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Effects of chitin nanofiber application on plant growth and its differences by soil type

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ABSTRACT
Large numbers of crab shells are disposed of as food waste in the food processing process. Chitin nanofiber (CNF) refers to chitin extracted from crab shells in the form of ultrafine fibers that enable uniform dispersion in water. We explored the feasibility of using CNF materials as plant growth promoters. We investigated the effects of CNF application with fertilizer and its various application methods on the growth of the komatsuna plant cultivated in Entisols and Andosols with the application of CNF materials. The surface application of CNF materials had plant growth-promoting effects in both Entisols and Andosols. The topdressing treatment was more effective in promoting plant growth in Entisols. The inorganic nitrogen content absorbed by plants in the topdressing treatment was lower than that in the basal application treatments because the CNF added during the latter half of the cultivation period was not fully decomposed before the completion of cultivation. The calcium content of plants in the basal application treatment of CNF/protein/calcium carbonate was higher than that in the topdressing treatments, indicating that the calcium encasing the CNF was absorbed by the plants. The cultivation of plants with the application of CNF materials promoted nitrogen utilization efficiency and plant growth.

Contribution/Originality: The study demonstrates that the application of chitin nanofiber materials produced using crab shells promotes plant growth. The plant growth-promoting effect of chitin nanofiber materials depends on the soil type and the method of application to the soil. The study proposes an effective method of recycling crab shells for use in agriculture.

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1. INTRODUCTION
Crabs are crustaceans with a single pair of pincers that live in all oceans of the world, in freshwater, and on land. Sakaiminato, located on the western border of Tottori prefecture, Japan, is a popular fishing port for crabs, and in particular the primary source of *Chionoecetes opilio* (red snow crab) in Japan (Motoh & Yamamoto, 1998). A seafood processing company that peels the crabs is located in the nearby area of Sakaiminato. Since only 40% of a crab's total weight is eaten, a large amount of crab shell is generated as food waste during processing. Crab shells have long been

considered waste and disposed of in landfills, causing environmental pollution and wasting biomass resources. Studies on the reuse of crab shells have considered ways to recycle such large quantities of crab shells (Jeon & Yeom, 2009; Rizzi et al., 2019; Rizzi et al., 2020). Mageshwaei and Thiripurasundari (2022) suggested that crab shell could be an effective organic fertilizer for improving soil quality, plant growth and mineral quality. It is necessary to further consider the reuse of crab shell waste from an agricultural standpoint against the increasing amounts being produced. Notably, crab shell is an organic source, with the major components being chitin, protein, and calcium carbonate ($CaCO_3$). The chitin, protein, and $CaCO_3$ content of crab shells is approximately 30%, 15%, and 55% w/w, respectively (Aklog et al., 2016). Chitin is a long-chain polymer of N-acetylglucosamine, an amide derivative of glucose. Therefore, this material cannot be dissolved in most solvents. However, it is possible to nanofabricate it into fibers the thickness of one ten-thousandth of a human hair by using a special technique to grind it (Ifuku & Saimoto, 2012). Thus, chitin nanofiber (CNF) is a nanofiber material made from chitin, which can be taken from the shells of crustaceans. CNF refers to chitin extracted from the outer shell of crustaceans, such as crab shells, in the form of ultrafine fibers (10–20 nm). CNF displays uniform dispersion in water, which is not possible with conventional chitin powder, and facilitates blending with other materials and molding. Thus, CNF is characterized by high dispersibility in water and a variety of potential applications. Its high concentration and translucency, as well as its fine structure, allow it to be mixed with other base materials, wetted, and molded for a wide range of applications (Ifuku & Saimoto, 2012). CNF is produced by processing the chitin contained in crab shells and is used in medical products (Azuma, Ifuku, Osaki, Okamoto, & Minami, 2014; Ifuku & Saimoto, 2012) and cosmetics (Triunfo et al., 2021). Aklog et al. (2016) prepared a CNF/protein/$CaCO_3$ complex by ejecting the slurry under a high pressure of 200 MPa through a small nozzle with a diameter of 100 μm. They prepared the CNF/protein complex by using a domestic blender under wet conditions with 2 M hydrochloric acid for 2 days at room temperature, with occasional stirring to remove mineral salts (primarily $CaCO_3$). The total nitrogen contents of CNF/protein/$CaCO_3$, CNF/protein, and CNF are 0.16%, 0.16%, and 0.11%, respectively. Its growth-promoting effect has been confirmed in plants grown under hydroponic cultivation conditions (Egusa, Parada, Aklog, Ifuku, & Kaminaka, 2019). The growth-promoting effect in hydroponic cultivation was confirmed using the CNF/protein complex and the CNF/protein/$CaCO_3$ complex as a liquid fertilizer. In particular, the CNF/protein/$CaCO_3$ complex resulted in a growth-promoting effect compared with other materials (Aklog et al., 2016). It has also been confirmed that the CNF/protein and CNF/protein/$CaCO_3$ treatments could reduce the costs associated with hydroponic cultivation because of the omission of certain preparation procedures during material preparation (Aklog et al., 2016). Egusa et al. (2020) also investigated the behavior of CNF applied to plants under hydroponic conditions based on fertilizer nitrogen and genetic information. They observed that the nitrogen utilization efficiency of plants increased, and the lower the concentration of liquid fertilizer applied, the greater the observed growth-promoting effect. Nevertheless, there is still limited information on the growth-promoting effects of CFN materials under soil cultivation conditions.

We conducted a plant rooting test with the application of CFN materials to determine the application rates for a cultivation test. Based on the plant rooting test, we applied each material to the soil to investigate the effect of CNF in soil on plants from the perspective of growth promotion. We also evaluated the effect of CNF in different soil types on plants to explore the effects of the application method in each.

2. MATERIALS AND METHODS

2.1. Preparation and Characterization of the CNF Materials

Our preparation of the CNF materials followed the procedure of Aklog et al. (2016).

