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Water-Soluble Carbon Nanoparticles Improve Seed Germination and Post-Germination Growth of Lettuce under Salinity Stress

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Abstract: Seed germination is a critical developmental phase for seedling establishment and crop production. Increasing salinity stress associated with climatic change can pose a challenge for seed germination and stand establishment of many crops including lettuce. Here, we show that water soluble carbon nanoparticles (CNPs) can significantly promote seed germination without affecting seedling growth. Twenty-seven varieties of lettuce (*Lactuca sativa*) were screened for sensitivity to germination in 150 and 200 mM NaCl, and six salt-sensitive varieties (Little Gem, Parris Island, Breen, Butter Crunch, Muir, and Jericho) were selected and primed with 0.3% soluble carbon nanoparticles. Pretreatment with CNPs significantly improved seed germination under 150 mM NaCl and high temperature. CNP treatment slightly inhibited the elongation of primary roots but promoted lateral root growth and accumulation of chlorophyll content of seedlings grown under salt stress. Despite different lettuce varieties exhibiting a distinct response to nanoparticle treatments, results from this study indicate that soluble nanoparticles can significantly improve lettuce seed germination under salinity stress, which provide fundamental evidence on the potential of nanoparticles in agricultural application to improve crop yield and quality under stressful conditions.

Keywords: lettuce; seed germination; salinity; nanoparticles

1. Introduction

Salinity stress is a major environmental factor that affects crop production. It is estimated that one-third of agricultural land is under salinity stress, partially due to the extensive use of brackish, saline, or reclaimed water for horticultural production [1]. Climatic warming trends have led to more frequent extreme weather events such as drought and heat, which limit the sustainability of water resources. In addition, intensive land use by human beings for urbanization and organic matter oxidation in waste water runoff have led to increased alkalization and salinization of soils [2].

Salinity stress can cause various adverse effects on plant growth and productivity that may influence food supplies [3,4]. One of the most common adverse effects is the osmotic stress imposed by the high concentration of salt ions, thus limiting the water availability for plants [5]. The limitation in water uptake can significantly delay or inhibit seed germination and early seedling growth, posing a challenge for uniform stand establishment that substantially affects crop production. Various treatments prior to seed sowing in the field have been undertaken to improve germination under saline

conditions including pretreatment or priming in polyethylene glycol, salts, and nutrients, which can stimulate the metabolic process of seeds for uniform and rapid germination [6].

Nanoparticles (NPs) are natural or engineered materials possessing high bioactivity and biosafety properties due to their small size (with a particle size less than 100 nm) and high surface-to-volume ratio [7,8]. NPs have been extensively used in a range of applications in different fields such as agriculture, pharmaceuticals, food technology, and environmental protection [7]. Due to their unique physical-chemical properties, NPs are also receiving attention in agricultural applications. Different types of engineered nanoparticles have been examined as nanofertilizers, nanopesticides, and nanosensors for their beneficial effects on growth, yield, and quality of important agricultural crops [9]. NPs have been evaluated for their effects on seed germination and post-germination seedling growth. However, contradicting results for the effects of NPs on seed germination have been reported across different plant species. For example, NPs containing copper oxide (CuO), zinc oxide (ZnO), and titanium oxide (TiO_2) were reported to enhance seed germination of oat, corn, black mustard, and cucumber seeds [10–13]. In others studies, these NPs were reported to inhibit seed germination of Arabidopsis, corn, cucumber, rice, and other species [14–19]. NP-mediated inhibition of seed germination might be due to excessive toxic metal ions and partial dissolution of NPs [17,20–22].

In addition to metallic nanoparticles, carbon nanomaterials are categorized into several forms such as nanotubes, graphenes, and fullerenes [8]. Carbon nanoparticles (CNPs) have been reported to have a positive impact on seed germination [23–25]. However, the effect of carbon nanomaterials is dependent on the size, concentration, and solubility of the applied nanomaterials [26], resulting in contradictory effects on seed germination. Additionally, carbon nanoparticles have been reported to mitigate the adverse effects of various abiotic stresses including salinity [27], indicating their potential for improving crop yield and quality under stressful conditions.

Lettuce is the most consumed leafy vegetable in the U.S. Lettuce is a seeded crop, and seed germination and early seedling growth are important determinants of successful stand establishment [28,29]. Salinity has been a major environmental factor that affects seed germination and stand establishment of lettuce in some regions [30]. In this study, we report that water soluble carbon nanoparticles can substantially enhance lettuce seed germination and post-germination growth under saline conditions.

2. Materials and Methods

2.1. Sensitivity of Different Lettuce Varieties

Twenty-seven lettuce varieties classified into Leafy, Romaine, Iceberg, and Butter head types were purchased from different seed companies (Table 1), except for varieties of "Salinas" and "PI251246" that were reproduced in our laboratory at the University of Florida in 2018. All varieties were examined preliminarily for their germination responses to 150 mM NaCl or 200 mM NaCl. To minimize fungal infection, all germination solutions used in this study contained 0.2% Plant Preservative Mixture (PPM) (5-chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone) (Caisson Labs, Utah, USA) unless otherwise stated. Thirty seeds per variety were placed on a germination blotter in a 4.7 cm Petri dish and wetted with three milliliters of germination solutions (DI H_2O +150 mM NaCl or 200 mM NaCl, and DI H_2O only as a control treatment). There were three replications per treatment. Seeds were incubated in a growth chamber with a constant temperature of 20 °C and a photoperiod of 16 h light/8 h dark unless otherwise stated.

