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Chitin-based biostimulant improves productivity and antioxidant activities of 'RD43' rice

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ABSTRACT: The rice variety 'RD43' produces white grains with superior dietary characteristics, medium to low levels of apparent amylose content and rapidly available glucose. In this study, we investigated the effects of chitin-based bio-stimulant (CB) on growth, yield components, starch properties, total protein content, and antioxidant capacity of 'RD43' rice grown in a pot system. Two types of CB were developed: Type I CB derived from fermented shrimp shell with *Bacillus licheniformis* SK-1 mixed (FC) with rice bran, and Type II CB derived from FC blended with rice bran and rice husk. Both Type I and Type II CB were applied to soil at concentrations of 0.01%, 0.1%, and 0.2% (w/w), whereas the general practice without soil supplements was used as a control. The application of 0.2% Type II CB resulted in the maximum tiller number and the rice yield enhancement of 69.8%, while 0.2% Type I CB application increased the yield by 56.8%. This was attributed to the increase in the number of spikelets and filled grains per panicle without the increase in panicle number per plant. While neither type of CB application affected starch properties or protein content, 0.2% Type II CB significantly increased the antioxidant activity of brown seeds. These findings reveal that CB application can boost the yield of 'RD43' rice and preserve starch quality and protein content. Moreover, the enhanced antioxidant activity further underscores the CB potential to elevate 'RD43' rice as a functional food, supporting both yield and health benefits in rice production.

KEYWORDS: amylose content, antioxidant activity, biostimulant, digestible starch, fermented chitin, rice yield

INTRODUCTION

Rice (*Oryza sativa* L.), a staple food for over 3 billion people, plays a critical role in global nutrition and serves as a primary energy source in many diets, emphasizing its role in human health. Rice cultivation is widespread across Asia, where carbohydrates as the primary component of Asian diets are typically obtained from rice [1]. However, modern diet is also strongly linked to chronic conditions like diabetes, obesity, and hypertension, with growing evidence connecting refined grains to metabolic disorders. For rice consumers, elevated glucose absorption is associated with a higher risk of cardiovascular diseases and diabetes [2]. As about 80% of the rice endosperm is made up of starch, consisting of 10–30% amylose and 70–90% amylopectin [3], eating quality of rice is influenced by the apparent amylose content (AAC) which determines the firmness and texture of the cooked grains [4]. Therefore, starch quality is a key consideration when selecting rice for consumption.

'RD43' is a white rice cultivar developed in Thailand from a cross between 'Khao Jow Hawm Suphan Buri' and 'Supan Buri 1'. It is considered rice for health as it has medium to low glycemic index. As compared to that of 'KDML105' or Hom Mali, 'RD43' flour has lower level of rapidly available glucose (RAG) and higher resistant starch (RS). It also has a higher capacity to disrupt cholesterol micellization and bind to bile acid [5]. Due to these characteristics, there is a potential to utilize 'RD43' rice flour for a variety of products, such as noodles, pasta, bread, and cakes, with low glycemic index and healthier outlook [6]. In addition, 'RD43' is photoperiod-insensitive, making it suitable for various growing conditions. However, 'RD43' yield is limited; it was reported by Thai Rice Department to give the yield of 3,500–3,625 kg/ha. Furthermore, as a white rice variety, RD43' has low level of antioxidants as compared to color rice. It would also add more health benefits if there were a cultivation practice that enhances the antioxidant capacity of 'RD43' rice grains.

To promote sustainable production of rice for health, including the variety 'RD43', we aimed to develop organic materials that possess plant growth stimulant properties. We considered shrimp shell as chitin source and rice husk (RH), as both are agro-industrial wastes with high potential for growth and yield promotion in rice production. Chitin is a polysaccharide polymer composed of N-acetyl-D-glucosamine

and D-glucosamine, which are attached to each other *via* β (1–4) linkages and can be further digested into chitosan [7]. Due to their high nitrogen content and low C/N ratio, chitin and chitosan can be used as natural fertilizers [8]. In rice, chitosan nanoparticles suppress rice blast disease by 77.7%, increasing antioxidant enzyme activity, enhancing photosynthesis, gas exchange, and nutritional profile of rice plants. According to Rassaei [9], chitosan can improve soil qualities by decreasing the reduction rate of nutrients and enhancing organic matter (OM) in the maize field.

RH is composed of 70–80% organic substances and the remaining are the mineralogical components, i.e., silica and trace elements. The highest SiO_2 content in rice is observed in rice husk, which varies from 8.7% to 12.1% [10]. Rice accumulates up to 10% of Si in the shoots on a dry weight basis, and Si is required for stable and high grain yield [11]. Tamai and Ma [12] used a low-silicon rice (*lsi1*) mutant to evaluate the impact of Si on rice growth and productivity. The results showed that low Si accumulation in the mutant had a negative impact on tiller number, chlorophyll content, and root growth, resulting in a 79–98% loss in grain yield. In addition, prior studies have indicated that Si may enhance antioxidant activity in a variety of plants [13]. Applying Si in cucumber cultivation influenced gene expression involved in the synthesis of Fe-mobilizing compounds. The increased accumulation of organic acids and phenolic compounds was then observed, which mediated the increase in Fe availability in the rhizosphere [14].