2.2. Plant Rooting Test with the Application of Chitin Nanofiber Materials

Root growth in the early growth stage with the application of CNF was examined using a seed pack growth pouch. Ten seeds of komatsuna (*Brassica rapa* var. *perviridis* cv. Saori) were sown in seed packs (177×163mm, Daiki Rika Kogyo Co., Ltd., Japan), and 15 mL of reverse osmosis (RO) water and 0.01%, 0.05% and 0.10% (chitin concentration) solutions of CNF, CNF/protein, and CNF/protein/$CaCO_3$ were added, respectively. The seed packs were placed in a thermostatic chamber in a dark place at 25°C for 8 days. Every two days, 5 mL of each solution was added to the pouches to maintain a constant solution volume during the experiment. The root length was measured every two days.

2.3. Plant Cultivation

2.3.1. Soils

Sandy soil (Entisols) and kuroboku soil (Andosols) (Soil Survey Staff, 2014) were collected from the Tottori Sand Dune, Japan, and Koyama, Tottori, Japan, respectively. The physicochemical properties of the soil used in this study are shown in Table 1.

2.3.2. Effect of the Combined Application of CNF Materials and Fertilizer on Plant Growth

Plant cultivation experiments were conducted for 34 days from June 3, 2020, to July 6, 2020, using komatsuna (*B. rapa* var. *perviridis* cv. Saori), a Japanese mustard spinach. It was cultivated in a greenhouse, and the seedlings were raised in nursery boxes for 2 weeks. Two of the raised komatsuna plants were transplanted into 1/10,000 Wagner pots filled with 1700 g/pot of Entisols and 900 g/pot of Andosols. The CNF, CNF/protein, and CNF/protein/$CaCO_3$ complexes were applied to the Wagner pots at 0.01% (w/v) chitin, respectively, and mixed thoroughly with the soil. No-fertilizer treatment and fertilizer treatment were set up for each soil type. In the

fertilizer treatment, a basal dose of N, P_2O_5, and K_2O was applied to each pot at 120, 120, and 120 kg ha^{-1} (120, 120, and 120 mg pot^{-1}) using chemical fertilizer (Asahi Ace, Jcam Agri. Co., Ltd., Japan), respectively. After transplanting, each komatsuna plant was grown for 20 days, and triple replicates were performed for each treatment. Water was applied to each pot in the morning and evening to maintain 60% of the maximum water-retaining capacity of the soils during the growing period.

Table 1. Physicochemical properties of Entisols and Andosols (mean ± standard deviation, n= 4).

Property	Entisols		Andosols	
pH (1:2.5, Soil:Water)	6.13	± 0.04	5.36	± 0.03
EC (1:5, Soil:Water; mS m^{-1})	0.57	± 0.09	5.03	± 0.11
Total C (g kg^{-1})	0.92	± 0.07	56.7	± 2.51
Total N (g kg^{-1})	0.16	± 0.01	4.38	± 0.17
NO_3-N (mg kg^{-1})	3.49	± 0.12	20.73	± 1.42
NH_4-N (mg kg^{-1})	0.35	± 0.09	1.60	± 0.09
Truog available P (mg kg^{-1})	0.73	± 0.04	2.34	± 0.16
Phosphate absorption coefficient	7	± 0.21	1964	± 38
Exchangeable Ca (cmolc kg^{-1})	0.23	± 0.04	0.59	± 0.08
CEC (cmolc kg^{-1})	0.97	± 0.08	29.48	± 1.26
Soil texture	S		LiC	

2.3.3. Effect of the Application Methods of CNF Materials on Plant Growth

Plant cultivation experiments were conducted for 34 days from April 23, 2021, to May 26, 2021, using komatsuna. The seedlings, amount of soil in pots, and quantities of fertilizer were identical to those described in Section 2.3.2. The CNF, CNF/protein, and CNF/protein/$CaCO_3$ complexes were applied to the surface in two treatments, respectively including 1) the basal application of 0.01 w/v% to the surface immediately before transplanting and 2) the topdressing treatments of 0.0005 w/v% to the surface every day (the total application rates were 0.01 w/v% throughout the growing period). After transplanting, each komatsuna plant was grown for 20 days, and triple replicates were performed for each treatment. Water was applied to each pot in the morning and evening to maintain 60% of the maximum water-retaining capacity of the soils during the growing period.

2.4. Statistical Analysis

Data were statistically evaluated using a one-way analysis of variance (one-way ANOVA) with a Tukey's honestly significant difference test ($P < 0.05$) using International Business Machines (IBM)® SPSS® statistics software (version 27.0).

3. RESULTS AND DISCUSSION

3.1. Plant Rooting Test with CNF Application

The results of plant root growth using the seed pack growth test are shown in Figure 1. Root lengths in the 0.01% and 0.05% CNF treatments were almost identical to those in the RO water treatment. However, root elongation was significantly suppressed in the 0.10% CNF treatment, reducing significantly from the 4th day onward. Treatment with 0.01% and 0.05% CNF/protein resulted in significantly increased root length compared with the RO water treatment, especially after the 4th day. In contrast, root elongation was suppressed in the 0.10% treatment, reducing significantly after the 4th day. Root length in the 0.01% CNF/protein/$CaCO_3$ treatment increased significantly compared to that in the RO water treatment, and root elongation was gradually promoted from the 4th day onward.

