vitro atmospheric environment but also at the sites of carboxylation inside the leaves.4 Many scientists at home and abroad have proved through genetic improvement experiments that the elevated carbon dioxide concentration in the environment can enhance the photosynthesis of plant leaves.5–7 Undoubtedly, high CO2 concentration contributes to crop yield.8

Since Saussure first reported the effect of CO2 fertilization on plant productivity in 1804,9 many agricultural scientists have studied the development and utilization of CO2 in depth. In developed countries, CO2 was first applied to cucumbers, tomatoes, and salads (western-style mixed vegetables) in greenhouse (enclosed space). In recent decades, the CO2 fertilizer (such as AGROSOL, a carbon dioxide gas fertilizer) was applied to promote field condition (open space). CO2 fertilization is based on the following process: the gas fertilizer enters the plant stoma and reacts with its acidic environment to slowly release CO2 to promote plant growth. However, the potential mechanism of CO2 gas fertilizers in plant photosynthesis is unclear.

In photosynthesis, CO2 diffuses from atmosphere to the substomata of leaves and then to the sites of carboxylation. Generally, enhancement of CO2 is accompanied by higher photosynthetic rate.5,8 However, under low light, the limiting factor of photosynthetic rate is the light energy rather than the available CO2.10 On the contrary, under high or saturation light, the photosynthetic rate will be saturated when the concentration of CO2 is beyond the CO2 saturation point.10 Thus, how to provide sustainable and sufficient concentration of CO2 is a key factor to enhance the crop yield.
In recent years, slow-release technology has been widely used in agriculture, medicine, and other aspects, and slow-release fertilizers have received enormous attention because of decreasing a fertilizer’s loss rate, supplying nutrients sustainably, lowering application frequency, and minimizing potential negative effects associated with overdosage. Coated fertilizers are fertilizers with a thin layer coated on their surface by physical coating to form a new fertilizer, so as to achieve the effect of sustained-release. Recently, Messersmith et al. have reported that a thin, surface adherent, and multifunctional biopolymer coatings can be formed by oxidative oligomerization of polyphenol precursors. Tannic acid (TA) was colorless, nontoxic, biodegradable, inexpensive, and easily available, and polyhydroxy can be oxidized in the air by self-polymerization to form a thin film, thus becoming the ideal coating material. In this work, inspired by the slow-release technology, we prepared sustained-release gas fertilizers to better adapt to growth needs of plants. Herein, micron-sized calcium carbonate particles with uniform size and morphology were prepared and utilized as carbon sources for plant photosynthesis. TA was chosen as a typical plant polyphenol precursor to illustrate the preparation and structural manipulation of the coated fertilizers. Subsequently, the as-formed coating was covalently cross-linked by polyethylenimine (PEI) through Schiff base/Michael addition reaction between amine groups of PEI and catechol/pyrogallol groups of TA to ensure slow-release property of CO2. PEI/TA-coated CaCO3 as slow-release CO2 gas fertilizer not only has better mechanical stability but also achieves the effect of sustained release to meet the growth needs of plants. Therefore, a new idea of improving plant photosynthetic efficiency and improving crop yield was established by the modification of fertilizer dosage forms.
RESULTS AND DISCUSSION

Morphology and Characteristics of PEI/TA-Coated CaCO₃ as Slow-Release CO₂ Gas Fertilizers. Figure 1 presented the schematic preparation process of PEI/TA-coated CaCO₃ inspired by polyphenol chemistry, which can be divided into three steps: (1) generation of poly(sodium 4-styrenesulfonate) (PSS)-doped CaCO₃ microspheres through a coprecipitation process; (2) oxidative oligomerization and deposition of polyphenol on the surface of the microspheres; (3) further cross-linking of the as-formed polyphenol coating with PEI through Schiff base/Michael addition reaction between the amine groups of PEI and the catechol/pyrogallol groups of polyphenol.