Table 1. Information on the 27 lettuce varieties used in this study.

Leafy	Romaine	Iceberg	Butterhead	Seed Company
Muir, Rouge D'hiver, Green Forest	Truchas, Dragoon Breen, Costal Star			Johnny's Seeds
Red Salad Bowl Black Seeded Simpson Salad Bowl, Red Sails, Lollo Rossa, Dark Lolla Rossa	Blushed Butter, Parris Island	Red Iceberg	Tom Thumb Butter Crunch	Fedco Seeds
Green Ice	Cimarron Jericho	Red Iceberg	Little Gem *, All Year Round	Pinetree Garden Seeds
Grand Rapids				Eden Brothers
Grand Rapids				Stokes Seeds
PI251246 **			Salinas	Self-produced

* Little Gem is a Romaine/Butterhead hybrid. ** PI251246 is a primitive accession whose seeds were historically used for oil production.

2.2. Pretreatments of Salt-Sensitive Lettuce Seeds with Carbon Nanoparticles (CNPs)

Based on the preliminary screening results, six lettuce varieties (Little Gem, Parris Island, Breen, Butter Crunch, Muir, and Jericho) with low germination percentage at both concentrations of NaCl were identified. They were considered to be sensitive to salt stress and were used for evaluating the effects of CNPs in the alleviation of germination inhibition caused by 150 or 250 mM NaCl.

The water-soluble CNP solution containing 0.3% (by weight) of carbon nanoparticles with an approximate particle diameter range of 90–110 nm was manufactured and kindly provided by Vulpes Corp Inc. (St. Louis, Missouri, USA). The treatments with CNPs were setup as follows: 30 seeds were treated with 1 mL of 0.3% CNPs for 2, 4, 6, 8, 10, 12, and 14 h with agitation (225 rpm) in a shaker at room temperature (24 ± 1 °C), respectively, followed by rinsing with DI water or salt solution twice prior to the germination tests. Another treatment was performed using 0.3% CNPs combined with 150 or 250 mM NaCl for 4 h. Seeds of six varieties treated only with water or 150 and 250 mM NaCl were also included as controls.

Additionally, seeds of the most salt sensitive "Little Gem" and least sensitive "Parris Island" were directly germinated in 0.015% (20-fold dilution of original CNP product) or 0.01% (30-fold dilution of original CNP product) of CNP solution containing 150 mM NaCl.

2.3. Seed Priming with CNPs

Seeds of six varieties were treated with 0.3% CNPs for 4 h in a shaker with 225 rpm agitation at room temperature; treated seeds were then rinsed with DI water twice and briefly dried with paper towels before being dried in a 33 ± 1 °C incubator for 72 h. Relative moisture contents of these dried seeds were measured through drying at 70 °C for 10 days. Primed seeds were germinated in H_2O or 150 mM NaCl.

2.4. Thermoinhibition Tests of CNP-Treated Seeds

Lettuce seeds were first treated with either H_2O or 0.3% CNPs for 4 h, followed by two rinses with DI water. Seeds were incubated in DI water at 34 °C to examine the CNP effect on the thermoinhibition of seed germination, and seed germination was examined nine days after imbibition.

2.5. Effects of CNPs on Post-Germination Growth

Seeds were surface-sterilized in 70% ethanol for 2 min, followed by 20% Clorox^{TM} bleach (containing 6.0% sodium hypochlorite) for 10 min with gentle agitation and then rinsed seven times with sterile DI H_2O. Sterilized seeds were incubated with sterile 0.3% CNPs for 4 h prior to placement on 1/2 strength of Murashige and Skoog (MS) medium (PhytoTechnology Laboratories, Lenexa, KS, USA). Germinated seeds were transferred onto either the same 1/2 MS medium or 1/2 MS medium supplemented with 150 mM NaCl and chlorophyll contents, lengths of leaves and roots, numbers of roots and leaves, dry and fresh weights were measured after 21 days of growth. Three replications with nine seedlings in each replication were measured for each variety.

2.6. Determination of Chlorophyll Contents

Chlorophyll was extracted with the methanol–HCl method as described by Porra et al. [31]. In brief, a single leaf or part leaf from each 21-day-old seedling was weighed and ground in a 1.5 mL microcentrifuge tube with a sterile plastic pestle, and 800 uL of the methanol with 1% HCl solution was added. This ground mixture was dark-incubated at 4 °C overnight. A total of 200 µL of extraction for each sample was used for chlorophyll determination with a Synergy H1 microplate reader (BioTek Instruments, Virginia, USA) at the O.D. of 652 and 665 nm with a reading path correction. Nine seedlings were applied to each sample per treatment. The following equations were used for calculating the content of *Chla*, *Chlb*, and total *Chla + Chlb: Chla = (16.29*[OD 665]) − (8.54*[OD 652])*, *Chlb = (30.66*[OD 652]) − (13.58*[OD 665])*, and *Total Chla + Chlb = (22.12*[OD 652]) + (2.71*[OD 665]*).

2.7. Statistical Analysis

Pairwise student's *t*-tests were performed to compare the control vs. CNP treated samples for statistical significance analysis.