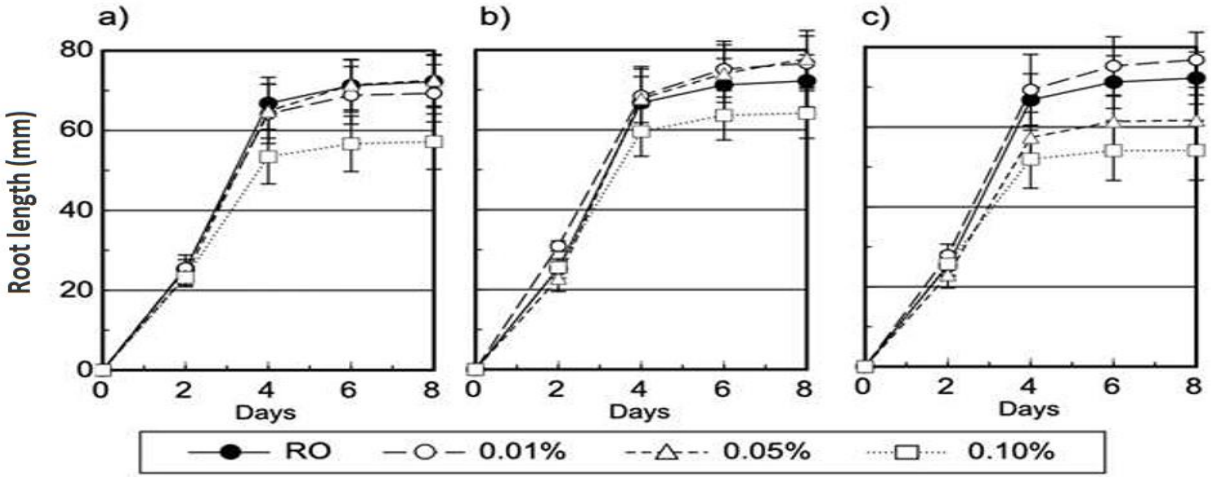

Figure 1. Root lengths in the seed pack growth test: a) chitin nanofiber, b) chitin nanofiber/protein, and c) chitin nanofiber/protein/$CaCO_3$. RO: reverse osmosis water.

Note: 0.01%, 0.05%, and 0.10% are solute concentrations of chitin nanofiber materials, respectively. Data are represented as means ± standard error (n=4).

However, root elongation was suppressed in the 0.05% and 0.10% treatments, which showed significant suppression after the 4th day. Root growth was inhibited with the increase in concentration from the 4th day onward in all treatments. This may be partially due to the concentration impairment caused by the high osmotic pressure around the roots, which prevented the roots from absorbing water. Root elongation was enhanced in the 0.01% CNF/protein and 0.01% CNF/protein/$CaCO_3$ treatments, suggesting that the protein and $CaCO_3$ covering the CNF were associated with root growth.

3.2. Effect of CNF on Plant Cultivation

3.2.1. Plant Growth

Plant height and plant dry matter weight are shown in Figures 2 and 3. Plant growth showed differences according to the soil type and treatment. Obviously, plant height and plant dry matter weight were low under the no fertilizer treatment. In Entisols, plant height was slightly higher in the CNF/protein/$CaCO_3$ treatment under the same conditions of the presence or absence of fertilizer. However, in Andosols, it was higher in the CNF, CNF/protein, and CNF/protein/$CaCO_3$ treatments without fertilizer, although there was no difference among the fertilizer treatments. The dry matter weight in Entisols was higher in the CNF/protein/$CaCO_3$ treatment under the same conditions of the presence or absence of fertilizer. However, there was no difference in the dry matter weight of the soil in Andosols without fertilizer. This result suggests that Entisols were significantly affected by the CNF material. In contrast, the dry matter weights in Andosols with fertilizer exhibited growth effects due to the application of CNF materials, although there was not much difference among the materials. However, the effect of CNF material application was significantly more pronounced with fertilizer. The nitrogen uptake by the plants exhibited a similar trend to that of dry matter weight (Figure 4), suggesting that CNF application promoted plant growth due to increased nitrogen uptake. The increase in the nitrogen utilization efficiency of plants with CNF application under the condition of soil cultivation supports the result of hydroponic cultivation reported by Egusa et al. (2020).

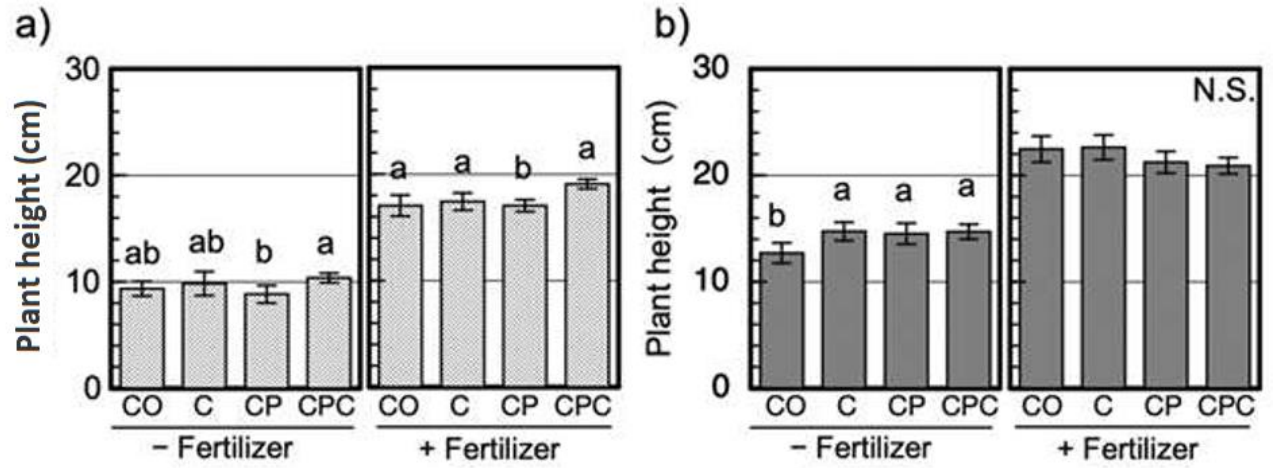

Figure 2. Plant heights: a) Entisols and b) Andosols. CO: control. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.

Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA). N.S.: Not significant.