Inspired by a commercially available gas fertilizer (AGRO-SOL), calcium carbonate was selected as the carbon source of CO₂ gas fertilizers. X-ray photoelectron spectroscopy (XPS) and X-ray energy dispersive spectroscopy (EDX) spectrum (Figure 2A,C) well proved that the essential components of AGRO-SOL are carbonate. In Figure 2B, scanning electron microscopy (SEM) image of AGRO-SOL is shown; it’s irregular and has uneven size of the industrial grade carbonate, and the particle size generally reaches tens microns. For that reason, about 4 μm particle size of PSS-doped CaCO₃ microspheres were prepared through a coprecipitation process (as shown in Figure 3A). PSS is a crystalline shape controller that makes the prepared calcium carbonate microspheres more orderly and more homogeneous. A natural polyphenol (TA) not only has low-cost and is easily available but also can be biodegradable. The pyrogallol groups of TA can be easily oxidized to reactive quinones that could connect with each other through oxidative coupling reactions, then forming the oligomers.

Considering the strong interfacial affinity of TA, the oligomers could be subsequently deposited on the surface of CaCO₃ microspheres. As shown in Figure 3B, a rougher surface with “massif-like” humps could be observed in TA-coated CaCO₃ microspheres. This evinced that TA oligomers were successfully deposited on the CaCO₃ microspheres. In addition, transmission electron microscopy (TEM) images of PSS-doped CaCO₃ microspheres, TA-coated CaCO₃ microspheres, and PEI/TA-coated CaCO₃ microspheres are also shown in Figure 3. Comparing with an uncoated fertilizer (Figure 3E), the light outer shell is clearly observed on the boundary of dark CaCO₃ microspheres (as is shown in Figure 3F), which also indicates that the TA is successfully coated on the CaCO₃ microspheres. However, the rougher surface was probably as a result of irregular stacking of TA oligomers. Owing to the weak interaction between TA oligomers (hydrogen bonds and π-stacking interactions), the TA coating would dissemble, which led to the failure of slow release. To strengthen the mechanical stability of the TA coating, PEI was introduced to chemically cross-link TA oligomers. As shown in Figure 3C, a much smoother and compacter surface was obtained after PEI cross-linking, which indicated that the interaction between TA oligomers were strengthened by PEI. Comparing with TA-coated CaCO₃ microspheres, more regular and smoother light outer shells were clearly observed on the boundary of dark CaCO₃ microspheres (Figure 3G), which also evinced that the interaction between TA oligomers were strengthened by PEI. The increase of N element in the EDX spectrum of PEI/TA-coated CaCO₃ also clearly demonstrated that PEI is cross-linked to TA oligomers (Figure 3D,H).

Thermogravimetric Analysis. The effect of introduced TA on the thermal stability of the resulting polytannic acid (PTA) was investigated with thermogravimetric analysis (TGA) and was depicted in Figure 4A. TGA curves also verified the successful coating of TA on CaCO₃ microspheres. The weight loss of the PSS-doped CaCO₃ microspheres is 47.74% at temperatures up to 1000 °C. After the oxidative oligomerization and deposition of polyphenol, the weight loss rate of TA-coated CaCO₃ is apparently faster than PSS-doped CaCO₃ microspheres. The corresponding weight loss values at temperatures up to 1000 °C with different deposition cycles are as follows: 49.57, 52.48, 55.30, 64.07, and 73.73%. This indicates that the percentage of TA of PTA-1, PTA-2, PTA-3, PTA-5, and PTA-10 is 1.83, 4.74, 7.56, 16.33, and 25.99%, respectively. The content of TA increases with the increasing number of deposition cycles. Thus, it is indicated that we successfully prepared TA-coated CaCO₃ as slow-release CO₂ gas fertilizers.