Based on these well-recognized benefits of chitin/chitosan and RH in promoting growth and plant protection, we reasoned that chitin- and RH-based materials have agronomic and economic potential to benefit rice farming by promoting growth and productivity. In addition, their unique bio-stimulant properties may also promote antioxidant capacity of rice grains, providing additional advantages to rice consumers with special interests in more health benefits from rice consumption. Therefore, this study investigated the practical utilization of CB products formulated with or without RH incorporation for the production of 'RD43' rice. We also explored how CB treatments affect starch properties, antioxidant ability, and total protein content of 'RD43' rice grains. We demonstrated that, at an appropriate concentration, these zero/low-cost materials provide promising benefits for boosting both grain yield and quality. This practical knowledge could be shared with farmers for high-quality rice production.

MATERIALS AND METHODS

Plant materials and CB production

The seeds of 'RD43' rice used for this study were obtained from the Pathum Thani Rice Research Center, Pathum Thani, Thailand. To create CB, 5 kg of shrimp shells were mixed with 500 ml of bacterial culture media at the ambient temperature for 4 days. The single colony of *B. licheniformis* SK-1 strain was cultured in a minimal medium containing amounts in w/v of: 0.25% yeast extract, 0.03% $MgSO_4$, 0.5% $(NH_4)_2S_2O$, 0.6% KH_2PO_4, 1% K_2HPO_4, and 0.02% colloidal chitin at 37 °C for 24 h [15]. From the bacterial treated CB, two types of fertilizer pellets were produced. Type I combined CB and rice bran at 1:1 ratio, while Type II used CB, rice bran, and rice husk at the ratio of 2:1:1. A pellet-forming machine was used to generate cylindrical pellets, measuring 6 mm in diameter and 1 cm in length.

Experimental design and rice cultivation

The study was conducted at the Pathum Thani Rice Research Center from April to July 2021. The experiment was performed in randomized complete block design (RCBD) with four replications, with each replicate containing 4 plants. Rice seeds were germinated for 7 days under natural light conditions. A single seedling was transplanted into a pot containing 8-kg of paddy soil supplemented with CB at one of the following concentrations: 0.01%, 0.1% or 0.2% w/w. The pot containing soil without CB was used as a control. The plants were grown under natural conditions with an average daytime temperature of 34.0 °C, nighttime temperature of 29.8 °C, 205.6 mm of rainfall, and 75.83% relative humidity. To each pot, including the control, one gram of fertilizer (N-P-K, 16-16-16) was applied before transplantation, and once again at 30 and 56 days after transplantation (DAT). Water levels were maintained at 4 cm above the soil surface until seed maturity, after which it was drained for harvest.

Soil physicochemical properties detection

Soil sampling and pre-analysis of soil properties

Prior to transplantation, soil samples were systematically collected from each pot to assess key physicochemical properties, including pH, electrical conductivity (EC), organic matter percentage (OM), and concentrations of total nitrogen (total N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), molybdenum (Mo), and silicon (Si). The soil samples were air-dried and carefully cleared of any contaminants. Subsequently, each sample was finely ground using a mortar and pestle and sieved through a 100-mesh filter. The samples were then used to determine soil pH [16], EC [17], OM, and total N levels [18] according to standard procedures.

Microwave acid digestion procedure

Two-hundred milligrams of finely ground soil were weighed into digestion vessels. Each vessel received 3.5 ml of 37.5% hydrochloric acid (analysis grade; Merck, Darmstadt, Germany), 3.5 ml of 65% nitric acid

(analysis grade; Merck) and 1 ml of 40% hydrofluoric acid (Thermo Fisher Scientific, Waltham, MA, USA). A blank was prepared with the same acid volumes but without soil. All vessels were sealed and digested using the ETHOS EASY microwave system (Milestone™ Srl, Sorisole, Italy), following the clay soil digestion protocols. Post-digestion, the clear solutions were diluted to a final volume of 100 ml with deionized water.

Analytical procedure for element analysis by ICP-OES

Elemental composition of soil samples was determined using the Inductively coupled plasma-optical emission spectrometry (ICP-OES; Thermo Scientific TM iCAPTM PRO Series). Clear solutions from samples and a blank were analyzed in triplicate. Standard curves for P, K, Ca, Mg, S, Fe, Zn, Cu, Mn, Mo, and Si were prepared with acids used in the digestion step.

Rice growth and yield assessment

Tiller numbers per plant were recorded at maximum tillering stage. After harvest, the yield components were evaluated, including number of panicles per pot, number of spikelets per panicle, number of filled grains per panicle, total grain yield per pot, and 100-grain weight.

Measurement of apparent amylose content (AAC) and starch properties

AAC

For each replicate, rice seeds from four plants were combined, dehulled, ground, and sieved through a 100-mesh sieve. The colorimetric change from the amylose-iodine complex according to the modified method of Juliano [19] by Praphasanobol et al [20] was used for the analysis of AAC. The absorbance was measured at 620 nm with a microplate reader (SpectraMax® M3, San Jose, USA). Three technical replicates per sample were performed. AAC was calculated using a potato amylose (Sigma-Aldrich, Co., Darmstadt, Germany) standard curve.