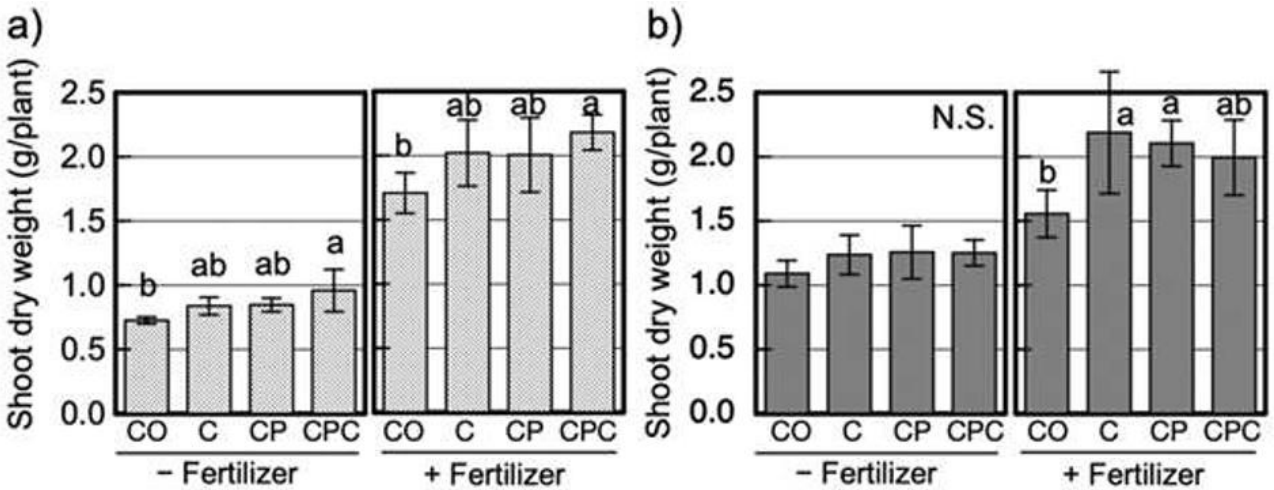

Figure 3. Shoot dry weights: a) Entisols and b) Andosols. CO: control. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.

Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA). N.S.: Not significant.

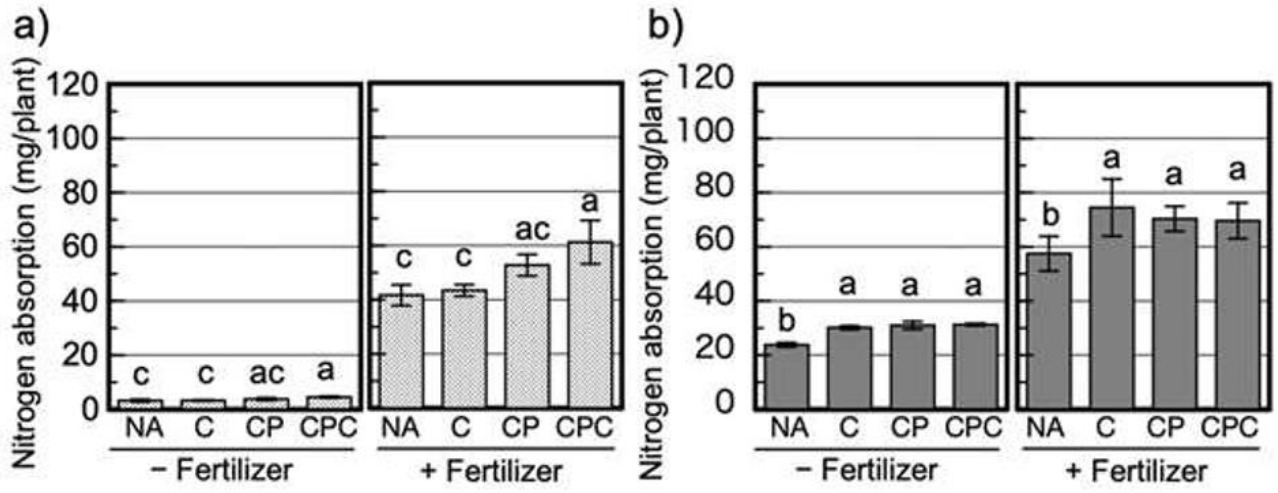

Figure 4. Nitrogen absorption by the plant: a) Entisols and b) Andosols. CO: control. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.

Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA).

3.2.2. Effect of CNF on Soils after Plant Cultivation

The physicochemical properties of the soil after plant cultivation are shown in Table 2. The soil pH after plant cultivation in Entisols with fertilizer was lower than that in Entisols without fertilizer, whereas the soil pH of Andosols showed no differences in the presence or absence of fertilizer. The differences may be due to the difference in the pH buffering capacity of the soils. There were also no differences in the soil pH among the treatments with CNF materials. The levels of ammonia nitrogen did not differ among treatments with CNF materials within the same soil type and with the presence or absence of fertilizer. The levels of nitrate nitrogen were higher in the CNF/protein/$CaCO_3$ treatment with fertilizer in both soil types. A slight decomposition of CNF and protein may be involved in the CNF/protein/$CaCO_3$ treatment with fertilizer in Entisols. Moreover, nitrification by soil bacteria may be involved in the nitrification of fertilizer in both soil types. The CNF might be decomposed at an early stage, and nitrate nitrogen levels might have once increased but been absorbed by the plants under CNF treatment with fertilizer in Entisols (Figure 4). In contrast, the rate of nitrogen absorption in Entisols without fertilizer was lower than that with fertilizer because of the lower abundance of soil microorganisms than in the soil with fertilizer. The levels of nitrate nitrogen did not decrease in the CNF treatment in Entisols with fertilizer. In Andosols, these levels increased due to an increase in the abundance of soil bacteria as a result of the decomposition of soil and CNF materials. This might be caused by the high nitrate nitrogen content in Andosols, due to which the effect of consumption by soil microorganisms was small. The level of exchangeable calcium was slightly higher in soils treated with CNF/protein/$CaCO_3$ than in soils with other treatments.