To improve the mechanical stability of TA-coated, PEI was introduced to chemically cross-link TA oligomers. The effect of introduced PEI on the thermal stability of the resulting PEI/TA-coated CaCO₃ was investigated with TGA and is depicted in Figure 4B. The weight loss of the PTA-1 is 49.57% at temperatures up to 1000 °C. After chemical cross-linking with different concentrations of PEI, the weight loss rate of PEI/TA-coated CaCO₃ is apparently faster than that of PTA-1. The corresponding weight loss values at temperatures up to 1000 °C with different concentrations of PEI are as follows: 51.53, 51.24, and 55.27%. This indicates that the percentage of PEI of 0.2 PEI/TA-coated CaCO₃, 1.0 PEI/TA-coated CaCO₃, and 2.0 PEI/TA-coated CaCO₃ is 1.74, 1.67, and 5.7%, respectively. TGA curves also proved the successful cross-
link of PEI with TA oligomers. Through the TGA data, we know the thickness of the coating of slow-release CO₂ gas fertilizers.

**Slow-Release Behavior of CO₂ Gas Fertilizers in Vitro.**

It is difficult to observe and detect the CO₂ release process of slow-release CO₂ gas fertilizers in the plant stomata, so we use the one-factor experimental method to simulate slow-release behavior of above-mentioned fertilizers in vitro. The amount of fertilizers per test is known by coating rate (obtained through TGA) because we need to ensure that the content of calcium carbonate of as carbon source is constant. We first used inorganic acid (dilute hydrochloric acid, pH 2) to simulate the acidity of plant stomata (in Figure 5A,B) and also try to use organic acids such as dL-tartaric acid (as shown in Figure 5C). The CO₂ release amount was continuously recorded using the Li-840A CO₂/H₂O gas analyzer. As shown in Figure 5, it recorded CO₂ cumulative release quantity in first 4 h, because of the instruments and other objective reasons, and did not continue to record. However, it is obviously observed that the trend of the CO₂ cumulative release quantity curves is infinitely close to the same level because the content of calcium carbonate of as a carbon source is constant. When the slow-release layer is coated, the release curve obviously has a slow-release step, and with the increase of deposition cycles, the slope of the release curve is more gentle and the release step is more longer (insets of Figure 5A−C). It can be attributed to the thicknesses of coated films. We presume that coated films have a “barrier-like” effect to hinder the release of CO₂. When the film is thicker, the barrier of CO₂ becomes bigger. From the previous SEM and TEM images, we can see that the coating formed by TA is unstable, so PEI was introduced to strengthen the mechanical stability of the TA coating by chemically cross-linking. As shown in Figure 5B, PEI/TA-coated CaCO₃ has a better sustained-release effect than TA-coated CaCO₃, and the mechanical stability is improved. To better simulate the acidic environment of plant leaf stomata, we do the same release experiments using dL-tartaric acid as an organic acid of common and high content in plant leaves. It also proved prepared fertilizers has good sustained-release properties.

**Application of PEI/TA-Coated CaCO₃ as Slow-Release CO₂ Gas Fertilizers.**

While spraying CO₂ gas fertilizers at 12:00 pm, we picked antepenultimate leaves of *Brassica chinensis*, rapidly prepared sample, fixed by stationary liquid, freeze dried, and then tested by SEM, finally got SEM images of *B. chinensis*’s stomata. Figure 6 shows SEM images of *B. chinensis*’s stomata, A is a blank control (CK) without fertilization just spray distilled water, and B is plant stomata after fertilizing slow-release CO₂ gas fertilizers. From the Figure 6, we can clearly see that the CO₂ gas fertilizers entered into the plant stomata.