3. Results

3.1. Distinct Responses of Lettuce Varieties to Salt Stress at Germination

Seeds of the 27 lettuce varieties were highly viable with germination rates greater than 90% in H_2O at 20 °C (Figure 1). However, seeds of these varieties exhibited distinct responses to two concentrations of NaCl. Eighteen varieties were tolerant to 150 mM NaCl, with a germination percentage of 75% or higher. Nine of these 18 varieties had a germination rate of 90% (Figure 1). However, sensitivity of these 18 varieties to 200 mM NaCl was not consistent with that to 150 mM NaCl. For example, Grand Rapids, PI251246, Red Salad Bowl, and Salad Bowl had germination rates greater than 80% at 150 and 200 mM NaCl. In contrast, germination of Costal Star, Black Seeded Simpson, Dragoon, Salinas, and All Year Round was less than 20% at 200 mM NaCl, despite germinating well at 150 mM NaCl (Figure 1). These data indicated that the upper limitation for salt tolerance significantly varied among these varieties. On the contrary, seeds of six varieties (Little Gem, Parris Island, Breen, Jericho, Muir, and Butter Crunch) had germination rates less than 35% at both salt concentrations, suggesting that they were highly sensitive to salt stress (Figure 1). Thus, these six varieties were selected to examine the effects of CNPs on alleviating the salt effects on seed germination.

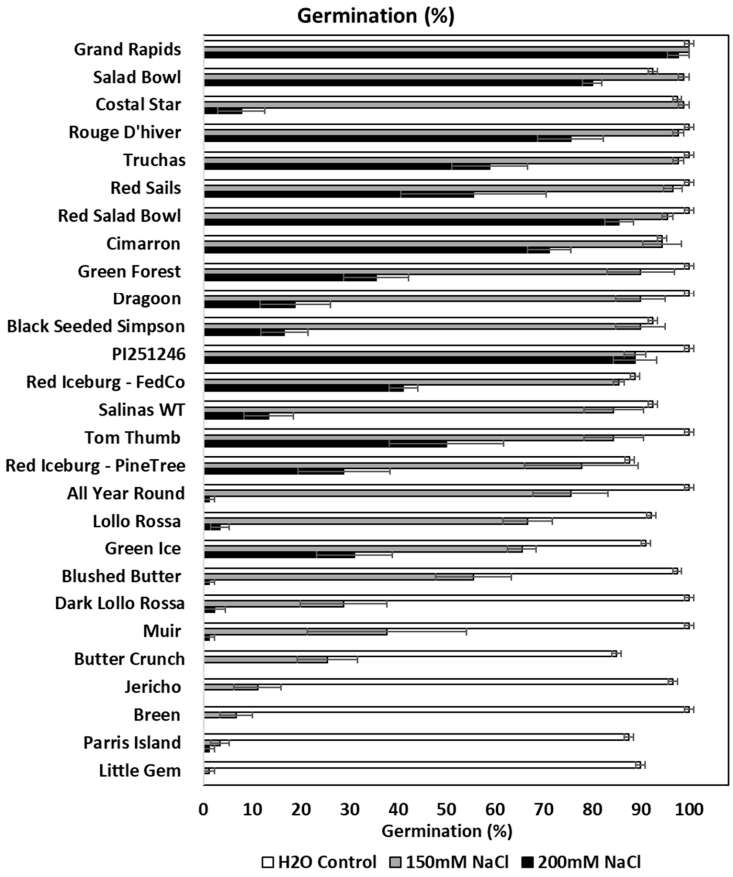

Figure 1. Germination sensitivity of 27 lettuce varieties to salt stresses. Seeds of 27 lettuce varieties were imbibed in H_2O, 150 mM NaCl, or 200 mM NaCl solution for nine days in 16 h light/8 h dark at 20 °C.

3.2. Effects of CNPs on Seed Germination of Salt-Sensitive Lettuce

To examine the effect of CNPs on alleviating salt stress on seed germination, seeds of six highly sensitive varieties (Little Gem, Parris Island, Breen, Jericho, Muir, and Butter Crunch) were treated for 2, 4, 6, 8, 10, 12, and 14 h prior to germination in 150 mM NaCl. Among these varieties, Little Gem, Parris Island, Breen, and Jeicho were extremely sensitive to 150 mM NaCl, with a germination rate of <10%, whereas Butter Crunch and Muir seeds could reach 25% and 37% germination rates, respectively (Figure 2A,H). Interestingly, a 2-h CNP treatment was sufficient to significantly alleviate the salt inhibition on Parris Island, Breen, Jericho, Muir, and Butter Crunch, resulting in germination rates of 91%, 72%, 55%, 100%, and 76%, respectively (Figure 2A,H). However, seeds of the most sensitive variety (Little Gem) exhibited no significant change in germination after 2, 4, 6, and 8 h of CNP treatment (Figure 2A–D). As CNP treatments extended to 10 h or longer, Little Gem seeds could have a germination rate up to 15%, but Parris Island, Jericho, and Muir seeds exhibited no or slight declines in their germination percentages (Figure 2E–G). In contrast, extended CNP treatments (10 h or longer) resulted in an obvious reduction in seed germination of Breen and Butter Crunch seeds (Figure 2E–G). The best overall germination results were observed in the 4-h treatment (Figure 2B,H).