This was due to the decomposition of $CaCO_3$ adhering to the CNF/protein/$CaCO_3$ complex. The amount of available phosphate was significantly higher in the CNF/protein/$CaCO_3$ treatment in Entisols with fertilizer. This may be because of the effect of CNF on the plants in the CNF/protein/$CaCO_3$ treatment in Entisols with fertilizer. The plant growth-promoting effect of the CNF/protein/$CaCO_3$ treatment in Entisols with fertilizer suggests that CNF, covered by protein and $CaCO_3$, exerted the growth-promoting effect on the plants. In other words, the CNF/protein/$CaCO_3$ treatment promoted root growth before decomposition, and the protein and $CaCO_3$ promoted plant growth after decomposition. In the CNF/protein treatment with fertilizer and the CNF/protein/$CaCO_3$ treatment in Entisols with fertilizer, the protein and nitrate nitrogen, the levels of which increased due to the partial decomposition of CNF, also contributed to growth promotion. The CNF materials were decomposed to some extent by soil microorganisms in Entisols without fertilizer, but the plants did not grow well because of the low nitrogen content. The CNF materials exerted a certain effect in the soil without fertilizer, but they decomposed early and were not highly effective. The abundance of soil microorganisms might have increased, and the CNF materials were decomposed by the fertilizer in Andosols.

Table 2. Physicochemical properties of Entisols and Andosols after cultivation.

Treatments	pH (1:2.5)		EC (1:5) (mS m^{-1})		NO_3-N (mg kg^{-1})		NH_4-N (mg kg^{-1})		Exchangeable Ca (mg kg^{-1})		Truog available P (mg kg^{-1})	
Entisols												
No fertilizer												
Control	7.07 ^{c}	± 0.07	1.84 ^{c}	± 0.02	0.0 ^{d}	± 0.00	0.2^{ns}	± 0.01	170.7 ^{c}	± 8.32	1.0 ^{d}	± 0.08
CNF	7.22 ^{b}	± 0.03	2.14 ^{b}	± 0.10	0.0 ^{d}	± 0.00	0.2^{ns}	± 0.02	169.5 ^{c}	± 3.96	1.2 ^{c}	± 0.05
CNF/Protein	7.30 ^{a}	± 0.01	2.23^{ab}	± 0.04	0.0 ^{d}	± 0.00	0.3^{ns}	± 0.07	171.6^{ac}	± 7.34	1.1^{cd}	± 0.09
CNF/Protein/$CaCO_3$	7.30 ^{a}	± 0.23	2.36 ^{a}	± 0.11	0.1 ^{a}	± 0.02	0.3^{ns}	± 0.04	182.7 ^{a}	± 4.70	1.3 ^{a}	± 0.07
With fertilizer												
Control	6.86 ^{a}	± 0.04	2.66^{ab}	± 0.21	4.9 ^{d}	± 0.42	2.8^{ab}	± 0.37	153.3 ^{d}	± 5.40	28.1 ^{c}	± 0.88
CNF	6.81 ^{a}	± 0.03	2.55 ^{b}	± 0.25	3.8 ^{d}	± 0.41	2.3 ^{c}	± 0.25	151.6 ^{d}	± 4.45	21.2 ^{d}	± 1.77
CNF/Protein	6.69 ^{b}	± 0.05	3.16^{ab}	± 0.54	13.3 ^{b}	± 0.91	2.5^{bc}	± 0.26	145.6 ^{d}	± 9.62	26.0 ^{c}	± 1.47
CNF/Protein/$CaCO_3$	6.78^{ab}	± 0.07	3.41 ^{a}	± 0.69	23.8 ^{a}	± 2.32	3.0 ^{a}	± 0.27	171.7 ^{a}	± 2.05	41.8 ^{a}	± 0.63
Andosols												
No fertilizer												
Control	5.41 ^{a}	± 0.05	9.05 ^{b}	± 0.74	36.9^{ns}	± 4.40	5.6^{ns}	± 0.25	554.3 ^{c}	± 6.67	3.1 ^{a}	± 0.61
CNF	5.39^{ab}	± 0.02	10.03^{ab}	± 0.50	34.0^{ns}	± 2.97	5.5^{ns}	± 0.48	535.1 ^{d}	± 6.80	1.0 ^{d}	± 0.65
CNF/Protein	5.35 ^{b}	± 0.03	10.99 ^{a}	± 0.73	33.8^{ns}	± 5.16	5.5^{ns}	± 0.50	545.6^{cd}	± 4.41	1.3 ^{d}	± 1.18
CNF/Protein/$CaCO_3$	5.39^{ab}	± 0.02	10.26 ^{a}	± 0.62	39.3^{ns}	± 6.61	5.0^{ns}	± 0.54	567.6 ^{a}	± 6.82	1.1 ^{d}	± 1.13
With fertilizer												
Control	5.32 ^{b}	± 0.02	9.18 ^{b}	± 0.16	46.3 ^{b}	± 2.26	6.1^{ns}	± 0.55	547.1^{ab}	± 7.98	8.8^{cd}	± 1.03
CNF	5.25 ^{c}	± 0.01	10.84 ^{c}	± 0.20	52.9^{ab}	± 1.82	6.3^{ns}	± 0.64	544.5^{ab}	± 20.9	9.6 ^{c}	± 1.95
CNF/Protein	5.42 ^{a}	± 0.04	10.53 ^{c}	± 0.79	55.5 ^{a}	± 3.80	6.1^{ns}	± 0.67	529.7 ^{b}	± 8.61	5.6 ^{d}	± 0.79
CNF/Protein/$CaCO_3$	5.40 ^{a}	± 0.03	11.99 ^{a}	± 0.47	56.5 ^{a}	± 8.47	6.7^{ns}	± 0.19	560.3 ^{a}	± 7.63	19.2 ^{a}	± 3.05

Note: The values in the table are means ± SD.
Different letters and ns within columns indicate significant differences ($P < 0.05$) and nonsignificant differences by Tukey's HSD test, respectively.