**Effects of Slow-Release CO₂ Gas Fertilizers on Plant Photosynthetic Characteristics of *B. chinensis*.** Net photosynthetic rate ($P_n$) has been widely used as an indicator of CO₂ gas exchange through the stomata pore of leaves, represented the capacity of photosynthetic CO₂ fixation used to generate sugars carbohydrates. As expected, compared with CK, $P_n$ was increased in all of CO₂ gas fertilizer.
treatments, thereby contributing to development and growth of the plants (Table 1). Those results can be further supported by higher chlorophyll content under all of CO₂ gas fertilizer treatments (Table 1). It’s well documented that chlorophyll is essential in photosynthesis, allowing leaves to absorb light energy. Generally, the increase of chlorophyll content is accompanied with the enhancement of photosynthetic capacity. Thus, application of CO₂ gas fertilizers can promote plant chlorophyll synthesis and have higher photosynthetic capacity. Furthermore, the treatment of PEI/TA-coated CaCO₃ has remarkable effects to the leaf area, leaf mass per area, and dry biomass, especially the latter two (Table 1). Leaf mass per area as an important physiological indicator of photosynthetic capacity reflected the dry matter accumulation of plant. In the treatment of PEI/TA-coated CaCO₃, the Pn, leaf area, and leaf mass per area significantly increased, indicating that slow-release CO₂ fertilizers could effectively improve the photosynthetic capacity of B. chinensis, increase dry matter accumulation, and consequently promote the enhancement of crop yield.

Given the slow-release CO₂ gas fertilizers diffused into the substomatal cavities (Figure 6), CO₂ was released at those sites and used to plant photosynthesis. We inferred that the photosynthetic capacity will be enhanced because of increased CO₂ supplied by slow-release CO₂ gas fertilizer in short-term.

Chlorophyll fluorescence is a probe of photosynthesis. Our results showed that no differences of the maximum quantum yield of PS II photochemistry (Fm/Fn) was observed among all the treatments, indicating that photosynthesis apparatus was not damaged by CO₂ release of CO₂ gas fertilizers. Compared with CK, the leaves spraying CO₂ gas fertilizers showed the similar of ETR(II) and Y(II). Considering higher Pn of leaves applied CO₂ gas fertilizers, it is implied that photorespiration may be inhibited during the photosynthetic process. As discussed above, CO₂ will be collected in the substomatal cavities, the enzyme RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase is an important carboxyl enzyme in Calvin cycle of photosynthesis and catalyzes the reaction between RuBP and carbon dioxide) will prefer to benefit CO₂ fixation and hardly combines with oxygen.

### CONCLUSIONS

In summary, we have successfully prepared the CO₂ gas fertilizers with sustained-release property and applied it to the B. chinensis growth. Through the systematic study, we have found that the slow-release CO₂ fertilizers not only are very stable core–shell structure but also have sustained-release property and most importantly can slowly release CO₂ in situ in plant stomata. Finally, plant physiological characteristics also proved that prepared slow-release CO₂ fertilizers can improve the photosynthesis efficiency of plants and ultimately can promote plant growth.

### MATERIALS AND METHODS

**Materials.** AGROSOL (a commercially available carbon dioxide gas fertilizer) was purchased from Austrian Agrosol Company. PSS (Mw ca. 70,000), TA, and tris-(hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich Company. PEI (Mw ca. 10,000 Da; branched) was purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China. DL-Tartaric acid, sodium carbonate anhydrous (Na₂CO₃), and calcium chloride anhydrous (CaCl₂) were purchased from Xi’ya Chemical Industry Co., Ltd., Shandong, China.
were obtained from Tianjin Sheng’ao Chemical Reagent Co., Ltd. The water used in all experiments was deionized. The pH values of solutions were measured with a PHS 25 pH-meter (Shanghai Instrument Electric Scientific Instrument Co., Ltd., Lei magnetic instrument factory, Shanghai, China) and adjusted by addition of HCl solution (10 mM).

Preparation of PSS-Doped CaCO₃ as CO₂ Gas Fertilizers. First, PSS-doped CaCO₃ microspheres (diameter: 3–4 μm) were prepared by a coprecipitation process and utilized as CO₂ gas fertilizers for plant photosynthesis. Specifically, 80 mL of 0.33 M Na₂CO₃ solution was rapidly added into 80 mL of 0.33 M CaCl₂ solution containing 240 mg of PSS under vigorous stirring for 30 s. After standing for 10 min, PSS-doped CaCO₃ microspheres were acquired through centrifugation and water washing. Then, the precipitate was dried under vacuum to a constant weight.

