Nanoceria Seed Priming Improves Salt Tolerance in Rapeseed Through Modulating ROS Homeostasis and α -Amylase Activities

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Keywords: Nanoceria, salt stress, seed priming, ROS homeostasis, α -amylase activity, Na^+/K^+ ratio

Posted Date: June 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-642905/v1>

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Abstract

Background: Salinity is a big threat to agriculture by limiting crop production. Nanopriming (seed priming with nanomaterials) is an emerged approach to improve plant stress tolerance; however, our knowledge about the underlying mechanisms is limited.

Results: We used cerium oxide nanoparticles (nanoceria) to prime rapeseeds and investigated the possible mechanisms behind nanoceria improved rapeseed salt tolerance. We synthesized and characterized polyacrylic acid coated nanoceria (PNC, 8.5 ± 0.2 nm, -43.3 ± 6.3 mV) and monitored its distribution in different tissues of the seed during the imbibition period (1, 3, 8h priming). Our results showed that compared with the no nanoparticle control, PNC nanopriming improved germination rate (12%) and biomass (41%) in rapeseeds under salt stress (200 mM NaCl). During the priming hours, PNC were located mostly in the seed coat, nevertheless the intensity of PNC in cotyledon and radicle was increased alongside with the increase of priming hours. During the priming hours, the amount of the absorbed water (52%, 14%, 12% increase at 1, 3, 8h priming, respectively) and the activities of α -amylase were significantly higher (175%, 309%, 295% increase at 1, 3, 8h priming, respectively) in PNC treatment than the control. PNC primed rapeseeds showed significantly lower content of MDA, H_2O_2 , and $\cdot O_2^-$ in both shoot and root than the control under salt stress. Also, under salt stress, PNC nanopriming enabled significantly higher K^+ retention (29%) and also significantly lower Na^+ accumulation (18.5%) and Na^+/K^+ ratio (37%) than the control.

Conclusions: Our results suggested that besides the more absorbed water and increased α -amylase activities, PNC nanopriming improves salt tolerance in rapeseeds through maintaining ROS homeostasis and Na^+/K^+ ratio. It adds more knowledge regarding the mechanisms underlying nanopriming improved plant salt tolerance.

Background

Rapeseed (*Brassica napus* L.) is one of the important oilseed crops in the world [1]. In the past decade, the rapeseed total harvested area, productivity, and annual grain yield increased by 17.6% (29.5–34.7 million ha), 50.3% (50.6–76.1 million tons), and 27.8% (1716–2194 kg ha) respectively, reflecting that the demand for rapeseed is continuing to increase [2]. However, stresses such as drought, salinity, and heat limit rapeseed production [3]. Generally, about 20% (~ 953 million ha) of the total global land is salt-affected [3]. Salinity is one of the major stresses impairing seed germination ability or resulting longer germination time [4, 5]. Besides seed germination, salinity stress affects the performance of rapeseed plants in early seedling growth stage, inhibits photosynthetic functions in vegetative growth stage, and reduces seed production [6, 7].

In general, seed germination is the first step of establishing a plant. The process of seed germination is categorized into three subsequent phases: (1) imbibition phase (water absorption), (2) lag phase (reserves metabolism), and (3) radical protrusion [8]. To improve seed germination under hostile conditions, many techniques have been applied in agricultural practice. Seed priming is one of the widely adopted techniques. During the process of priming, seeds are imbibed in a solution containing certain solutes for the activation of pre-germinative metabolism while not allowing the seed to fully germinate [1]. Among the priming techniques, seed nanopriming which uses nanomaterials to prime seeds, has been reported to be successfully applied for improving germination and stress tolerance of seedlings [9]. Nanoparticles (NPs) such as AgNPs and Fe₂O₃ were documented to improve seed development by enhancing starch metabolism and triggering Fe acquisition in wheat and rice [10]. Seed priming in lupine with ZnO NPs played an important role in NaCl stress tolerance via enhancing antioxidant activities and Na⁺ adjustment [11]. Similarly, in wheat crops, AgNPs modulated hormonal balance while improved seed germination and salinity tolerance [12].

Cerium oxide nanoparticles (nanoceria, CeO₂-NPs), due to their unique catalytic ROS (reactive oxygen species) scavenging properties, are widely used in industry, medical research and plant science [13, 14]. Cerium oxide nanoparticles were reported to reduce overaccumulated ROS, thus improving plant tolerance to stress such as salinity [15, 16] light and temperature stress [16]. Further studies showed that after scavenging of ROS, nanoceria could modulate the activities of channel proteins related to K⁺ efflux to enable better mesophyll K⁺ retention, and upregulate the expression of *HKT1* gene to allow better shoot Na⁺ exclusion, thus improving plant salt tolerance [17, 18]. To our surprise, to date, no PNC nanopriming was tested on rapeseeds. Whether PNC nanopriming would improve rapeseed salt tolerance is still unknown. Also, the mechanisms underlying PNC nanopriming improved plant salt tolerance are ambiguous. Our previous study showed that under paperroll condition, PNC nanopriming improved salt tolerance in cotton seedlings by modulating ROS homeostasis and Ca²⁺ signalling [19]. However, whether the underlying mechanisms such as nanoparticle distribution in PNC primed seeds, and the maintenance of ROS homeostasis and Na⁺/K⁺ ratio in the consequent established seedlings will be different between rapeseed and other crops e.g. cotton are still unknown. Also, the effect of PNC nanopriming on the relative gene expression level and activities of α-amylase and thus the soluble sugar content in seeds during the priming hours was largely overlooked. In the present study we will try to address above questions to investigate the mechanisms regarding PNC nanopriming improved plant salt tolerance.