3.3. Effect of Different Application Methods of CNF on Plant Cultivation

3.3.1. Plant Growth

Plant height and plant dry matter weight are shown in Figures 5 and 6. There was a significant increase in plant height under some CNF treatments compared to that under treatment with fertilizer alone in Entisols. However, there were no significant differences in Andosols. Plant dry matter weight was highest in the CNF/protein/$CaCO_3$ treatment in both the basal application and the topdressing treatments in both Entisols and Andosols. In Entisols, the basal application resulted in greater plant dry matter weights than the topdressing treatment with all material treatments. However, in Andosols, the plant dry matter weight showed an increasing tendency in all treatments compared with the fertilizer-only treatment, but no difference was observed between the basal application and topdressing treatments. This result suggests that the basal application treatment was more affected by the CNF material than the topdressing treatments in Entisols. In contrast, there was no difference between the basal application and topdressing treatments in Andosols despite the effect of CNF materials. Moreover, the effect of CNF materials was probably due to the surface application of CNF rather than its broadcast application. The amount of nitrogen absorbed by the plants is shown in Figure 7. The total nitrogen uptake was highest with the basal application of the CNF/protein/$CaCO_3$ treatment in Entisols. In Andosols, the amount of total nitrogen was higher in the topdressing treatment than in the basal application treatment. The cultivation of plants with the application of CNF materials promoted nitrogen utilization efficiency and plant growth. A comparison of the calcium content in plants among treatments with the CNF material revealed a higher calcium content in the basal application treatment than in the topdressing treatment among all treatments (Figure 8). Furthermore, the maximum amount of calcium was observed in the CNF/protein/$CaCO_3$ treatment in both Entisols and Andosols, suggesting that the added $CaCO_3$ was decomposed and absorbed by the plant. Furthermore, according to Kishore, Pande, and Podile (2005) and Egusa et al. (2019), the application of CNF materials increases plants' self-protection function against diseases and pests. It has also been inferred that CNF is degraded by chitinase in the plant and acts as an elicitor inducer, contributing to plant growth promotion (Erwig et al., 2017).

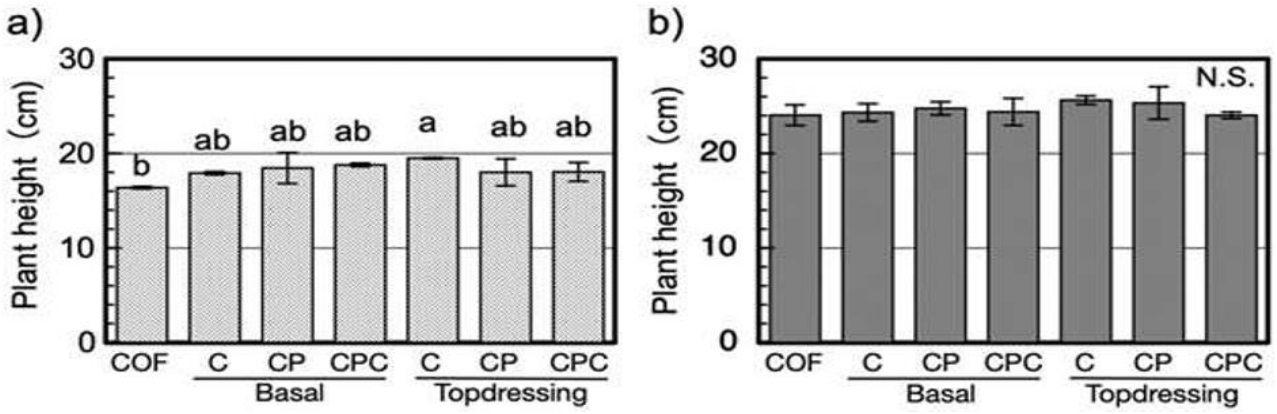

Figure 5. Plant heights: a) Entisols and b) Andosols. COF: only fertilizer. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.

Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA). N.S.: Not significant.

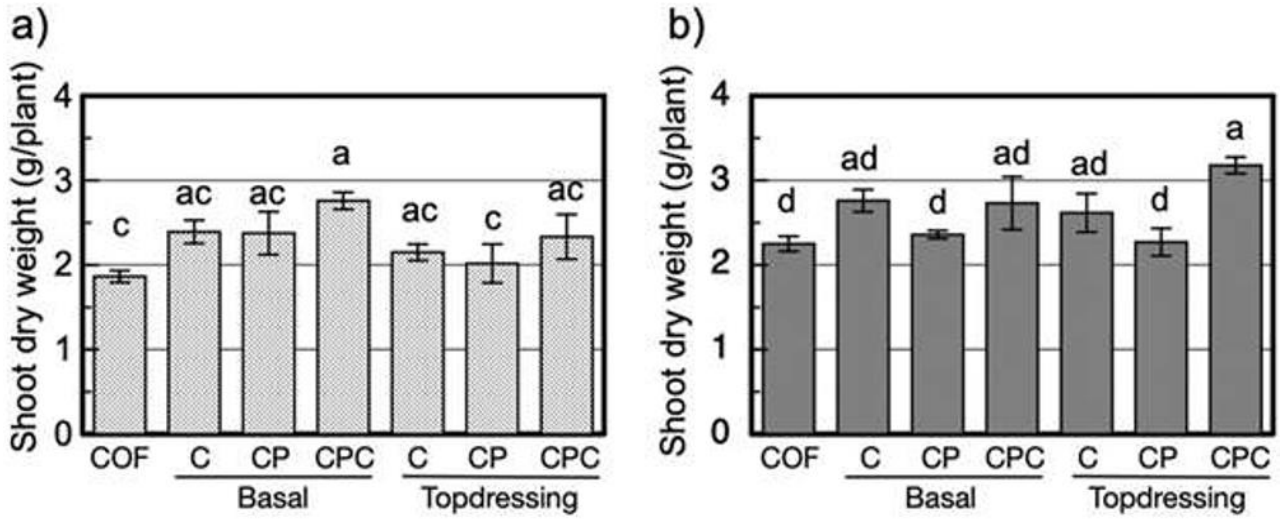

Figure 6. Shoot dry weights: a) Entisols and b) Andosols. COF: only fertilizer. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.

Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA).

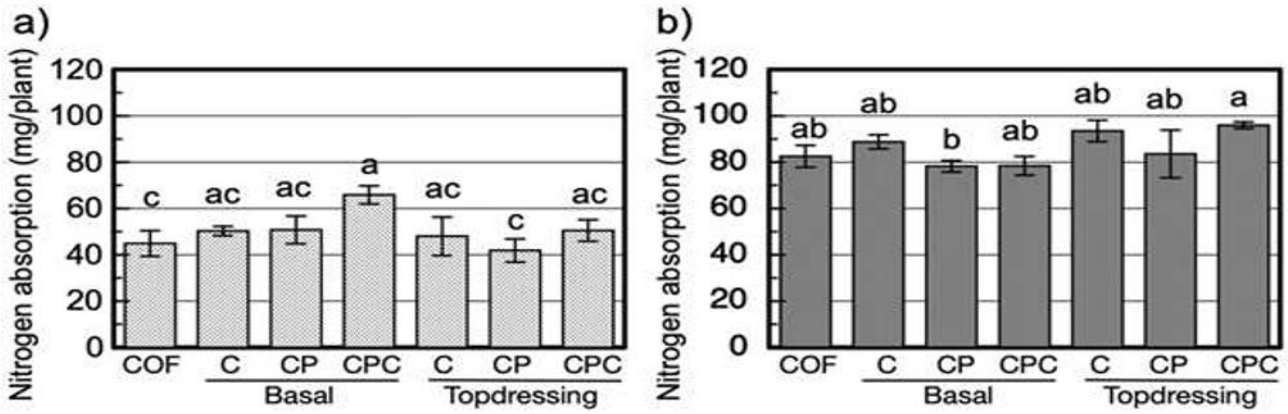

Figure 7. Nitrogen absorption by the plant: a) Entisols and b) Andosols. COF: only fertilizer. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.
Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA).

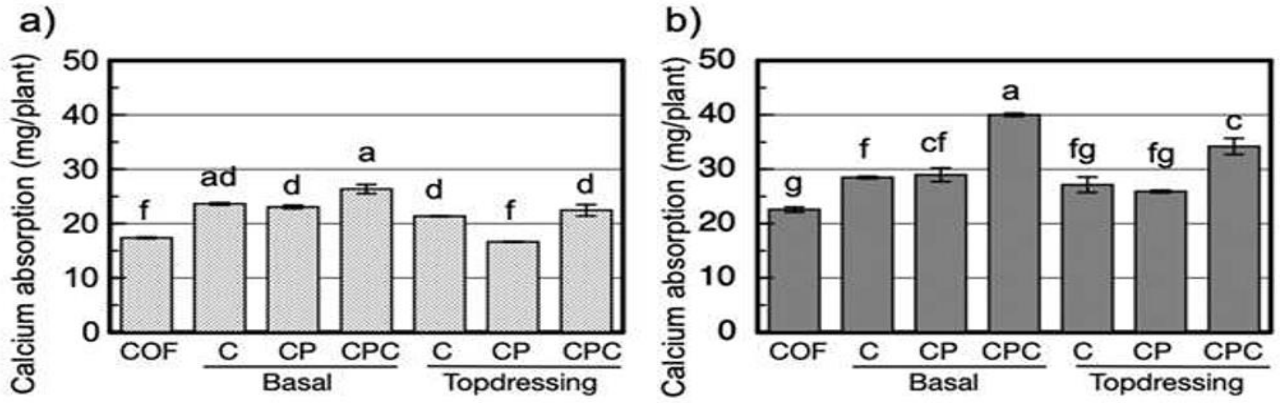

Figure 8. Calcium absorption by the plant: a) Entisols and b) Andosols. COF: only fertilizer. C: chitin nanofiber. CP: chitin nanofiber/protein. CPC: chitin nanofiber/protein/$CaCO_3$.
Note: Different letters indicate significant differences at $P \leq 0.05$ by Tukey's HSD (honestly significant difference) test of variance (ANOVA).