Preparation of TA-Coated CaCO₃ as Slow-Release CO₂ Gas Fertilizers. In a typical procedure, 2 g of PSS-doped CaCO₃ microspheres as mentioned above were dispersed in 200 mL of Tris·HCl buffer (50 mM, pH 8.0) containing 1.0 mg mL⁻¹ TA. After gently stirring at room temperature for 2 h, the microspheres were collected by centrifugation and washed with water three times. The fertilizers were dried to a constant weight under vacuum at 40 °C, and the resulting products were obtained. To get PSS-doped CaCO₃ with different thicknesses of TA-coated, the number of deposition cycles was designed at 1, 2, 3, 5, and 10, respectively. The deposition procedure was repeated in the same way as mentioned above. The resulting products are denoted as PTA-1, PTA-2, PTA-3, PTA-5, and PTA-10 where 1, 2, 3, 5, and 10 refer to the number of deposition cycles.

Preparation of PEI/TA-Coated CaCO₃ as Slow-Release CO₂ Gas Fertilizers. The 0.5 g acquired TA-coated CaCO₃ microspheres were redispersed in 50 mL of Tris·HCl buffer (50 mM, pH 8.0) with 1.0 mg mL⁻¹ PEI, which was gently stirred at room temperature for 0.5 h. Then, the microspheres were centrifuged and washed with water three times, and then the precipitate was dried under vacuum to a constant weight.

Characterization Techniques. SEM (Hitachi SU8010) and the attached EDX and XPS were performed to measure the morphology and elemental composition of AGROSOIL, PSS-doped CaCO₃ microspheres, TA-coated CaCO₃ microspheres, and PEI/TA-coated CaCO₃ microspheres. TEM (Hitachi H-600) was used to observe the morphology and thickness of the TA-coated CaCO₃ microspheres and PEI/TA-coated CaCO₃ microspheres. Thermal stability was determined by a thermogravimetric analyzer (TGA, Netzsch STA449F3) over a temperature range of 25–1000 °C at a heating rate of 20 K min⁻¹ under a N₂ atmosphere.

Release Behavior of TA-Coated CaCO₃ and PEI/TA-Coated CaCO₃ as Slow-Release CO₂ Gas Fertilizers. The release behavior of AGROSOIL, PSS-doped CaCO₃, TA-coated CaCO₃, and PEI/TA-coated CaCO₃ was detected as follows: for example, 25 mg of PTA-1 was encapsulated at 500 mL round-bottom flask under 50 rpm string, and then 70 mL of pH 2 hydrochloric acid solution (used to simulate plant stoma acidic environment) was slowly added to sealed round-bottom flask. The CO₂ release quantity was sustained record by LI-840A CO₂/H₂O gas analyzer (Li-6400, Li-Cor Inc., Lincoln, NE, USA). The specific amount of fertilizer was obtained by calculating the coating rate of the slow-release fertilizers by TGA.

To better simulate the acidic environment in the stomata of plant leaves, a common and high content organic acid (dl-tartaric acid) was used for similar release experiments. The specific method is as follows: for example, weigh 10 mg of PTA-1 in a 500 mL round-bottomed flask and weigh the same amount of dl-tartaric acid under 50 rpm string, and then slowly add 50 mL of distilled water through a separatory funnel to a closed round-bottom flask. The CO₂ release quantity was continuously recorded through the LI-840A CO₂/H₂O gas analyzer. The specific amount of fertilizer was obtained by calculating the coating rate of the slow-release fertilizers by TGA. Other slow-release CO₂ gas fertilizers are tested in the same manner as described above.