In this work, we studied the distribution of PNC in the seed coat, cotyledon, and radical during the priming hours. We analyzed the activities and relative gene expression of α-amylase in rapeseeds with PNC nanopriming. Furthermore, after priming, we compared the activities of antioxidant enzymes and the ROS level between PNC nanoprimed rapeseeds and the control one under salinity stress. We also investigated the Na⁺ content, K⁺ content, and Na⁺/K⁺ ratio in shoot and root in salt (200 mM NaCl) stressed rapeseeds with or without PNC nanopriming. Our results add more knowledge to nanopriming improved plant salt tolerance.

Materials And Methods

Synthesis and characterization of nanoceria (PNC)

Following our previous paper [20], 1.08 g of cerium (III) nitrate (Sigma Aldrich, 99%) and 4.5 g poly (acrylic) acid (1800 MW, Sigma Aldrich) were dissolved in 2.5 mL and 5 mL ddH₂O in a 50 mL conical tube, respectively. These solutions were thoroughly mixed by using a vortex mixture at 2,000 rpm for 15 minutes. To a 50 mL glass beaker, 15 mL of 30% ammonium hydroxide solution (Sigma Aldrich) (7.2 M) was added. The mixture of cerium (III) nitrate and poly (acrylic) acid was added dropwise into the ammonium hydroxide solution while stirring at 500 rpm overnight at room temperature in a fume hood. After 24h, to remove any possible debris and large agglomerates, the final solution obtained was transferred to 50 mL conical tube and centrifuged at 4,000 x g for 1h. The obtained supernatant was transferred into three 15 mL 10 kDa filters (MWCO 10 K, Millipore Inc.) and centrifuged at 4,500 rpm for at least six cycles (45 min each cycle) for further purification. The freshly synthesized PNC was stored at 4 °C until further use. The final concentration of the synthesized PNC solution was calculated by recording the absorbance at 271 nm using an UV-VIS spectrophotometer according to Beer-Lambert's law (Figure S1). The zeta potential and size of PNC dispersed in DI water were measured by dynamic light scattering instrument (Malvern Zetasizer, Nano). FEI Talos microscope operating at 300 kV was used for recording transmission electron microscopy (TEM) images.

Seed materials, seed priming, stress treatments, and growth conditions

Seeds of rapeseed salt-sensitive variety “Zhongshuang 11” (ZS 11) were used in this experiment [21]. After concentration screening experiment (Figure S2), 0.1 mM PNC was used as a priming agent in this experiment. For pH maintenance, PNC in 10 mM TES buffer (pH 7.5 adjusted by HCl) [20] was used. 10 mM TES buffer alone was used as a control group. Seeds were immersed in PNC+TES or TES solution. The conical flasks containing the seeds and priming solution were put on a mechanical shaker (50 rpm) under dark conditions with constant gentle agitation for 8 hours, and the seed to solution ratio was 1:5 (w/v) [1]. After 8h priming, the seeds were rinsed with DI water and the seeds were kept in dark at ambient room temperature to dry-back. The primed seeds were sown in polyethylene boxes (12 × 12 × 6 cm length, breadth, and height, respectively). The boxes contained three sterilized germination papers moistened with 10 mL of 200 mM NaCl solution or DI water. Every second day the bottom two germination papers were replaced with two new papers and 7 mL of salt solution or DI water was added to the corresponding boxes. The boxes were exposed to 14h light (200 μmol m⁻² s⁻¹) and 10 h dark duration with 25 ± 1 and 20 ± 1 °C, respectively. The germination rate was recorded on daily basis and the germination trail was terminated 7 days after sowing. Then, the biomass was recorded immediately.

Localization of nanoceria in rapeseed seeds

To visualize the localization of nanoceria in the different tissues of seed, i.e. seed coat, cotyledon, and radical, during priming hours, PNC was labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) fluorescent dye following the standard protocols as described in previous

study [20]. Briefly, 4 mL of 0.5 mM (5.8 mg/L) PNC was mixed with 200 μ L Dil dye solution [0.3 mg/L, in dimethylsulfoxide (DMSO)] at 1,000 rpm continuous stirring for 1 min at ambient temperature. The obtained mixture was then purified at 4,500 rpm for five cycles (5 min each cycle) using a 10K Amicon cell (MWCO 30K, Millipore Inc.). Eventually, final concentration of Dil-PNC solution was calculated using the same method as described in section 2.1 (Figure S1).

Rapeseed seeds were immersed in the Dil-PNC+TES solution for the 1, 3, and 8 hours with TES (10 mM) as a control group. At each time point, the seeds were sliced into seed coat, cotyledon, and radical (~200 μ m size). The sliced seed tissues at different time points were mounted on the glass slides. A drop of perfluorodecalin (PFD) was applied to each slice for better quality of confocal imaging. A square coverslip was placed on the mounted sample and was gently pressed to well-cover the sample with observation gel and remove the air bubbles trapped underneath. The prepared sample slide was placed on the microscope and imaged by a Leica laser scanning confocal microscope (TCS, SP8). The imaging settings are as follows: 514 nm laser excitation; Z-Stack section thickness: 4 μ m; PMT1, 550-615 nm, for Dil-PNC fluorescence; PMT2, 700-750 nm, for the possible fluorescence of different seed tissues. 3-4 biological replicates were used for confocal imaging.

Measurement of α -amylase activity, water content, and total soluble sugar content in rapeseeds

For the imbibition experiments, 0.5 g seeds were immersed in the respective solutions. The water absorbance by the seeds was calculated from difference in initial weight and the water absorbed during the particular hour of priming. The activity of α -amylase was determined by 3, 5-dinitrosalicylic acid (DNS) method [22]. For each treatment, 1g seeds was grinded in liquid nitrogen with the help of mortar and pestle. The grinded samples were transferred into centrifuge tubes containing 10 mL of phosphate buffer (pH 7). Pipetted 100 μ L enzyme solution into tubes containing 1 mL DNS reagents and 1 mL distilled water. All the tubes were incubated in hot water bath for 10 minutes, and then were allowed to cool down at ambient temperature. Finally, the samples were well-mixed and the absorbance was recorded at 540 nm while tarring the spectrophotometer with a blank sample. The total soluble sugar content was determined by following the instructions provided by Suzhou Biotechnology Co., Ltd. "Kit for total soluble sugar determination" (item number: G0501W).