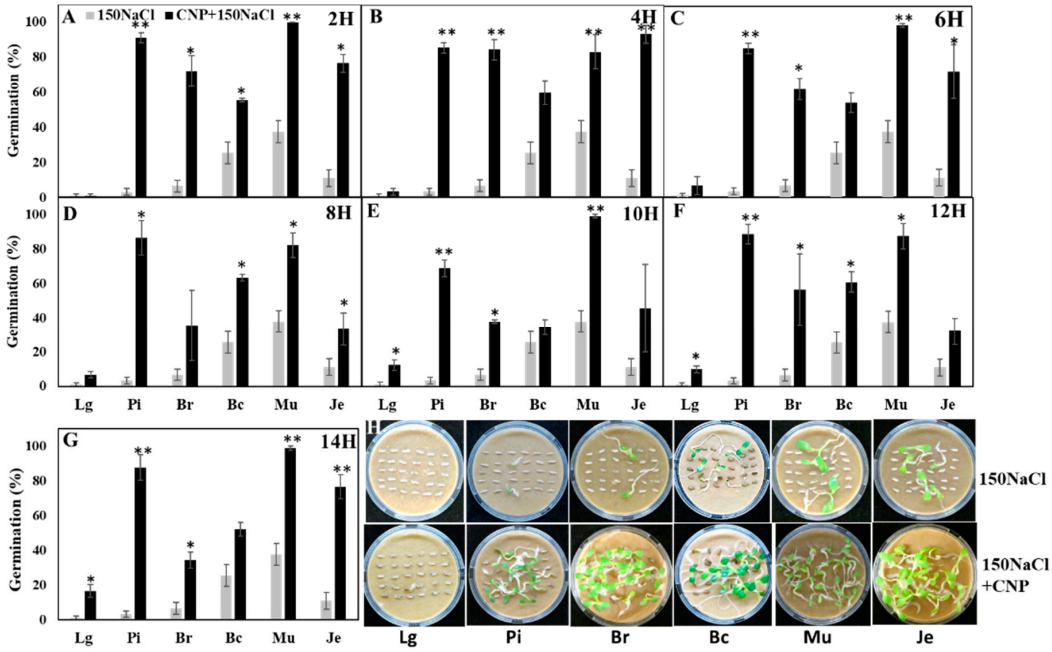

Figure 2. Germination of lettuce seeds pretreated without (■) or with carbon nanoparticles (CNPs) (■) in 150 mM NaCl solution. 2H in (**A**), 4H in (**B**), 6H in (**C**), 8H in (**D**), 10H in (**E**), 12H in (**F**), and 14H in (**G**) indicate CNP treatment period in hours. (**H**) Representative images for seed germination of different varieties pretreated with 150 mM NaCl or 150 mM NaCl +0.3% CNP for 4 h, followed by imbibition in the 150 mM NaCl solution for six days. Lg (Little Gem); Pi (Parris Island); Br (Breen); Bc (Butter Crunch); Mu (Muir); Je (Jericho). * and ** denote the statistical significance at the $p < 0.05$ and $p < 0.01$ level, respectively.

Regarding germination rates, CNP treatments generally expedited seed germination under salinity stress. Without CNP treatments, five of the six tested varieties (except Muir) were not able to germinate within three days of imbibition in 150 mM NaCl (Figure 3H). Even after nine days of imbibition in 150 mM NaCl, the germination percentages of the tested varieties were very low. With CNP treatments, Muir germinated significantly quicker and attained a complete germination within three days of imbibition with most CNP treatments (Figure 3A–G). Similarly, after 2 and 4 h of CNP treatment, Parris Island, Breen, Jericho, and Butter Crunch could reach >70% of their maximum germination within six days (Figure 3A,B).

In addition to the pretreatment of seeds with CNPs, we tested how the direct application of CNPs during seed imbibition affected seed germination. As suggested by the manufacturer's instruction, we used 20× (containing 0.015% CNPs) or 30 × (containing 0.01% CNPs) diluted CNP for imbibing seeds of Little Gem and Parris Island. Germination of Parris Island seeds improved from 3% at 150 mM NaCl to 38%, 56%, and 75% in solutions containing 150 mM NaCl and 20×, 30×, 1× CNPs, respectively (Figure 3I). However, direct imbibition with the CNP solution did not obviously improve the seed germination of Little Gem. Compared to pretreatment with CNP, direct imbibition of seeds was shown to be less effective, suggesting that continued exposure to high concentrations of nanoparticles may be detrimental to plant growth as also observed in other studies. For example, both prolonged exposure to gold and silver nanoparticles were found to inhibit seed germination and growth or increase phototoxic effects in aged rice seeds [32]. Treatment with high concentrations of titanium nanoparticles also contributed to decreased shoot and seedling length in wheat [33].