Digestible starch measurement

The digestible starch fractions including rapidly available glucose (RAG), slowly available glucose (SAG), and total glucose (TG) were analyzed using a method adapted from Englyst et al [3] and modified by Prapasanobol et al [21]. Absorbance readings were taken at 510 nm against a reagent blank using a microplate reader. Three technical replicates per sample were performed. The starch fractions were calculated as follows:

$$\begin{aligned} \mathrm{RAG} &= \mathrm{G}_{20}, \\ \mathrm{SAG} &= \mathrm{G}_{120} - \mathrm{G}_{20}, \\ \mathrm{RS} &= (\mathrm{TG} - \mathrm{G}_{120}) \times 0.9. \end{aligned}$$

Determination of antioxidant activity

Antioxidant activity was measured using three radical scavenging assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and Ferric ion reducing antioxidant power (FRAP), following the modified methods of Molyneux [22], Thaworn et al [23], and Benzie and Strain [24], respectively. Three technical replicates per sample were performed. Rice flour was prepared by dehulling, grinding, and sieving through a 100-mesh screen. A 100 mg sample was extracted with 500 µl of 80% methanol at room temperature for 24 h, then centrifuged at 4,000×g at 4 °C for 15 min. The supernatants from two extractions were combined, filtered through Whatman® Grade 542, and stored at 4 °C for antioxidant activity analysis [25].

DPPH radical scavenging assay

In a 96-well plate, 20 µl of the extract was mixed with 200 µl of 0.2 mM DPPH solution and incubated for 20 min at room temperature. Absorbance was measured at 515 nm using a microplate reader, with results expressed as ascorbic acid (Acros Organics, Geel, Belgium) equivalent antioxidant capacity (mg AEAC/g fresh weight).

ABTS radical scavenging assay

ABTS·+ solution was prepared by dissolving 7 mM ABTS in 2.45 mM potassium persulfate. It was then diluted to an absorbance of 0.700 ± 0.02 at 734 nm. In a 96-well plate, 200 µl of this solution was combined with 20 µl of sample extract and incubated in the dark for 30 min. Absorbance was measured at 734 nm using microplate reader. The results were expressed as Trolox (Sigma-Aldrich) equivalent antioxidant capacity (mmol TEAC/g fresh weight).

Ferric ion reducing antioxidant power

The working solution was made by mixing acetate buffer (pH 3.6), 10 mM TPTZ (in 40 mM HCl), and 20 mM $FeCl_3 \cdot 6\,H_2O$ at 10:1:1 ratio and incubating at 37 °C for 10 min. In a 96-well plate, 200 µl of the FRAP solution was combined with 20 µl of the extract and incubated for 6 min at 37 °C. The absorbance was measured at 570 nm using a microplate reader. The results were expressed as $FeSO_4$ (QRëC, Auckland, New Zealand,) equivalent antioxidant capacity (mg $FeSO_4$/g fresh weight).

Isolation and measurement of total protein content from rice seeds

Rice seeds were ground into fine powder using a mixer mill MM400 (Retsch, Haan, Germany) and sieved through a 100-mesh screen. Following the instruction of Quick Start™ Bradford Protein Assay [26], 0.2 g of rice powder was mixed with 500 µl of extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl

(pH 7.5), 50 mM EDTA, 2% v/v 2-mercaptoethanol, 1% w/v PVPP), vortexed, and incubated at 4 °C for 30 min. The mixture was centrifuged at 12,000 rpm (13,800×g) for 10 min, and 250 µl of the supernatant was combined with 300 µl of 0.6 M ammonium acetate (Merck) and incubated at −20 °C for 30 min. Next, the proteins were precipitated by centrifugation for 10 min, then washed with 300 µl of 70% ethanol before being centrifuged at 12,000 rpm for 5 min (3 times). After drying the protein pellet for 10 min, 300 µl of extraction buffer was added and vortex. The protein solution was quantified by Bradford assay [27] using Bovine Serum Albumin (BSA) (Sigma-Aldrich, Co.) as the protein standard.

Statistical analysis

The experiments were conducted under RCBD with four replications (4 plants per replication). Analysis of variance (ANOVA) was performed, and the mean of each parameter was compared with Duncan's multiple range test (DMRT) using the IBM SPSS statistic software and accepting the significant level at $p < 0.05$.

RESULTS

Application of Type I and Type II CB positively influences soil physicochemical properties

The effects of CB application on soil physicochemical properties were determined before transplantation (7 days after the soil application). Both Type I and Type II CB increased soil OM in a concentration-dependent manner, with minimal impact on the EC (Table 1). Notably, soil pH was significantly raised to a maximum of 7.14 ± 0.09 by 0.2% Type II CB supplementation, compared to the lowest pH of 6.37 ± 0.35 found in the control. After harvest, soil pH and EC increased across the treatments as compared to the respective pre-transplantation levels. Moreover, OM content generally increased, with the most pronounced enhancements observed in soils treated with 0.1% and 0.2% Type II CB (Table 1).

The application of CB significantly enhanced soil macronutrient levels, including total N, P, K, Ca, Mg, and S, particularly when 0.1% or 0.2% of Type I or Type II CB were applied (Table 2). However, after harvest, an overall reduction in all macronutrients was observed across all the treatments, with the control showing the most pronounced decline (Table 2).

In terms of micronutrient availability, the application of Type I and Type II CB led to the increase of Fe, Mn, and Mo (Table 3). Type I CB did not affect the content of Zn and Si, while Type II CB at 0.1 and 0.2% significantly increased Zn and Si in soil. Especially, 0.2% Type II CB supplementation caused more than 36 times increase in Si concentration than the control treatment. After harvest, a decrease in all micronutrient levels was noted across the treatments, particularly for Fe and Si, when compared to the pre-transplantation soil (Table 3). Together, these findings indicate that CB application has the potential to improve the physicochemical properties of the soil under pot experiments, contributing to enhanced nutrient availability and soil quality.