Quantification of ROS levels and antioxidants enzymes assay

For the determination of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) assay, kits from "Nanjing Jiancheng Biotechnology Co., Ltd (item number: A04-1-1)" and "Beijing Leigen Biotechnology Co., Ltd. (item number: T01123)" were used respectively. H_2O_2 and O_2^- contents were measured according to the instructions provided by the manufacturer. MDA content was determined by homogenizing 0.1 g fresh sample with 1 mL of 5% (w/v) trichloroacetic acid at 10,000 g for 10 min [23]. Subsequently, 0.4 mL of 5% of trichloroacetic acid (TTCA) containing 0.67% (w/v) thiobarbituric acid (TBA) was added to 0.4 mL of the supernatant. The absorbance was recorded via spectrophotometer at 450 nm, 532 nm, and 600 nm. MDA was quantified as follow: MDA concentration ($\mu\text{mol/L}$) = $6.45(\text{A}_{532}-\text{A}_{600})-0.56\text{A}_{450}$, where A=

absorbance at different wavelengths. Finally, MDA content ($\mu\text{mol/g}$) = $C \times V / (1000 \times W)$, C = concentration of MDA, V = sample extraction liquid (mL), W = weight of sample.

The activities of superoxide dismutase (SOD) was measured by homogenizing 0.1 g fresh sample in 1 mL of phosphate buffer (pH 7.8) having 0.1 mM EDTA [24]. The homogenate was centrifuged at 12,000 rpm at 4 °C. In a 10 mL tube, 0.2 ml of the enzyme extract, 0.3 mL 130 mmol/L Met (methionine) buffer, 0.3 mL 750 $\mu\text{mol/L}$ NBT (nitroblue tetrazolium), 0.3 mL 100 $\mu\text{mol/L}$ EDTA- Na_2 , and 0.3 ml of 20 $\mu\text{mol/L}$ flavin were added. At the end of the reaction, dark control tube was used as a blank control, and the absorbance was recorded at 560 nm using spectrophotometer. SOD activity (U/g) = $2(\text{ACK}-\text{AE}) \times V / (\text{ACK} \times W \times V_t)$, ACK = dark control absorbance, AE = sample absorbance, V = volume of sample (mL), W = weight of sample (g), V_t = extract liquid volume (mL). The catalase (CAT) activity was measured according to the standard procedures of [25]. Samples were ground with PBS buffer (pH 7.8) followed by centrifugation at 4,000 rpm (15 min). After the centrifugation the collected supernatant was vortexed with PBS buffer (pH 7.8) and H_2O_2 (10 mM). At 240 nm (1 record/ 1 min, 4 min), the average decrease in the absorbance was recoded and the absorbance coefficient of $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ was used. The final value of CAT was expressed as $\text{mmol H}_2\text{O}_2/\text{mg protein}/\text{min}$. Peroxidase (POD) activity was determined using guaiacol method [23]. The reaction mixture was comprised of 10 mM guaiacol, 5 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) heated at 25 °C. in 10 mL tube, 0.2 mL enzyme extract and 2.8 mL of the reaction mixture was added and mixed subsequently while the absorbance was recorded at 470 nm. Using the absorbance coefficient of $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$, the POD activity was calculated via analyzing the averaged decrease of the recorded absorbance value at 470 nm (1 record/1 min, 3 min). The final value of POD was expressed as $\mu\text{mol tetra-gualacol}/\text{mg protein}/\text{min}$.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

RNAprep Pure Plant Kit (RN38, Aidlab, Beijing, China) was used for the total RNA isolation. Using the TRUEScript first Strand cDNA Synthesis Kit (PC5402, Aidlab, Beijing, China), 2 μg of total RNA was reverse transcribed into cDNA. According to the manufacturer's instructions, the amplification of qRT-PCR products was performed in a reaction mixture of 12.5 μL SYBR Green qPCR Mix (PC3302, Aidlab, Beijing, China). The qRT-PCR analysis was performed on the Bio-Rad CFX Connect Real-Time PCR System (Bio-Rad, California, USA). For each treatment, three technical and three biological replicates were used. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. The primers used for qRT-PCR are shown in Table S1[26].

Statistical analysis

Means were compared using a one-way ANOVA based on Tukey and Duncan test in SPSS software. Different lowercase alphabets indictate significant difference at $P = 0.05$. Error bars are standard error. The graphs were plotted with Excel 2016.

Results

Characterization of PNC

A clear peak at 271 nm was observed in the absorbance curve of PNC (Figure S1). The images from transmission electron microscopy (TEM) shows an average PNC core size of 4.7 ± 0.9 nm (Figure 1a). Analysis from a dynamic light scattering instrument (Malvern Zetasizer, Nano) showed that the average size of PNC by intensity was 8.5 ± 0.2 nm (Figure 1b), and the average zeta potential was -43.3 ± 6.3 mV (Figure 1c).

Influence of PNC priming on rapeseed germination and phenotype

The germination rate of rapeseed was not significantly affected by PNC+TES priming under normal growing conditions as compared to TES (or control) priming (Figure 1d). However, a significant difference of the germination rate was observed between PNC+TES priming and the control/TES priming under 200 mM salinity stress from Day2 to Day7 (trial terminated), showing the final germination rate for PNC priming (84%) and TES priming (76%) ($P < 0.05$). Compared with the TES priming, PNC+TES priming markedly increased fresh weight (54.4 ± 0.3 vs 34.4 ± 0.7 mg/seedling, 41% increase) of the rapeseed seedlings under salt stress (Figure 1e).