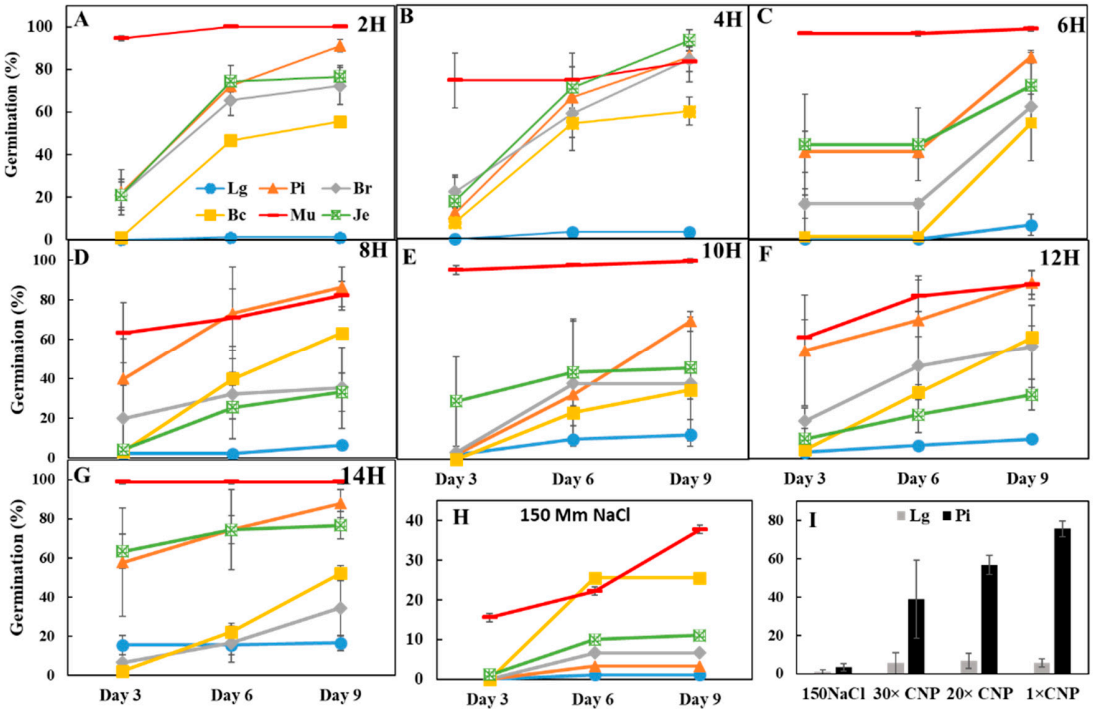

Figure 3. Germination rates of lettuce seeds pretreated for 2 (**A**), 4 (**B**), 6 (**C**), 8 (**D**), 10 (**E**), 12 (**F**), or 14 h (**G**) in 150 mM NaCl. (**H**) Germination percentages of lettuce seeds without CNP treatment in 150 mM NaCl. (**I**) Germination of Little Gem (Lg) and Parris Island (Pi) in 150 mM NaCl and 150 NaCl solution containing the original CNPs (1×) or 20× or 30× diluted CNPs.

3.3. Pretreatment with CNP But Not H_2O Improved Lettuce Seed Germination

To test whether the improvement in seed germination under salt conditions could be mainly derived from water absorption during CNP pretreatment, we pretreated lettuce seeds with H_2O, 150 mM NaCl, 250 mM NaCl, CNP, CNP plus 150 mM NaCl, and CNP plus 250 mM NaCl for 4 h before incubating them for nine days in the 150 NaCl solution. After four hours of H_2O pretreatment, seeds of Lg, Br, and Je still barely germinated, while Pi, Bc, and Mu seeds were able to germinate around 35% (Figure 4A). Compared to the control (without any pretreatment), Pi was the only variety that exhibited an increase in its germination after 4 h of H_2O pretreatment (Figure 4A). As seed priming with salt may alleviate the inhibitory effects of salinity stress on seed germination [34,35], we pretreated seeds with NaCl alone or with a combination of NaCl and CNPs. Although pretreatments with 150 mM NaCl alone promoted seed germination of Pi and Bc, pretreatment with NaCl did not generate beneficial effects on seed germination of four varieties (Lg, Br, Mu, and Je) (Figure 4A). Compared to the controls (no pretreatment), pretreatment with 250 mM NaCl showed more inhibition on the seed germination of Muir and Breen in the 150 mM NaCl solution (Figure 4A). Interestingly, combining 150 mM NaCl with CNPs improved seed germination to the level comparable to those from CNP-treated seeds of Parris Island, Breen, Jericho, Muir, and Butter Crunch, although 250 mM NaCl combined with CNP did not improve seed germination of Breen, Muir, and Jericho as much as the treatments of 150 mM NaCl with CNPs (Figure 4B). Collectively, these results indicate that the improvement in seed germination by CNP treatment is not simply due to water absorption during the pretreatments, and that pretreatment with salt solutions did not improve the seed germination of tested lettuce varieties.

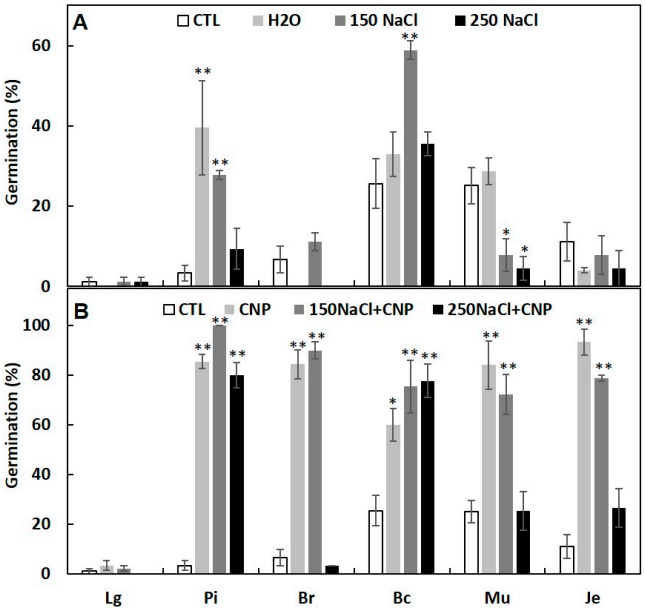

Figure 4. (**A**) Germination of lettuce seeds pretreated with H_2O, 150 mM NaCl, 250 mM NaCl for 4 h prior to an imbibition in 150 mM NaCl solution for nine days; (**B**) Germination of lettuce seeds pretreated with CNP, 150 mM NaCl + CNP, 250 mM NaCl + CNP for 4 h prior to an imbibition in 150 mM NaCl solution for nine days. CTL: seeds without any pretreatment. * and ** denote the statistical significance compared to CTL at the $p < 0.05$ and $p < 0.01$ level, respectively.