Plant Materials and Experimental Design. Our study was conducted at Shihzei University, Xinjiang, China (45°19′N, 86°03′E). B. chinensis (Brassical rapa L. chinensis) were grown in plastic pots (19 cm in diameter, 28 cm in height) filled with substrates (soil vs vermiculite vs perlite = 1:1:1). All pots were exposed to full sunlight (the maximum intensity is 2000 μmol·m⁻²·s⁻¹) during their growth period. Five or six seeds were sown in each pot. By the time the first true leaf had expanded, only one plant per pot was left for uniformity. The seedlings, with the growth of three true leaves, were watered with the nutrient solution (contained enough Ca²⁺ and Mg²⁺) three times a day and seedlings, with four leaves, were then divided into four groups for different fertilizer treatments. Four treatments were used: blank control (CK), just spray the distilled water, AGROSOIL, PSS-doped CaCO₃, and PEI/TA-coated CaCO₃. We used spray bottle and sprayed fertilizer on the abaxial surface of leaves at 12:00 pm every day. It’s been sprayed for half a month, and then, the third upper leaf was measured. Every treatment had at least three replicates.

Measurement of Photosynthetic Characteristics. The morphology of processed plant stomata was observed by SEM (Hitachi SU8010). Gas exchange measurements were carried out between 11:00 am and 13:00 pm using a portable gas exchange analyzer system (Li-6400, Li-Cor Inc., Lincoln, NE, USA) equipped with a blue-red LED light source (Li-6400-02). During the measurements, the reference CO₂ concentration was set as 400 μmol mol⁻¹ and the photosynthetic active radiation (PAR) was maintained at 1800 μmol·m⁻²·s⁻¹. When measuring CO₂ assimilation rates, the leaf temperature was (20±2) °C and relative humidity is (56±2)%.

Leaf chlorophyll fluorescence was measured using the IMAGING-PAM (Walz, Effeltrich, Germany). Fully dark-adapted plants (2 h) were used to determine the maximum quantum yield of PS II (Fv/Fm). The leaf was illuminated with an actinic light of 1051 μmol·m⁻²·s⁻¹ for 5 min; the light-adapted maximum and minimum fluorescence (Fm and Fo) were recorded after the fluorescence signal reached a steady state (Fm). The maximum quantum yield of PS II was calculated as Fv/Fm = (Fm – Fo)/Fm (Fm and Fo are the dark-adapted maximum and minimum fluorescence, respectively). Actually, PS II quantum efficiency (Y(II)) was determined by the equation 18 (Fm' – Fv')/Fm. The rate of electron transport was estimated from (Fm' – Fv')/Fm' × 0.5 × leaf absorbance × PAR (the coefficient 0.5 is because an electron transfer needs to absorb two quanta, and photosynthesis includes two optical systems; leaf absorbance refers to the ratio of light intensity absorbed to plant leaves).

Chlorophyll (Chls) contents were determined in discs from leaves, removed by a punch (total of about 1 cm²). Discs of the
leaves were extracted in 80% (v/v) acetone for 24 h at room temperature in the dark.28,29 The absorbance of an extract was measured with a UV-2041 spectrophotometer (Shimadzu, Japan) at 663 and 645 nm. The concentrations of Chls per unit leaf area were determined, using the equations according to Lichtenthaler.19

\[
C_{\text{Chl}} = 12.7 \times A_{663} - 2.69 \times A_{645} \\
C_{\text{Chl}} = 22.9 \times A_{645} - 4.68 \times A_{663} \\
C_{\text{Chl}} = (20.21 \times A_{645} + 8.02 \times A_{663})
\]

Pigment content = \( C_n \times V/S \)

Here, \( A_n \) is the absorbance at a given wavelength (\( n \)), \( V \) is the volume of each extracting solution, and \( S \) is the area of leaf discs.

Total leaf area of the whole plant was measured with a leaf area meter Li-3100 (Li-COR, Lincoln, USA). After that, antepenultimate leaf of each plant was immediately punched five leaf discs by a punch (diameter of 0.8 cm), and then the overground part of plant and the discs were heated to de-enzyme at 105 °C for 30 min and dried at 80 °C until a constant weight (dry weight, DW) was reached in an oven. Finally, the dry biomass and the leaf mass per area were achieved. Leaf mass per area was calculated as dry weight/area of the leaf discs.

**Statistical Analysis.** All data were tested by analysis of variance (ANOVA). The significance of differences between treatment means were separated by using Student–Newman–Keuls (S–N–K) test at the 0.05 probability level.

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**Notes**
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