Localization of PNC in seeds during priming hours

Seeds primed with Dil-PNC in 10 μ M TES buffer for one, three, and eight hours was sampled for visualizing the distribution of PNC in seed coat, cotyledon and radical. The sampling hours (1h, 3h, and 8h) were based on a preliminary experiment in which we measured the water absorbance by seeds. A clear increase of water content was observed in rapeseed seeds during priming hours, showing a sharp increase from 1h to 3 h, and a steady increase from 4h to 8h (Figure 1f). The results indicated that the seed begins to absorb water from the first hour of priming, whereas the peak difference in water absorbance was recorded at 3h and 8h. PNC priming enabled better water absorbance in seeds than the control at 1, 3 and 8 hours (Figure 1g). During the first hour of priming, Dil-PNC signal was only detected in the seed coat (Figure 2a), compared with no Dil-PNC signals were detected in cotyledon (Figure 2b) and radical (Figure 2c). At 3h priming, Dil-PNC signal was observed in both the seed coat (Figure 2a) and cotyledon (Figure 2b), while no Dil-PNC signal was detected in radical (Figure 2c). Further, during 8h priming, Dil-PNC was found in all tissues of the seed, i.e., seed coat, cotyledon, and radical, showing the signal intensity as seed coat > cotyledon > radical (Figure 2a, b, and c). No signals were detected in the control group at 1h, 3h, and 8h priming (Supplementary Figure S3-5).

PNC nanoprimering regulated relative expression of *AMY* genes to enhance α -amylase activity and total soluble sugar content in rapeseeds

Significant differences in α -amylase activity were recorded during the priming hours of PNC priming ($P < 0.05$). Seed priming with PNC+TES increased α -amylase activity by 175%, 309%, and 295% at 1h (0.130 ± 0.038 vs 0.047 ± 0.003 mg g⁻¹ min⁻¹), 3h (0.341 ± 0.005 vs 0.0883 ± 0.001 mg g⁻¹ min⁻¹), and 8h (0.357 ± 0.048 vs 0.090 ± 0.002 mg g⁻¹ min⁻¹) priming, respectively, compared to the TES priming

control (Figure 3a). Consistently, at 1h and 8h, the relative expression level for *AMY1* gene was significantly higher in PNC+TES primed seeds than the TES priming control, while a downregulation of *AMY1* gene was found at 3h (Figure 3b). The upregulation of *AMY2* gene was found at 8h priming with PNC+TES than the TES control, compared with no difference at 1 and 3h priming (Figure 3c). The highest increase in the relative expression values for *AMY1* and *AMY2* was recorded at 8h priming, showing 169% and 68% increase respectively (Figure 3b and 3c). Compared to TES priming, PNC+TES priming significantly increased total soluble sugar content (TSS) by 177%, 64%, and 58% during 1h (8.8 ± 0.1 vs 3.2 ± 0.2 mg g⁻¹ FW), 3h (10.4 ± 0.2 vs 6.4 ± 0.2 mg g⁻¹ FW), and 8h (13.1 ± 0.2 vs 8.3 ± 0.2 mg g⁻¹ FW), respectively (Figure 3d). This is in accordance with the increased activities of α -amylase in PNC+TES primed seeds than the TES control.

PNC nanopriming modulates ROS level in the seeds and seedlings of rapeseeds

During the first-hour, compared to TES priming, PNC priming showed significantly increased MDA contents (2555 ± 206 vs 2228 ± 39 mg g⁻¹ FW), H₂O₂ (18.2 ± 1.3 vs 14.2 ± 0.2 μ mol g⁻¹ FW), and $\cdot$ O₂⁻ (22.3 ± 0.3 vs 19.1 ± 0.3 μ mol g⁻¹ FW) by 15%, 28%, and 15%, respectively (Figure 4a-c). Whereas, at 3h and 8 h, PNC priming reduced the ROS contents. At 3h, PNC priming significantly reduced MDA contents (1428 ± 156 vs 1849 ± 132 mg g⁻¹ FW), H₂O₂ (7.3 ± 0.1 vs 10.5 ± 0.5 μ mol g⁻¹ FW), and $\cdot$ O₂⁻ (9.3 ± 0.4 vs 18.1 ± 0.3 μ mol g⁻¹ FW) by 23%, 31%, and 48% than the TES priming, respectively (Figure 4a-c). Similarly, at 8h, the MDA contents (1144 ± 14 vs 1625 ± 156 mg g⁻¹ FW, 30% decrease), H₂O₂ (3.0 ± 0.1 vs 10 ± 0.3 μ mol g⁻¹ FW, 70% decrease), and $\cdot$ O₂⁻ (5.2 ± 0.2 vs 14.1 ± 0.3 μ mol g⁻¹ FW, 64% decrease) was significantly lower in PNC+TES primed seeds than the TES control (Figure 4a-c). A significant increase of SOD and POD activities was found in seeds primed with PNC+TES than the TES control (Figure 4d and 4e). PNC priming increased SOD activities in seeds than the control by 52%, 127%, and 53% at 1h (92.6 ± 1.3 vs 60.8 ± 1.3 U g⁻¹ FW), 3h (156.2 ± 1.3 vs 68.7 ± 1.3 U g⁻¹ FW), and 8h (172.1 ± 1.3 vs 112.5 ± 1.3 U g⁻¹ FW), respectively (Figure 4d). PNC priming increased POD activities in seeds than the control by 76% and 53% and 60% at 1h (6.4 ± 1.0 vs 3.6 ± 0.1 μ mol tetra-gualacol /mg protein /min), 3h (5.9 ± 0.4 vs 3.8 ± 0.1 μ mol tetra-gualacol /mg protein /min) and 8h (7.1 ± 0.3 vs 4.5 ± 0.1 μ mol tetra-gualacol /mg protein/min), respectively (Figure 4e). In contrast to the changes of SOD and POD activities, PNC priming resulted significantly lower CAT activity in seeds than the control, showing 43% and 55% decrease at 3h (0.85 ± 0.07 vs 1.49 ± 0.09 mmol H₂O₂/mg protein/min), and 8h (0.84 ± 0.07 vs 1.88 ± 0.15 mmol H₂O₂/mg protein/min), respectively (Figure 4f).