3.4. Seed Priming with CNPs Can Enhance Seed Germination under Salinity and High Temperature Stresses

To determine whether seed priming with CNPs (i.e., pretreated with CNP and seeds dried back) will still enhance their germination under abiotic stresses, we treated lettuce seeds for 4 h followed by 72 h of drying at 33 °C, resulting in a 4.4–8.1% of relative moisture contents for these dried seeds. All CNP-primed seeds could germinate well in DI H_2O (CNP-Dry-H_2O), indicating that seed viability was not reduced by these treatments (Figure 5A). However, different varieties exhibited various responses to salinity stresses after CNP treatment and drying. CNP-primed seeds (CNP-Dry + 150 NaCl) of Parris Island and Butter Crunch could germinate to the same level as those treated with CNP only (CNP + 150 NaCl) in 150 mM NaCl solution, whereas germination of CNP-primed seeds of Breen, Jericho, and Muir was much lower compared to their counterparts treated with CNP without drying (Figure 5A). Surprisingly, Muir seeds seemed to be very sensitive to drying after CNP treatment, because its CNP-primed seeds (CNP-Dry + 150 NaCl) exhibited even lower germination than the control seeds (150 NaCl). These results indicate that seed priming with CNPs is genotypic-dependent.

Thermoinhibition is one type of seed germination inhibition caused by high temperature and is one of the crucial challenges for lettuce production. We tested the seed germination of six varieties at 34 °C, and found that Muir seeds with no treatment (CTL-34 °C) were the only ones that could germinate well (82%) at 34 °C. Priming with H_2O only (H2O-34 °C) exhibited no obvious effect on seed germination at 34 °C except for Butter Crunch, with an increase from 2.5% (34 °C-CTL) to 49% (H2O-34 °C) (Figure 5B). Interestingly, seeds pretreated with CNP only (CNP-34 °C) of all six varieties germinated better at 34 °C compared to the control seeds (CTL-34 °C) (Figure 5B). CNP-primed seeds of (CNP-Dry-34 °C) did not germinate as well as CNP-pretreated seeds. Generally, the beneficial effect of CNPs on alleviating lettuce seed thermoinhibition was much less than its effect on reversing the germination inhibition by NaCl.

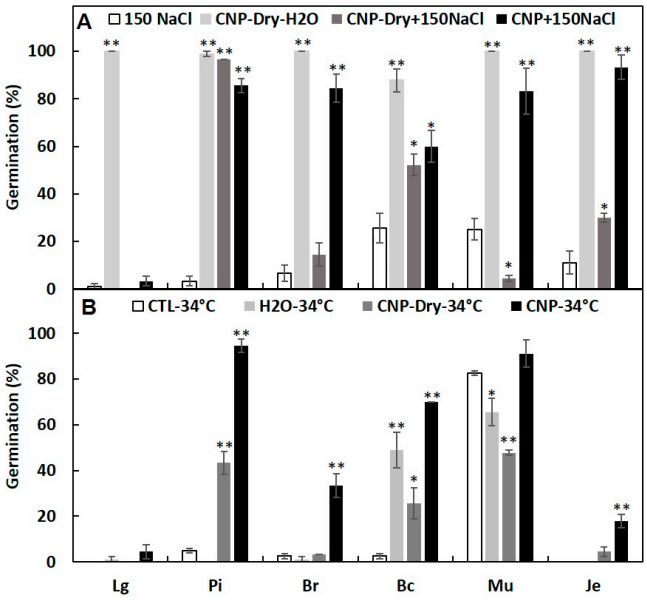

Figure 5. Effects of priming with CNPs on lettuce seed germination. (**A**) Germination of CNPs-treated seeds with (CNP-Dry) or without drying (CNP) in H_2O or 150 mM NaCl. 150 NaCl: direct germination of seeds without any treatment in 150 mM NaCl. (**B**) Germination of CNPs-treated seeds with (CNP-Dry) or without drying (CNP) in H_2O at 34 °C. 34 °C-CTL: germination of seeds without any treatment in H_2O at 34 °C; H_2O-34 °C: germination of seeds pretreated in H_2O at 34 °C. * and ** denote the statistical significance compared to 150 NaCl (A) or CTL-34 °C (B) at $p < 0.05$ and $p < 0.01$ level, respectively.