CB application significantly enhances tillering and grain yield of 'RD43' rice

Comparison of plant growth and total grain yield unveiled that both Type I and Type II CB treatments increased tiller number and yield per plant collectively in a concentration-dependent manner (Fig. 1). Both types of CB boosted tiller number up to 25.6% (Fig. 1A). Supplementation of 0.2% Type I or Type II CB led to more than 11 tillers, while 'RD43' rice without CB treatments had approximately 9.3 tillers. Additionally, total grain yield per plant was substantially elevated following the application of 0.1% or 0.2% Type I and Type II CB (Fig. 1B). As compared to the control, soil supplemented with 0.2% Type I or Type II CB increased grain yield by 58.6% and 69.8%, respectively. The highest yield per plant (56.88 g) was observed in plants treated with 0.2% Type II CB (Fig. 1B).

Interestingly, when different yield-related parameters were explored, it was found that the increase in grain yield by CB was due to the increase in the number of spikelets and filled grains per panicle rather than the increase in panicle number per plant (Table 4). The significantly higher number of filled grains per panicle could be detected with the treatment of 0.1% or 0.2% CB. Moreover, application of 0.2% Type II CB significantly increased spikelet number per panicle, leading to the enhancement of seed yield. Notably, the application of CB had no significant effect on flowering time, number of panicles per plant, or seed weight (Table 4). Collectively, it can be concluded that CB treatments enhance tillering capacity and increase grain yield of 'RD43' rice, specifically by promoting spikelet and filled grain per panicle.

CB application maintains starch properties of 'RD43' rice seeds

Given that 'RD43' is a rice cultivar with consumer preference for healthier starch properties, we determined whether AAC, RAG, SAG and RS of 'RD43' rice seeds were altered by CB treatments. Starch properties measured from cooked rice showed no significant differences among the treatments (Table 5). The AAC of 'RD43' seeds ranged from 18.4% to 19.23%, which is considered as medium levels. High concentrations of Type I CB tended to lower RAG. However, these were not significantly different from the control treatment. The percentages of SAG and RS in 'RD43' seeds were also not different among the treatments (Table 5). These data indicate that CB application has negligible impact on the key starch properties of 'RD43' rice.

Table 1 Physicochemical properties of the soil at 7 days after CB supplementation (pre-transplantation) and after harvest: the soil pH, EC, and OM.

Treatment	Pre-transplantation			After harvest		
	Soil pH	Soil EC (dS/m)	OM (%)	Soil pH	Soil EC (dS/m)	OM (%)
Control	6.37 ± 0.35^{g}	0.24 ± 0.05^{cd}	1.46 ± 0.07^{h}	7.80 ± 0.42^{a}	0.27 ± 0.02^{a}	1.47 ± 0.05^{gh}
0.01% Type I	6.73 ± 0.31^{efg}	0.24 ± 0.29^{cd}	1.48 ± 0.05^{gh}	7.62 ± 0.32^{ab}	0.25 ± 0.02^{bc}	1.58 ± 0.06^{efg}
0.1% Type I	6.70 ± 0.42^{fg}	0.24 ± 0.15^{cd}	1.66 ± 0.04^{def}	7.67 ± 0.22^{ab}	0.25 ± 0.01^{bc}	1.74 ± 0.23^{d}
0.2% Type I	6.91 ± 0.38^{def}	0.23 ± 0.25^{d}	2.36 ± 0.17^{b}	7.83 ± 0.20^{a}	0.26 ± 0.02^{ab}	2.07 ± 0.12^{c}
0.01% Type II	6.81 ± 0.04^{ef}	0.25 ± 0.18^{bc}	1.64 ± 0.05^{def}	7.50 ± 0.43^{abc}	0.26 ± 0.02^{ab}	2.37 ± 0.12^{b}
0.1% Type II	6.91 ± 0.33^{def}	0.24 ± 0.39^{cd}	1.73 ± 0.02^{d}	7.54 ± 0.20^{abc}	0.25 ± 0.02^{bc}	2.72 ± 0.38^{a}
0.2% Type II	7.14 ± 0.09^{cde}	0.24 ± 0.16^{cd}	2.68 ± 0.12^{a}	7.26 ± 0.11^{bcd}	0.25 ± 0.01^{bc}	2.72 ± 0.10^{a}

Note: data are shown as mean ± SD, derived from 4 replications. Means in a column with different superscript lowercase letters are significantly different ($p < 0.05$; DMRT) compared within the same period.

Table 2 Soil element composition: total N, P, K, Ca, Mg, and S contents of the soil at 7 days after CB treatments (pre-transplantation) and after harvest.