Compared with the non-stress condition, MDA, H₂O₂, and $\cdot$ O₂⁻ contents were significantly increased in salt stressed (200 mM NaCl, 7 days) seedlings of rapeseeds with or without PNC priming (Figure 5a-e). Under non-saline condition, no difference of MDA, H₂O₂, and $\cdot$ O₂⁻ content was found in either shoot or root seedlings of rapeseeds (7 days old) with or without PNC priming. However, under salt stress, PNC primed seedlings significantly reduced MDA (5234 ± 165 vs 6918 ± 100 mg g⁻¹ FW, 24% decrease), H₂O₂ (23.4 ± 0.6 vs 69.8 ± 1.8 μ mol g⁻¹ FW, 66% decrease), and $\cdot$ O₂⁻ (3.4 ± 0.3 vs 6.7 ± 0.3 μ mol g⁻¹ FW, 50%

decrease) content in shoot than the control (Figure a, 5c and 5e). Similarly, compared with the control, 27%, 39%, and 37% decrease of MDA (3395 ± 47 vs 4656 ± 122 mg g⁻¹ FW), H₂O₂ (13.2 ± 0.7 vs 21.6 ± 1.6 μmol g⁻¹ FW), and 'O₂⁻ (6.1 ± 0.7 vs 9.7 ± 1.8 μmol g⁻¹ FW) content was found in the root of PNC primed seedlings (Figure 5b, 5d and 5f). Similar to the results of the seeds during priming hours, compared with a decrease in CAT activities (0.94 ± 0.05 vs 1.78 ± 0.03 μmol H₂O₂/mg protein/min for shoot, and 1.10 ± 0.02 vs 1.60 ± 0.02 μmol H₂O₂/mg protein/min for root), a significant increase of SOD (183.9 ± 6.6 vs 151.3 ± 8.2 U g⁻¹ FW for shoot, and 131.7 ± 1.2 vs 107.6 ± 5.7 U g⁻¹ FW for root) and POD (6.5 ± 0.6 vs 5.53 ± 0.05 μmol tetra-gualacol /mg protein /min for shoot, and 8.8 ± 0.3 vs 5.6 ± 0.2 μmol tetra-gualacol /mg protein /min for root) activities was found in shoot and root of seedlings primed with PNC+TES than the TES control under salt stress (Figure 6a-e). Under non-saline condition, no significant difference was found between seedlings primed with and without PNC priming (Figure 6a-e). It shows that PNC priming enhanced shoot and root SOD (21% and 22%, respectively) and POD (17% and 49%, respectively) activities in contrast to the control under salt stress conditions (Figure 6a-d), while a decrease of CAT activity by 82% and 31% was observed in the shoot and root compared to the control under salt stress (Figure 6e-f).

PNC nanopriming affected Na⁺/K⁺ ratio in rapeseed under salt stress

Compared to non-saline conditions, an increase of Na⁺ content was found in salt stressed (200 mM NaCl, 7 days) seedlings of rapeseeds with or without PNC priming (Figure 7a-b). However, seedlings with PNC priming showed increased shoot Na⁺ content by 13% (17.5 ± 0.4 vs 15.6 ± 0.7 mg g⁻¹ DW, Figure 7a) and decreased root Na⁺ content by 52% (7.0 ± 0.5 vs 14.5 ± 0.6 mg g⁻¹ DW, Figure 7b) than the control under salt stress. An overall decrease of Na⁺ content was observed in seedlings primed with PNC than the control under salt stress (24.5 ± 0.6 vs 30.8 ± 0.6 mg g⁻¹ DW, Figure S6a). In comparison with non-saline conditions, K⁺ content in shoot and root of seedlings with or without PNC priming was significantly reduced under salt stress (200 mM NaCl, 7 days) (Figure 7c and 7d). While seedlings primed with PNC maintained higher K⁺ content in the shoot and root by 31% (3.80 ± 0.06 vs 2.91 ± 0.27 mg g⁻¹ DW, Figure 7c) and 29% (2.9 ± 0.2 vs 2.3 ± 0.1 mg g⁻¹ DW, Figure 7d), respectively than the control (seedlings primed with TES) under salt stress. An overall better maintained K⁺ content was observed in seedlings primed with PNC than the control under salt stress (6.7 ± 0.2 vs 5.1 ± 0.2 mg g⁻¹ DW, Figure S6b). Not surprisingly, compared to the TES control, seedlings primed with PNC showed significantly reduced Na⁺/K⁺ ratio by 10% and 62% in the shoot (4.6 ± 0.2 vs 5.1 ± 0.2 , Figure 7e) and root (2.4 ± 0.2 vs 6.3 ± 0.2 , Figure 7f) under salinity stress, respectively. An overall decreased Na⁺/K⁺ ratio was observed in seedlings primed with PNC than the control under salt stress (3.65 ± 0.07 vs 5.81 ± 0.08 , Figure S6c). No significant difference of Na⁺/K⁺ ratio in either shoot or root was found in seedlings primed with and without PNC under non-saline condition (Figure 7e and 7f).

Discussion

PNC nanopriming improves rapeseed salt tolerance through modulating α -amylase activity