3.3.2. Effect of CNF on Soils after Plant Cultivation

Table 3 shows the physicochemical properties of the soil after plant cultivation. The soil pH showed no differences with the different application methods of CNF materials in both soil types. The ammonia nitrogen content was higher in the soil subjected to the topdressing treatment than in the soil subjected to the basal application treatment among all treatments. This might be because the application of CNF materials over time resulted in late nitrification from the ammonia form of nitrogen into nitrate nitrogen. The amount of ammonia nitrogen was the highest in the CNF/protein treatment in both soil types. In Entisols, the amount of nitrate nitrogen in the soil exposed to the topdressing treatment was higher than in the soil exposed to the basal application treatment. This result suggests that plants' nitrogen uptake is promoted in the pre-mediated soil because CNF has been decomposed into a form that can be easily used by plants after CNF addition. The higher amount of nitrogen remaining in the soil exposed to the topdressing treatment might be due to the slower rate of nitrogen absorption by the plant compared to the basal application treatment (Figure 7). The nitrate nitrogen content in Andosols was higher in the order of CNF/protein/$CaCO_3$ > CNF/protein > CNF in both the basal application and topdressing treatments. The amount of exchangeable calcium in Andosols did not differ according to the CNF material in the basal application treatment, although it did in the CNF/protein/$CaCO_3$ topdressing treatment. The highest amount of calcium was found in the CNF/protein/$CaCO_3$ treatment in Andosols. The calcium content was also highest in the basal application treatment, indicating that calcium was decomposed by soil microorganisms after the addition of CNF materials. Consequently, the amount of exchangeable calcium that was absorbed by the plants and remained in the soil was low and did not differ from that in the other treatments. However, in the topdressing treatment, the calcium that was not absorbed by the plants remained in the soil because of the insufficient time for decomposition by the soil microorganisms after the addition of CNF materials. The amount of available phosphate was lower in all treatments in Andosols than in Entisols. The phosphate absorption coefficient in Andosols might be high, and the phosphoric acid of the applied fertilizer might be fixed to the soil in Andosols. We analyzed how the different application methods of CNF influenced the impact of the material type on the growth-promoting effect. It is preferable to effectively recycle crab shells than to dispose of them. The recycling method proposed in this study uses CNF materials to promote plant growth. The results of this study should contribute to the promotion of agriculture in areas where crustacean waste is generated, such as in the areas adjacent to crab and shrimp fisheries in Asia.

Table 3. Physicochemical properties of Entisols and Andosols after cultivation.

Treatments	pH (1:2.5)		EC (1:5) (mS m^{-1})		NO_3-N (mg kg^{-1})		NH_4-N (mg kg^{-1})		Exchangeable Ca (mg kg^{-1})		Truog available P (mg kg^{-1})	
Entisols												
Control (Only fertilizer)	8.02^{ns}	± 0.03	4.78^{ns}	± 0.57	68.5 ^{a}	± 6.87	10.9^{ab}	± 0.57	165.3 ^{f}	± 5.27	25.7 ^{a}	± 0.90
Basal applications												
CNF	7.97^{ns}	± 0.07	4.86^{ns}	± 0.66	46.2 ^{d}	± 2.73	11.2^{ab}	± 0.46	164.0 ^{f}	± 4.29	24.2 ^{a}	± 1.04
CNF/Protein	7.96^{ns}	± 0.02	4.08^{ns}	± 0.21	40.7 ^{d}	± 6.87	11.8^{ab}	± 0.07	206.3 ^{a}	± 5.96	16.2 ^{c}	± 1.37
CNF/Protein/$CaCO_3$	8.00^{ns}	± 0.01	3.79^{ns}	± 0.34	39.5 ^{d}	± 5.46	7.9 ^{b}	± 0.04	201.0 ^{f}	± 5.61	16.2 ^{c}	± 0.52
Topdressing applications												
CNF	8.06^{ns}	± 0.01	4.52^{ns}	± 0.86	85.2 ^{a}	± 5.68	12.9^{ab}	± 0.25	151.2 ^{f}	± 1.31	25.3 ^{a}	± 1.37
CNF/Protein	7.96^{ns}	± 0.03	5.71^{ns}	± 0.69	84.1 ^{a}	± 3.15	15.3 ^{a}	± 0.26	213.3 ^{a}	± 9.38	23.1 ^{a}	± 1.37
CNF/Protein/$CaCO_3$	7.99^{ns}	± 0.00	4.63^{ns}	± 0.55	67.4 ^{a}	± 4.17	10.8^{ab}	± 0.27	210.0 ^{f}	± 9.95	24.9 ^{a}	± 1.87
Andosols												
Control (Only fertilizer)	6.15^{ns}	± 0.02	6.03^{ns}	± 0.27	44.0 ^{c}	± 3.15	24.5 ^{a}	± 0.87	597.0^{def}	± 7.49	9.6 ^{a}	± 0.52
Basal applications												
CNF	6.17^{ns}	± 0.01	5.86^{ns}	± 0.13	66.3 ^{cd}	± 5.46	16.2^{de}	± 0.50	596.3 ^{ef}	± 12.23	9.2^{ab}	± 0.45
CNF/Protein	6.14^{ns}	± 0.00	6.53^{ns}	± 0.46	68.5 ^{ac}	± 3.15	21.8^{bc}	± 0.56	630.9 ^{de}	± 6.80	9.9 ^{a}	± 0.52
CNF/Protein/$CaCO_3$	6.15^{ns}	± 0.01	6.11^{ns}	± 0.16	70.7 ^{ac}	± 3.15	16.0 ^{e}	± 0.84	630.0 ^{de}	± 5.56	8.5^{ab}	± 0.26
Topdressing applications												
CNF	6.16^{ns}	± 0.04	6.51^{ns}	± 0.31	50.7 ^{de}	± 6.30	23.3^{ab}	± 1.17	568.1 ^{f}	± 9.65	7.7 ^{b}	± 0.52
CNF/Protein	6.12^{ns}	± 0.01	6.35^{ns}	± 0.49	55.1^{cde}	± 6.30	24.6 ^{a}	± 0.66	635.6 ^{d}	± 13.26	8.8^{ab}	± 0.52
CNF/Protein/$CaCO_3$	6.16^{ns}	± 0.02	6.37^{ns}	± 0.30	85.2 ^{a}	± 5.68	19.3^{cd}	± 0.02	719.4 ^{a}	± 18.58	9.6 ^{a}	± 0.52

Note: The values in the table are means ± SD. Different letters and ns within columns indicate significant differences ($P < 0.05$) and nonsignificant differences by Tukey's HSD test, respectively.

4. CONCLUSION

The study found a growth-promoting effect with the surface application of CNF compared with its broadcast application in Andosols. The CNF/protein/$CaCO_3$ treatment exerted a growth-promoting effect in both Entisols and Andosols. This result suggests that CNF application to the surface of each soil type exerts a growth promotion effect, which could be because the $CaCO_3$ and protein encapsulating the CNF exert a favorable effect on the growth of plants.

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Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.

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