Condition	Treatment	Total N (%)	P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	S (mg/g)
Pre-transplantation	Control	0.07 ± 0.01^{f}	5.698 ± 0.049^{f}	4.359 ± 0.059^{f}	0.520 ± 0.015^{h}	0.340 ± 0.020^{e}	0.623 ± 0.010^{f}
	0.01% Type I	0.09 ± 0.01^{e}	6.275 ± 0.100^{e}	4.601 ± 0.014^{e}	1.213 ± 0.028^{d}	0.380 ± 0.018^{e}	0.697 ± 0.026^{e}
	0.1% Type I	0.10 ± 0.01^{d}	8.456 ± 0.154^{c}	5.178 ± 0.014^{d}	1.708 ± 0.050^{c}	0.606 ± 0.015^{c}	1.082 ± 0.036^{c}
	0.2% Type I	0.12 ± 0.01^{bc}	9.127 ± 0.080^{b}	6.198 ± 0.059^{b}	2.422 ± 0.099^{a}	1.077 ± 0.031^{b}	1.177 ± 0.017^{b}
	0.01% Type II	0.09 ± 0.10^{e}	7.080 ± 0.557^{d}	4.332 ± 0.058^{f}	1.250 ± 0.021^{d}	0.528 ± 0.051^{d}	0.938 ± 0.009^{d}
	0.1% Type II	0.10 ± 0.01^{d}	8.878 ± 0.068^{bc}	5.301 ± 0.050^{c}	1.611 ± 0.054^{c}	0.664 ± 0.029^{c}	1.124 ± 0.013^{c}
	0.2% Type II	0.13 ± 0.01^{ab}	9.408 ± 0.128^{a}	7.342 ± 0.037^{a}	2.070 ± 0.084^{b}	1.208 ± 0.039^{a}	1.455 ± 0.016^{a}
After harvest	Control	0.07 ± 0.05^{f}	0.572 ± 0.024^{g}	0.597 ± 0.020^{h}	0.804 ± 0.080^{efg}	0.078 ± 0.005^{f}	0.123 ± 0.006^{g}
	0.01% Type I	0.08 ± 0.05^{ef}	0.629 ± 0.006^{g}	0.720 ± 0.021^{g}	0.890 ± 0.141^{e}	0.067 ± 0.005^{f}	0.133 ± 0.008^{g}
	0.1% Type I	0.07 ± 0.01^{f}	0.651 ± 0.035^{g}	0.618 ± 0.007^{gh}	0.842 ± 0.036^{ef}	0.064 ± 0.005^{f}	0.143 ± 0.002^{g}
	0.2% Type I	0.11 ± 0.01^{cd}	0.625 ± 0.023^{g}	0.697 ± 0.017^{gh}	0.673 ± 0.063^{fgh}	0.063 ± 0.002^{f}	0.159 ± 0.014^{g}
	0.01% Type II	0.07 ± 0.01^{f}	0.556 ± 0.084^{g}	0.670 ± 0.042^{gh}	0.637 ± 0.015^{gh}	0.059 ± 0.006^{f}	0.123 ± 0.004^{g}
	0.1% Type II	0.12 ± 0.02^{bc}	0.593 ± 0.017^{g}	0.616 ± 0.013^{gh}	0.673 ± 0.033^{fgh}	0.058 ± 0.003^{f}	0.147 ± 0.009^{g}
	0.2% Type II	0.14 ± 0.05^{a}	0.736 ± 0.044^{g}	0.639 ± 0.025^{gh}	0.687 ± 0.015^{fgh}	0.062 ± 0.002^{f}	0.151 ± 0.009^{g}

Note: data are shown as mean ± SD, derived from 4 replications. Means in a column with different superscript lowercase letters are significantly different ($p < 0.05$; DMRT) compared within the same period.

Table 3 Contents of Micronutrient elements (Fe, Zn, Cu, Mn, Mo, and Si) of the soil at 7 days after CB treatments (pre-transplantation) and after harvest.

Condition	Treatment	Fe (mg/g)	Zn (mg/g)	Cu (mg/g)	Mn (mg/g)	Mo (mg/g)	Si (mg/g)
Pre-transplantation	Control	5.850 ± 0.140^{d}	0.026 ± 0.001^{cd}	0.014 ± 0.001^{a}	0.052 ± 0.008^{cd}	0.028 ± 0.005^{bc}	0.211 ± 0.026^{fg}
	0.01% Type I	6.115 ± 0.250^{d}	0.024 ± 0.002^{cd}	0.014 ± 0.008^{a}	0.059 ± 0.007^{b}	0.032 ± 0.001^{ab}	0.253 ± 0.008^{fg}
	0.1% Type I	7.198 ± 0.416^{c}	0.025 ± 0.032^{cd}	0.015 ± 0.002^{a}	0.061 ± 0.005^{b}	0.032 ± 0.002^{ab}	0.264 ± 0.006^{fg}
	0.2% Type I	8.690 ± 0.132^{a}	0.025 ± 0.002^{cd}	0.013 ± 0.001^{a}	0.063 ± 0.011^{b}	0.036 ± 0.002^{a}	0.271 ± 0.006^{fg}
	0.01% Type II	6.964 ± 0.117^{c}	0.024 ± 0.001^{cd}	0.014 ± 0.001^{a}	0.065 ± 0.008^{a}	0.034 ± 0.002^{a}	2.618 ± 0.153^{c}
	0.1% Type II	8.256 ± 0.185^{b}	0.030 ± 0.045^{b}	0.015 ± 0.002^{a}	0.066 ± 0.006^{a}	0.036 ± 0.002^{a}	6.233 ± 0.165^{b}
	0.2% Type II	8.945 ± 0.097^{a}	0.032 ± 0.001^{a}	0.015 ± 0.001^{a}	0.068 ± 0.009^{a}	0.038 ± 0.002^{a}	7.733 ± 0.412^{a}
After harvest	Control	0.065 ± 0.004^{e}	0.017 ± 0.001^{f}	0.011 ± 0.001^{ab}	0.049 ± 0.003^{cd}	0.020 ± 0.003^{de}	0.157 ± 0.007^{g}
	0.01% Type I	0.062 ± 0.004^{e}	0.018 ± 0.002^{ef}	0.013 ± 0.002^{a}	0.044 ± 0.008^{cd}	0.028 ± 0.002^{bc}	0.172 ± 0.007^{g}
	0.1% Type I	0.060 ± 0.001^{e}	0.021 ± 0.001^{e}	0.013 ± 0.002^{a}	0.039 ± 0.002^{d}	0.022 ± 0.002^{cde}	0.168 ± 0.005^{g}
	0.2% Type I	0.062 ± 0.001^{e}	0.020 ± 0.002^{e}	0.005 ± 0.028^{c}	0.058 ± 0.008^{bc}	0.022 ± 0.001^{cde}	0.170 ± 0.016^{g}
	0.01% Type II	0.063 ± 0.001^{e}	0.020 ± 0.001^{e}	0.009 ± 0.002^{bc}	0.042 ± 0.002^{d}	0.016 ± 0.002^{e}	0.583 ± 0.019^{ef}
	0.1% Type II	0.068 ± 0.003^{e}	0.023 ± 0.002^{de}	0.007 ± 0.002^{bc}	0.045 ± 0.004^{cd}	0.026 ± 0.002^{bcd}	0.703 ± 0.031^{e}
	0.2% Type II	0.074 ± 0.002^{e}	0.028 ± 0.001^{c}	0.009 ± 0.002^{bc}	0.047 ± 0.007^{cd}	0.021 ± 0.002^{de}	1.261 ± 0.031^{d}