In terms of nano-enabled agriculture, nanopriming technique could be a good candidate to improve plant tolerance to salinity stress. In wheat, seed priming with silver nanoparticles (AgNPs) improved the germination and growth parameters by regulating hormonal balance and photosynthetic efficiency under salt stress [27]. Under salt stress, nanopriming with calcium silicate (Ca_2SiO_4) in lettuce improved seed germination through triggering antioxidant enzymes including SOD, CAT and GR (glutathione reductase) to effectively scavenge the over-produced ROS [28]. Water soluble carbon nanoparticles (CNP) promoted lettuce germination and lateral root growth under salt stress [29]. Titanium dioxide (TiO_2) nanopriming positively affected germination and seedling growth under salt stress by promoting antioxidant enzymes, relative water content, proline content, K^+ content, and reduced Na^+ content in maize crop [18]. Compared with the water control, soaking cotton seeds with nanoceria showed increased tolerance (46% increase of fresh weight) to salt stress, whereas no significant difference of germination rate was found between the treatments [19]. During the imbibition of water, nanoparticles enter the seed via the void spaces in the seed coat while some nanoparticles may also tend to adsorb at the surface of seed coat [30]. However, the surface adsorptions of the nanoparticles at seed coat may pose negative effects on germination, seedling growth, and enzymatic activities [31]. Here, we show that during priming hours, most of PNC is located at the surface of seed coat (Fig. 2), indicating a possible interaction between PNC and seed coat which might benefit seed performance under salt stress. PNC nanopriming significantly improved rapeseed tolerance to salinity stress, showing not only the increase of germination rate (12%) but also the fresh weight (41%) in rapeseed primed with PNC + TES than the TES buffer under salt stress (Fig. 1d and 1e). Our results showed that PNC nanopriming could help to improve salt tolerance in rapeseeds.

Generally, seed germination begins with imbibition (water uptake by seed) and terminates with the protrusion of radicle and plumule through the seed envelope [32]. During the germination, α -amylase is the key enzyme responsible for the degradation of starch [33]. Thus, under stress conditions the enhancement of α -amylase activity during the priming hours is associated with plant stress tolerance [34–36]. The results of our experiment revealed that PNC priming showed increased α -amylase activity than the control during 1h, 3h, and 8h priming hours, which is further confirmed by the upregulation of relative gene expression of *AMY1* and *AMY2* in PNC + TES primed seeds than the TES priming (Fig. 3). Interestingly, we noticed that at 3h priming, no upregulation of *AMY1* and *AMY2* was observed in PNC primed seeds than the control, while the α -amylase activity is increased in PNC group (Fig. 3), showing that the α -amylase activity could not be fully reflected by the relative expression of α -amylase genes (*AMY1* and *AMY2* in this study). This could be due to posttranscriptional regulation on α -amylase [37] or some other factors which could affect the activities of α -amylase. Another reason of the enhanced α -amylase activity in PNC + TES primed seeds might be associated with higher uptake of water during the imbibition period (Fig. 1g). During seed priming, higher amount absorbed water is always associated with the increased total soluble sugars [38]. Not surprisingly, significant higher content of total soluble sugars was found in rapeseed seeds primed with PNC + TES than TES alone, further supporting the higher α -

amylase activity in PNC primed seeds (Fig. 3a). This is similar to previous studies showing that nanopriming enhanced α -amylase activity and enhanced soluble sugar content to improve seed germination [33]. The amount of available soluble sugars in seeds are of importance for the following buildup of seedlings and even its stress tolerance [39]. Previous studies showed nanopriming [Zinc oxide (ZnO-NPs) nanoparticles, 50 ppm, 50 nm, 23 mV] enabled a positive correlation between improved α -amylase activity and seed germination and seedling vigor of lettuce plants [40]. Researchers found that seeds with higher amount of total soluble sugars showed better performance in seedling buildup and also the tolerance to drought stress [39]. Overall, our results showed that in rapeseeds, PNC nanopriming upregulated the relative expression of *AMY1* gene, showing increased α -amylase activity and thus significant higher total soluble sugar content than the control (TES priming). It then enabled better establishment of seedlings and also its tolerance to salinity stress in rapeseed primed with PNC + TES than TES priming.

PNC nanopriming reduces ROS over-accumulation to maintain better Na^+ / K^+ ratio homeostasis to improve rapeseed salt tolerance

ROS accumulation is always observed in primed seeds [30]. While over-accumulation of ROS could induce toxic effects to plants [17, 19]. PNC are known as potent ROS scavenger. Here, compared with higher ROS (H_2O_2 , O_2^-) and MDA content in PNC + TES treated seeds than the TES group during 1h priming, PNC + TES treatment significantly reduced the over-production of ROS than the TES control in seeds during 3h and 8 h priming (Fig. 4a-c). The higher ROS content in PNC + TES than TES treatment at 1h priming could be related to more absorbed water (Fig. 1g) which could trigger ROS accumulation in primed seeds [41, 42]. During priming, cotyledon and radicle are the main source for the accumulated ROS [43–45]. While, at 1h priming, PNC were mainly distributed at the seed coat (Fig. 2a-c), thus it might not be able to scavenge the higher amount of accumulated ROS than the control due to the better water absorbance in PNC primed seeds than the control (Fig. 1g). At 3h and 8h priming, PNC were also found in cotyledon and radicle (Fig. 2a-c), which helped to scavenge more the accumulated ROS in primed seeds. More interestingly, the amount of ROS in seeds are reduced alongside the priming hours, while PNC + TES group showed higher reduced amount of ROS than the TES control (Fig. 4b and 4c). Maintenance of ROS homeostasis during seed priming is of importance to seed germination and consequent seedling establishment [46]. Our results suggest that PNC nanopriming could enable better ROS scavenging ability in seeds during the priming hours (except the first hour) than the control.

After 1h priming, the activities of antioxidant enzymes are associated with ROS level in seeds, showing a significant higher SOD and POD activities in PNC + TES primed seeds than the TES priming at 3h and 8h priming (Fig. 4d-e). Similarly, the activity of SOD and POD was reported to increase and thereby controlling the over-production of H_2O_2 and O_2^- radicals in different crops due to nanopriming [47–49]. Nevertheless, in our experiment PNC reduced the activity of CAT during the priming hours (Fig. 4f). The lower activity of CAT in our experiment could be due to the fact that PNC can mimic CAT activity [16]. These findings are strongly supported by former studies which used spectrofluorometric using the