3.5. CNPs Treatment Positively Affected Post-Germination Seedling Growth under Salinity Stress

Uniformly germinated seeds with ~3 mm radicles were transferred onto 1/2 MS + 150 mM NaCl media to examine the CNP effects on post-germination seedling growth. Effects of CNP treatments varied among varieties. Butter Crunch had no response to CNP pretreatment, exhibiting no change in all measured seedling parameters (Table 2). In contrast, Parris Island seedlings were significantly responsive to CNP pretreatment, which greatly improved leaf length, leaf number, number of lateral roots, and fresh and dry weight, although root elongation was inhibited (Table 2, Figure 6C,D). In addition, CNP pretreatment also promoted leaf elongation and increased leaf number in Little Gem. However, CNP-pretreated Breen seeds exhibited inhibition in root elongation, fresh and dry weight, although the number of its lateral roots increased (Table 2, Figure 6A,B). CNP pretreatment also exhibited negative effects on growth of Muir and Jericho seedlings, with decreases in their leaf number and fresh and dry weight.

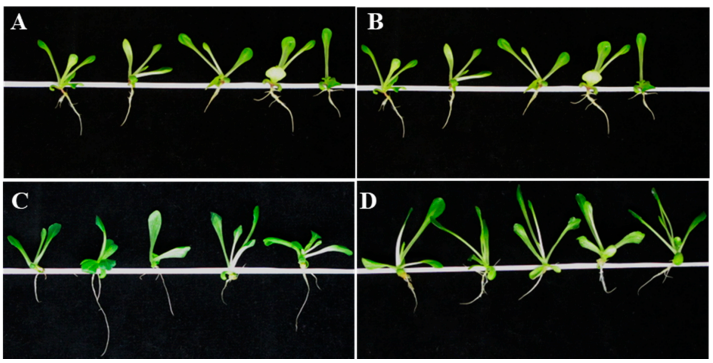

Figure 6. Representative images of Non-CNP pretreated and CNP-pretreated seedlings grown on MS media with 150 mM NaCl. (**A**) Non-CNP pretreated Breen; (**B**) CNP-pretreated Breen CNP; (**C**) Non-CNP pretreated Parris Island (**D**) CNP-pretreated Parris Island. Uniformly germinated seeds were chosen and grown on the MS media containing 150 mM NaCl for 21 days in the 16 h light/8 h dark.

Table 2. Effects of carbon nanoparticle (CNP) pretreatment on post-germination growth of seedlings grown under salt stress.

Variety	Treatment	Leaf Length (cm)	Root Length (cm)	No. of Leaf	No. of Lateral Roots	Fresh Weight (mg)	Dry Weight (mg)
Lg	control	2.68 ± 0.47	2.27 ± 0.62	4.42 ± 0.67	4.08 ± 4.27	125.33 ± 10.01	7.53 ± 0.52
	CNP	3.22 ± 0.36 *	2.21 ± 0.46	5.17 ± 0.58 **	7.17 ± 2.62	151.9 ± 10.97	8.6 ± 0.49
Pi	control	2.91 ± 0.45	2.71 ± 0.97	4.33 ± 0.89	2.17 ± 1.03	91.83 ± 5.29	4.98 ± 0.34
	CNP	4.18 ± 0.43 **	2.13 ± 0.51 *	4.83 ± 0.39 *	5.58 ± 1.08 **	166.68 ± 9.71 **	8.83 ± 0.4 **
Br	control	3.45 ± 0.67	3.06 ± 0.78	4.25 ± 0.87	2.83 ± 1.4	149.04 ± 7.71	6.78 ± 0.35
	CNP	2.96 ± 0.38	1.96±0.55 **	4.85 ± 0.79	4.42 ± 1.93 *	111.58 ± 7.98 **	6.35 ± 0.35 *
Bc	control	3.26 ± 0.49	2.3 ± 0.43	4.58 ± 0.51	2.92 ± 2.15	123.84 ± 7.19	7.26 ± 0.46
	CNP	2.98 ± 0.58	2.31 ± 0.53	4.75 ± 0.97	2.92 ± 2.91	131.22 ± 10.5	7.13 ± 0.51
Mu	control	4.35 ± 1.15	2.77 ± 0.77	6.33 ± 1.15	5.33 ± 1.77	362.28 ± 82.74	13.32 ± 1.66
	CNP	4.38 ± 0.62	2.65 ± 0.37	4.42 ± 0.67 **	5.67 ± 1.49	182.18 ± 71.9 *	6.88 ± 0.62 **
Je	control	3.55 ± 0.68	2.09 ± 0.64	3.83 ± 0.58	3.33 ± 1.3	57.12 ± 2.61	2.62 ± 0.21
	CNP	3.63 ± 0.24	2.18 ± 0.57	3.25 ± 0.45 **	2.58 ± 1.31	70.65 ± 27.8	2.2 ± 0.13 *

* and ** denote the statistical significant difference compared to the control counterparts at the $p < 0.05$ and $p < 0.01$ level, respectively.

We also examined whether there was any effect of CNP pretreatment on the chlorophyll content of seedlings grown on MS media containing 150 mM NaCl. We found that the chlorophyll b and total chlorophyll content were significantly increased in all six CNP-pretreated lettuce varieties (Table 3). Interestingly, CNP-pretreatment did not increase the content of chlorophyll a in most tested varieties except Muir, suggesting that this increment of total chlorophyll content was mainly contributed by chlorophyll b.

Table 3. Effects of CNP pretreatment on chlorophyll content of seedlings grown under salt stress.