Note: data are shown as mean ± SD, derived from 4 replications. Means in a column with different superscript lowercase letters are significantly different ($p < 0.05$; DMRT) compared within the same period.

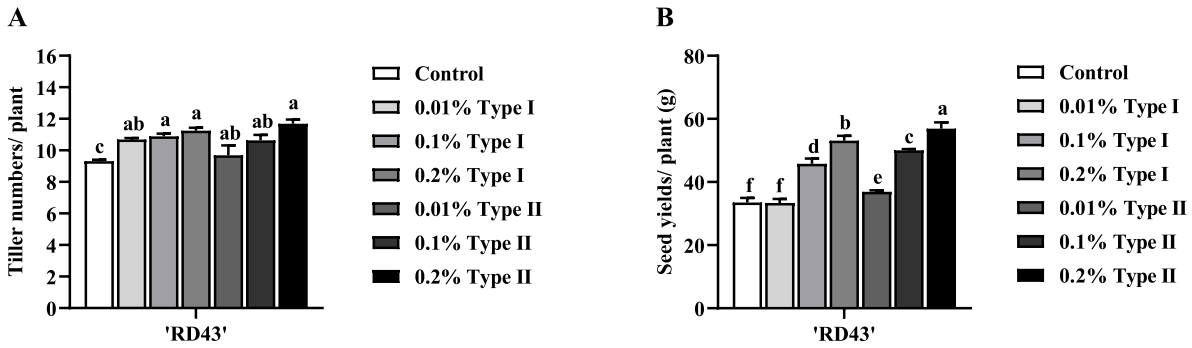

Fig. 1 Promotion of tiller numbers (A) and seed yield (B) of 'RD43' rice by CB application. Tiller numbers were recorded at 49 DAT (maximum tillering stage). Each value represents mean ± SD ($n = 16$). The different letters above the bars indicate significant statistical difference by DMRT at $p < 0.05$.

Table 4 The effect of various concentrations of Type I and Type II CB as a soil supplement on flowering time and yield components. The yield components include number of panicles per pot, spikelets per panicle, filled grains per panicle, grain yield per pot, and 100-grain-weight.

Treatment	Day of flowering (d)	Panicles/pot	Spikelets/panicle (seeds)	Filled grain/panicle (seeds)	100-grain weight (d)
Control	66.00 ± 1.53	8.33 ± 0.58	181.67 ± 6.02^{bc}	101.67 ± 5.51^{f}	2.26 ± 0.02
0.01% Type I	66.33 ± 1.53	9.05 ± 0.58	181.67 ± 6.43^{bc}	117.11 ± 6.25^{ef}	2.22 ± 0.02
0.1% Type I	65.00 ± 0.58	9.67 ± 0.58	180.33 ± 3.51^{bc}	122.09 ± 4.58^{e}	2.18 ± 0.06
0.2% Type I	65.67 ± 0.58	9.07 ± 1.00	189.23 ± 5.29^{bc}	175.03 ± 3.22^{ab}	2.21 ± 0.65
0.01% Type II	65.33 ± 0.58	9.67 ± 0.58	182.67 ± 6.66^{bc}	110.67 ± 7.09^{ef}	2.25 ± 0.01
0.1% Type II	65.67 ± 0.58	9.33 ± 0.58	188.33 ± 4.47^{bc}	174.33 ± 1.53^{ab}	2.31 ± 0.65
0.2% Type II	66.00 ± 1.53	9.67 ± 0.58	210.33 ± 4.57^{a}	183.33 ± 5.77^{a}	2.13 ± 0.17

Note: data are shown as mean ± SD, derived from 16 plants per treatment (four replications with four plants/replicate). Means in a column with different superscript lowercase letters are significantly different ($p < 0.05$; DMRT).