Amplex-Red reagent assay to confirm nanoceria (PNC) catalase-like catalytic activity [50]. Interestingly, the ROS level and the maintenance of ROS homeostasis showed similar trends between the priming hours experiments and the post-germination experiment. PNC priming significantly reduced the over-production of ROS (H_2O_2 and O_2^- radicals) in the shoot and root of rapeseed seedlings grown under 200 mM salt stress (Fig. 5a-f), suggesting that PNC nanopriming helped to maintain ROS homeostasis in the consequent established seedlings. Also, in contrary to the lower CAT activities, under salinity stress, SOD and POD activities are higher in rapeseeds seedling originated from PNC primed seeds than the control, regardless of shoot or root (Fig. 6a-f), showing that PNC nanopriming could affect the efficacy of antioxidant enzyme system in established seedlings. This might be associated with possible epigenetic effects enabled by nanomaterials in seeds [46, 51]. The successful savaging of ROS by PNC under sub-optimal growing conditions were also reported by several other studies [15, 17, 19, 52]

Besides osmotic stress and ROS over-accumulation, salinity also cause Na^+ over-accumulation and K^+ loss in plants [53–56]. The ability of plants to maintain Na^+ and K^+ homeostasis is a critical factor for its salt tolerance [57]. As usual, salt stress increased Na^+ accumulation in shoot and root; however, PNC priming accumulated more Na^+ in shoot while less in the root (Fig. 7a-b). This is similar to previous study. CeO_2 NPs allowed more Na^+ to accumulate in shoot compared to root by decreasing root apoplastic barriers to facilitate Na^+ transportation to shoot to improve rapeseed salt tolerance [15]. The overall Na^+ content in seedlings established from PNC primed seeds is significantly lower than the control under salt stress (Figure S6), suggesting that PNC nanopriming could help to reduce Na^+ over-accumulation in rapeseed under salt stress. This is different with the effect of nanoceria nanopriming in cotton, which shows that no significant difference of Na^+ content in cotyledon, hypocotyl and root was found between the seedlings established from nanoceria primed seeds and the control under salt stress [19].

Furthermore, compared with the non-saline condition, salt stress decreased K^+ content in shoot and root of rapeseed seedlings established with or without PNC nanopriming. Nevertheless, seedlings established from PNC primed seeds showed significantly lower K^+ loss from the shoot and root than the control under salt stress (Fig. 7c-d), suggesting that PNC nanopriming enabled better K^+ retention in rapeseed under salt stress. Previous studies showed that higher K^+ retention is associated with better salt tolerance [17, 19, 58]. In cotton under salt stress, PNC nanopriming resulted in significant lower K^+ content in root while no difference in cotyledon and hypocotyl [19]. These results suggest that the mechanisms employed in PNC nanopriming improved salt tolerance in rapeseed and cotton are different, indicating the complexity of mechanisms associated with nanopriming improved plant salt tolerance. Moreover, salt stress increased Na^+/K^+ ratio in the shoot and root of rapeseed seedlings established with or without PNC nanopriming, while PNC priming enable a reduced Na^+/K^+ ratio in the root and shoot than the control under salt stress (Fig. 7e-f), suggesting that PNC helped to maintain better Na^+/K^+ ratio than the control under salt stress. Na^+/K^+ ratio is a hallmark of plant salt tolerance [59]. Together with the Na^+ and K^+ content data, our results suggest that PNC nanopriming could help to maintain Na^+/K^+ ratio in rapeseed by reduce Na^+ over-accumulation and K^+ loss.

Nanopriming could be a promising way to improve plant salt stress tolerance

To improve crop production in lands affected by salinity, different strategies have been practiced [60]. Strategies such as water saving irrigation, drainage system, and soil management practices have been applied to promote agricultural production [61, 62]. However, these approaches are expensive which may cost more money on its implementation. Similarly, approaches i.e. screening and breeding of salt tolerant varieties [63], potential use of halophytes [64], and the use of beneficial soil microorganisms [65] could also promote agricultural production in salt affected soils. While these applications require long time. New approaches which are affordable and no time consuming are encouraged to address salinity issue in agricultural production.

Nanotechnology has potential to provide effective solutions to agriculture-related problems [66, 67]. Since the last couple of decades, a significant amount of research has been carried out on the application nanoparticles in agriculture under hostile environmental conditions. For example, to improve salt tolerance in crops, nanoparticles were applied as foliar spray or mixed with soil [68, 69]. Application of cerium oxide nanoparticles improved stress tolerance in *Moldavian balm* [foliar spray application, 50 mg L⁻¹, [70]], soybean [addition to dry soil at the rate of 2000 mg L⁻¹, [71]], and lettuce [addition to the soil at the rate of 100 mg Kg⁻¹ soil, [72]] plants by enhancing antioxidant enzymes activities, osmoregulation, photosynthesis, and water relation. However, foliar or soil application of nanoparticles have some obstacles such as possible high cost and environmental pollution, hindering the adaptation of nanotechnology and its widespread application. Economic viability and biosafety issues are considered as the major obstacles due to the reason that the higher dosage application of nanoparticles could cost more money and also the application process in agricultural land could lead to serious health and environmental risks [73, 74]. Therefore, nanopriming could be a sustainable strategy which entails a minimum use of nanoparticles by not only reducing the cost due to very small dosage application, but also less risk of nanomaterials on environment. For the current rapeseeds planting mode in China, estimated seed rate for rapeseed in the field is ~ 5 Kg/hectare. For the nanopriming, we need 25 mL (1:5 Kg/L) of priming solution for 5000 g seeds. Thus, we need about 0.138 L of PNC (0.1 mM, equal to 11.1 mg/L [16] for the seed rate/hectare. Thus, the amount of PNC for rapeseed nanopriming (1.5 mg PNC for one hectare) is far less than the soil application (1000 mg Kg⁻¹ dry sand and clay mixture, [69] or foliar spray (3400 mg PNC for one hectare, personnel communication to Dr. Lan Zhu at Huazhong Agricultural University) for improving rapeseed salt tolerance. It thus not only reduces the cost, but largely alleviates the concerns about biological effects of nanomaterials in environment.