Sample	*Chl a* −CNP (*ug/g* FW)	*Chl a* +CNP (*ug/g* FW)	*Chl b* −CNP (*ug/g* FW)	*Chl b* +CNP (*ug/g* FW)	Total *Chl* −CNP (*ug/g* FW)	Total *Chl* +CNP (*ug/g* FW)
Little Gem	20.50 ± 5.18	25.60 ± 9.97	188.00 ± 62.19	399.19 ± 163.7 *	231.54 ± 64.63	424.79 ± 172.87 *
Parris Island	29.58 ± 6.79	30.07 ± 12.46	548.52± 104.29	776.15 ± 123.07 **	593.41 ± 107.90	806.22 ± 131.44 **
Breen	16.37 ± 4.02	23.64 ± 8.38	163.80 ± 63.02	295.85 ± 96.60 *	200.48 ± 66.78	319.46 ± 103.98 *
Buttercrunch	24.77 ± 3.73	23.66 ± 13.52	674.52 ± 139.01	889.80 ± 118.60 **	700.89 ± 139.54	913.46 ± 106.67 **
Muir	18.25 ± 6.97	26.71 ± 5.99 *	248.96 ± 106.79	801.35 ± 212.21 **	299.18 ± 113.33	828.06 ± 215.44 **
Jericho	37.53 ± 11.39	39.33 ± 9.66	554.44 ± 184.83	939.01 ± 125.84 **	624.78 ± 194.19	978.35 ± 132.15 **

* and ** denote the statistical significant difference compared to the control (−CNP) counterparts at the $p < 0.05$ and $p < 0.01$ level, respectively. –CNP: no CNP treatment; +CNP: treated with CNP; FW: fresh weight.

4. Discussion

Salinity stress is a critical environmental issue that affects crop production globally. Salinity stress can particularly inhibit seed germination and early seedling development of seeded crops like lettuce, resulting in poor stand establishment. Our study shows that there is a significant variation in sensitivity to salt stress among the 27 lettuce varieties (Figure 1). The identified six most sensitive varieties fell into different categories (Leaf, Butter head, Romaine), indicating that sensitivity to salt stress is not specific to a certain category. Seed priming or pretreatment with nanoparticles have been shown to promote seed germination of various crops [11,23,24,27]. Here, we found that pretreatment with CNPs for only 2–4 h could significantly improve seed germinations of five of the six tested varieties under salinity and high temperature stresses. However, the effect of CNP is variety-dependent. Parris Island seeds exhibited high responsiveness to CNP treatment at germination for both salinity and temperature stresses, whereas there was no effect on Little Gem seeds. The results from Little Gem were not due to its hypersensitivity to salt, because no improvement in seed germination was observed even when the NaCl concentration was lowered to 100 mM (data not shown). In addition, pretreatment with CNPs had more beneficial effects on seed germination under salt stress than under high temperature stress.

Lettuce seed germination is inhibited by salt-induced osmotic stress rather than ion toxicity, since these seeds could fully germinate after removal of salt solutions. The salt-induced osmotic imbalance can reduce seed water uptake, and nanoparticles have been reported to enter plant cells to adjust osmotic imbalance for enhancing water uptake [36]. Increased water content was observed in the carbon nanomaterial treated seeds during germination when compared to the controls [25]. Therefore, the CNP-derived osmotic adjustment between the plant and the substrate may maintain water uptake and cell turgor for growth. Unlike salt stress, lettuce seed thermoinhibition is mainly due to the de novo synthesis of Abscisic Acid (ABA) induced by high temperature [37]. The mechanism underlying the effect of CNP pretreatment on alleviating thermoinhibition remains unclear at this time. However, several studies have shown that nanoparticles can upregulate heat shock proteins and mediate antioxidative properties, which play important roles in thermotolerance [38,39].

Reports of inhibition of root growth due to NPs have varied greatly among nanoparticles and plants [8,40]. For example, CNP stimulated root growth of cucumber (*Cucumis sativus*) and onion (*Allium cepa*), but inhibited root elongation in tomato (*Solanum lycopersicum*) and lettuce [41], indicating that the response to nanomaterials could be species or genotype dependent. Additionally, the conflicting results from the same species may be caused by the method of NP application (e.g. as supplements of culture media or direct addition into soil or foliar spray, etc.) and distinct physicochemical properties of different nanoparticles (e.g., application concentration, particle size,

and degree of dispersion, etc.) [8,40]. In this study, we observed that the CNP treatment may inhibit root elongation (Table 2). Increases in chlorophyll content were observed in three lettuce varieties treated with CNPs (Table 3). It was reported that CNP treatment could also increase chlorophyll content in tobacco and Arabidopsis, but contradicting effects were also observed in Arabidopsis and duckweed [8]. Interestingly, the increment in chlorophyll content is derived primarily from the increase in Chl b (Table 3). An increase in chlorophyll b is an adaption to shade, as it allows the plant to absorb a broader range of wavelengths of light. Silver nanoparticle and soluble carbon dot particles have been reported to alleviate dark stress to reduce petal and leaf abscission of geranium and promote wheat seedling growth [42,43].

5. Conclusions

We have shown that pretreatment of lettuce seeds with soluble carbon particles can significantly alleviate deleterious effects of salt stress on their germination. However, sensitivity to CNPs is genotype-dependent, and the effect of CNP on seed germination, and particularly on post-germination growth, varies among varieties. Thus, pre-testing for sensitivity prior to application of these soluble carbon particles is recommended.

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