CB treatments increase antioxidant levels of 'RD43' rice seeds

As chitin and chitosan are known to induce antioxidant systems in plants, we investigated whether CB application could increase antioxidant levels of 'RD43' rice seeds. To this end, antioxidant activity of milled grains was measured by DPPH, ABTS, and FRAP assays. Indeed, soil supplementation with 0.1% or 0.2% of Type II CB improved antioxidant capacity of 'RD43' rice seeds when detected with DPPH assay (Fig. 2A), while 0.1%, 02% Type I CB and 0.01–0.2% Type II CB showed a significant increase in antioxidant levels with ABTS assay (Fig. 2B). For DPPH assay, seeds obtained from 0.2% Type II CB application showed the highest level of antioxidant capacity of 0.641 mg AEAC/g FW, which was increased by 16.5%, compared to the control (Fig. 2A). For ABTS assay, the antioxidant capacity increased from 0.0152 mg TEAC/g FW in the control to 0.0175 mmol TEAC/g FW in 0.2% Type II CB

Table 5 Starch properties: AAC, RAG, SAG, and RS of milled 'RD43' rice seeds, obtained from control, Type I CB and Type II CB treatments.

Treatment	'RD43'			
	AAC (%)	RAG (%)	SAG (%)	RS (%)
Control	19.23 ± 0.65	22.06 ± 1.25	12.28 ± 1.90	3.23 ± 0.59
0.01% Type I	18.40 ± 0.64	20.99 ± 1.07	12.22 ± 1.74	3.84 ± 0.49
0.1% Type I	18.05 ± 0.21	21.24 ± 0.98	12.45 ± 1.42	3.41 ± 0.40
0.2% Type I	19.01 ± 0.11	20.02 ± 1.03	12.28 ± 1.29	3.66 ± 0.16
0.01% Type II	18.59 ± 0.32	21.27 ± 0.79	15.14 ± 1.11	3.53 ± 0.36
0.1% Type II	18.95 ± 0.32	20.60 ± 0.47	13.89 ± 1.02	3.17 ± 0.21
0.2% Type II	18.46 ± 0.28	21.69 ± 1.21	14.49 ± 1.03	3.45 ± 0.80

Note: data are shown as mean ± SD, derived from 16 plants per treatment (four replications with four plants/replicate). Means in a column with different superscript lowercase letters are significantly different ($p < 0.05$; DMRT).

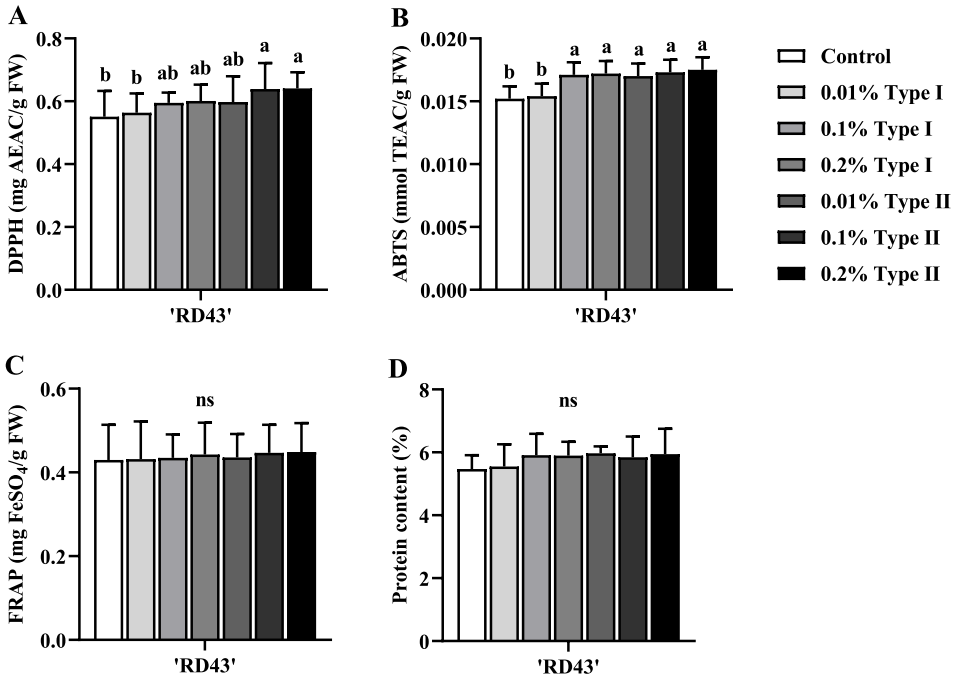

Fig. 2 Antioxidant activity of milled grains detected via DPPH (A), ABTS (B) and FRAP (C) assays, and whole grain protein content (D) of 'RD43' rice grown under control, Type I CB and Type II CB supplements. Data represent mean ± SD ($n = 16$). Different letters above the bars indicate significant difference by DMRT at $p < 0.05$.

treatment, accounting for 15.13% elevation (Fig. 2B). However, no significant changes in antioxidant levels were observed between the treatments when measured with FRAP assays (Fig. 2C). Additionally, CB application had no impact on seed protein contents (Fig. 2D). Based on these findings, it can be concluded that CB application significantly enhances the antioxidant properties of 'RD43' seeds without any alteration in the total protein content.