Conclusion

In summary, during the priming hours, compared the seeds primed with TES control solution, uptake of PNC by seeds allows more water absorbance, higher antioxidant enzyme (SOD and POD) activities, lower ROS accumulation, higher α -amylase activity (also the upregulation of *AMY1* gene) and total soluble sugar contents. We also found that during the first 1h priming, PNC are mainly located at the seed coat. While, at 3h and 8h, PNC are gradually absorbed into the cotyledon and the radical (only observed at 8h

priming). After seedling establishment, PNC nanopriming enabled higher antioxidant enzyme (SOD and POD) activities, lower ROS accumulation, and better maintenance of Na^+/K^+ ratio than the TES control under salt stress. Our results add more knowledge to PNC nanopriming improved salt tolerance in rapeseed. Overall, this could be the first study which investigated the downstream events from nanoparticle distribution, ROS level and antioxidant enzyme activities, and α -amylase activities and its gene expression in seeds during priming hours to the maintenance of ROS homeostasis and Na^+/K^+ ratio in salt stressed seedlings primed with nanocerium. However, it remains unclear whether the PNC nanopriming improved salt tolerance could last until plant harvest or not. Field or glasshouse experiments are required in further studies. Whether PNC nanopriming could improve the quality of rapeseed such as oil, protein and fatty acid contents or not is also worthy to be studied in future work.

Declarations

Acknowledgments

We thank A/Prof. Bo Wang for providing rapeseed seeds, and Mr. Jianbo Cao and Mr. Limin He for their help in TEM imaging at Public Laboratory of Electron Microscopy, Huazhong Agricultural University. This work was supported by the NSFC grant (No. 32071971, 31901464), project 2662020ZKPY001 supported by the Fundamental Research Funds for the Central Universities, and joint project SZYJY2021008 from Huazhong Agricultural University and Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences to H.W.

Author Contributions

Conceptualization, H.W., M.N.K, and Z.L.; writing-original draft preparation, H.W., and M.N.K.; conducting experiment, M.N.K, Y.L., Z.K., and L.C.; data analysis; H.W., M.N.K, Y.L., Z.K., L.C., and J.H.; and all authors have read and agreed to the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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Figures

Figure 1

Figure 1

Characterization of cerium oxide nanoparticles (PNC) and the effect of PNC nanopriming on seed germination and fresh weight of rapeseed under salt stress. a, TEM image of PNC, b, PNC size by intensity, c, zeta potential of PNC, d and e, the effect of PNC priming on rapeseed germination rate and fresh weight of rapeseed seedlings under salt stress or non-saline condition, f, water uptake by rapeseed seed from 1 to 8 priming hours with an interval of 1 hour, g, comparison of absorbed water content

between PNC priming and control during 1h, 3h, and 8h. Different lowercase alphabets on the vertical bars or * indicates significant difference at $P < 0.05$. Error bars are the representative of standard deviation of three biological replicates (one batch as one biological replicates) ($n = 3$).

Figure 2

Figure 2

Confocal imaging of Dil-PNC distribution in seed coat of the PNC primed seeds. Dil-PNC signal in seed coat (a), cotyledon (b), and radical (c) of rapeseed with PNC priming at 1h, 3h, and 8h of priming. Scale

bar: 10 μm .

Figure 3

Figure 3

PNC nanoprimer modulates the activities of α -amylase in seeds. a, α -amylase activity in seeds primed with or without PNC at 1, 3, and 8 priming hours, b and c, relative expression level of AMY1 and AMY2 genes in seeds primed with or without PNC at 1, 3, and 8 priming hours, and d, total soluble sugar content in seeds primed with or without PNC at 1, 3, and 8 priming hours. Different lowercase alphabets on the vertical bars or * indicates significant difference at P < 0.05. Error bars are the representative of standard error of three biological replicates (one batch as one biological replicates) (n = 3).

Figure 4

Figure 4

PNC nanoprimer modulates ROS homeostasis and antioxidant enzymes activities in seeds. MDA content (a), H₂O₂ content (b), •O₂⁻ content (c), SOD activity (d), POD activity (e), and CAT activity (f) in seeds primed with or without PNC at 1, 3, and 8 priming hours. Different lowercase alphabets on the vertical bars indicates significant difference at $P < 0.05$. Error bars are the representative of standard error of three biological replicates (one batch as one biological replicates) ($n = 3$).

Figure 5

Figure 5

PNC nanoprimer modulates ROS level in salt stressed seedlings established from the primed seeds. MDA content (a for shoot, b for root), H_2O_2 content (c for shoot, d for root), and $\text{O}_2^{\cdot-}$ content (e for shoot, f for root) in seedlings established from seeds primed with or without PNC under 200 mM NaCl stress (7 days) or non-saline condition. Different lowercase alphabets on the vertical bars indicates

significant difference at $P < 0.05$. Error bars are the representative of standard error of three biological replicates (one batch as one biological replicates) ($n = 3$).

Figure 6

Figure 6

PNC nanoprimer modulates antioxidant enzyme activities in salt stressed seedlings established from the primed seeds. Activities of SOD enzyme (a for shoot, b for root), POD enzyme (c for shoot, d for root), and CAT enzyme (e for shoot, f for root) in seedlings established from seeds primed with or without PNC

under 200 mM NaCl stress (7 days) or non-saline condition. Different lowercase alphabets on the vertical bars indicates significant difference. Error bars are the representative of standard error of three biological replicates (one batch as one biological replicates) (n = 3).

Figure 7

PNC nanoprimering modulates Na⁺ and K⁺ content and Na⁺/K⁺ ratio in rapeseed shoot and root under salt stress. Na⁺ content (a for shoot, b for root), K⁺ content (c for shoot, d for root), and Na⁺/K⁺ ratio (e for

shoot, f for root) in seedlings established from seeds primed with or without PNC under 200 mM NaCl stress (7 days) or non-saline condition. Different lowercase alphabets on the vertical bars indicates significant difference at $P < 0.05$. Error bars are the representative of standard error of three biological replicates (one batch as one biological replicates) ($n = 3$).

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