DISCUSSION

The application of CB in pot experiments was shown to enhance soil physicochemical properties, including OM content (Table 1), macronutrient levels (Table 2), and micronutrient availability (Table 3). This suggests that CB, as an OM rich in essential nutrients, benefits plant growth and productivity. OM in soil serves as a nutrient reservoir, boosting microbial activity, promoting nutrient cycling, and improving soil physical, chemical, and biological properties [28]. The observed increase in OM following CB application can be attributed to its direct carbon contribution from shrimp shells [29] and its role in stimulating microbial biomass, which enhances the decomposition of native OM and the release of microbial byproducts that aid in soil aggregation and carbon stabilization [30]. Addition of OMs in wetland rice soil has been shown to increase N-supplying capability, soil pH, resulting in improved nutrient release and availability [31].

Our findings indicate that CB enhances yield components and overall grain yield of 'RD43' rice. Maintaining macronutrient levels is crucial for crop yield and soil fertility. This study demonstrated that CB application, particularly at 0.2% Type II, significantly increased nitrogen levels (Table 2), which was related to higher tiller numbers and increased yield of 'RD43' rice (Fig. 1). Nitrogen is vital for rice production, influencing tiller development, leaf area, biomass accumulation, and spikelet formation [32]. Additionally, CB enhanced soil P and K levels, leading to increased spikelets and filled grains per panicle (Table 4). Phosphorus is essential for energy storage, cell division, and tissue development, and its uptake during late growth stages (heading and maturity) promotes grain production in rice [33]. Phosphorus has been shown to affect the number of floral buds, overall biomass production, and crop yield in cotton plants [34]. Potassium is involved in facilitating energy transfer, water uptake, nutrient transport, and other physiological activities [35].

The application of CB also improved the levels of Ca, Mg, and S in soil (Table 4). CB formulations containing chitin, such as those with shrimp shells, provide 17–21% chitin along with Ca, K, Mg, and Fe [36]. Magnesium, a critical component of chlorophyll, supports photosynthesis, and application of Mg and Si fertilizers could enhance plant growth by increasing stem strength and lodging resistance

of rice plants [37]. Together with other elements, CB may offer additional Mg, which helped to support vegetative growth. Micronutrients like Zn, Mn, and Fe could activate essential enzymatic processes in plants [38]. Zinc, for example, is vital for metabolic processes, energy production, and improving phosphorus availability in rice [39]. A previous study has also highlighted the benefits of zinc sulfate to increase crop yield in potatoes [40].

White rice contains health-promoting compounds, including phenolic compounds, sterols, γ-oryzanol, tocotrienols, and tocopherols, which are concentrated mainly in the outer layers of the grain, such as the pericarp and aleurone. Such antioxidants can impact the cellular redox status in human plasma, potentially protecting against or reducing the risk of chronic diseases linked to oxidative stress [41]. Yuliana and Akhbar [42] reported antioxidant levels in Cianjur white rice at 0.56 mg AEAC/g in raw rice and 0.33 mg AEAC/g in cooked rice. Similarly, the antioxidant activity in 'RD43' rice under control conditions, as measured by DPPH, was 0.55 mg AEAC/g FW. Generally, the antioxidant capacity of white rice was notably lower than colored rice. The average antioxidant capacity for white rice cultivars was 186.78 µg AEAC/g, ranging from 78.32 to 349.33 µg AEAC/g [23]. Antioxidant levels are influenced by genetics, environmental factors (e.g., temperature, light, precipitation), cultural practices (e.g., irrigation, fertilization), and post-harvest processes like drying and milling [43]. Moreover, prebiotic properties of rice, could be enhanced for functional food characteristics by *Pleurotus ostreatus* fermentation after germination [44]. In this study, we showed that Type II CB can improve the antioxidant activity of 'RD43' seeds up to 0.641 mg AEAC/g FW (Fig. 2), which was higher than the range of antioxidant levels in white rice. CB is primarily composed of shrimp shells, containing chitin and potential chitosan from *B. licheniformis*' chitinase and deacetylase activities. These molecules are recognized as microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), which activate plant immunity and disease resistance by interacting with chitin elicitor binding proteins (CEBiP) and lysin motif (LysM) receptors. These interactions initiate intracellular signaling pathways mediated by MAP kinases. Additionally, chitosan as a signal molecule could activate cellular radical scavenging activity [45]. Previous studies showed that, apart from promoting growth, chitin application also enhances secondary metabolites and antioxidant activity in plants under abiotic stress [46]. This may explain how CB application enhances the antioxidant capacity of 'RD43' rice seeds.

Although amylose and protein content have been reported to affect the texture of cooked rice [47], our study showed that the application of CB did not alter starch properties (Table 5) or protein content (Fig. 2D) of 'RD43' seeds. As appropriate starch properties are crucial for health, 'RD43' rice, with its moderate amylose content and resistant starch, remains suitable for diabetes patients [48]. Overall, the use of CB promotes vegetative growth and grain yield without affecting the texture of cooked rice or protein content. Therefore, CB can be considered a promising method for enhancing rice production while improving antioxidant levels in white rice.

CONCLUSION

In this study, CB treatments were shown to enhance rice production in the 'RD43' cultivar by improving nutrient availability. Soil supplementation with 0.2% Type II CB resulted in maximum yield production, characterized by an increased number of spikelets and filled grains per panicle. While the starch characteristics of rice plants treated with 0.2% Type II CB remained unaffected, antioxidant levels of brown seeds were significantly elevated. Consequently, CB soil supplements offer an effective approach to enhancing both the quantity and quality of 'RD43' rice.

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