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Response of soybean nodules to exogenously applied caffeic acid during NaCl-induced salinity

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ABSTRACT

Caffeic acid acts as an antioxidant to scavenge reactive oxygen species, but its influence on plant responses to abiotic stresses is only partially understood. Here, we investigated the influence of exogenously applied caffeic acid on soybean during NaCl-induced salinity. Exogenously applied caffeic acid reduced the deleterious effects of salinity stress on soybean plants and increased nitric oxide content in root nodules and this corresponded with elevated cyclic guanosine monophosphate content in the nodules. Salinity stress reduced nodule legheamoglobin content and nitrogenase activity whereas exogenous application of caffeic acid to NaCl-treated plants reversed these negative effects of NaCl on legheamoglobin content and nitrogenase activity. Hydrogen peroxide (H_2O_2) and malondialdehyde contents in soybean root nodules from plants exposed to salinity were lower when these plants were supplemented with exogenous caffeic acid than when no caffeic acid was supplemented. We suggest that caffeic acid enhances nitric oxide biosynthesis, which possibly acts to reduce salinity-induced oxidative stress through a mechanism that involves nitric oxide signaling coupled with cyclic guanosine monophosphate-mediated signaling to scavenge reactive oxygen species.

The ability of caffeic acid to reduce salinity-induced oxidative stress via regulation of nitric oxide signaling has implications for genetic improvement of crop to enhance their tolerance against salinity. This can be achieved by identifying genes, namely genes encoding *p*-coumarate 3-hydroxylases, responsible for the biosynthesis of caffeic acid and modulating their expression under salinity. Such improvement would impact positively on food security as it would limit the detrimental effects of salinity of crop productivity.

1. Introduction

Caffeic acid (CA) has emerged as an inhibitor of root growth in plants, exerting its growth-inhibiting effects by modulating the generation of reactive oxygen species (ROS) and increasing lignification (Singh et al., 2009; Bubna et al., 2011). Recently, the role of CA in alleviating salinity stress has been associated with enhanced scavenging of the superoxide ($O_2^{\cdot-}$) via the augmentation of superoxide dismutase (SOD, EC 1.15.1.1) activity (Klein et al., 2013). The salinity tolerance induced by exogenous CA is proposed to be resulting from the reduction of the extent of cell death caused by $O_2^{\cdot-}$ accumulation during salinity stress (Klein et al., 2013).

It is well-established that nitric oxide (NO) signaling is a component of plant responses to salinity stress, as indicated by changes in NO content in plants in which salinity stress is imposed (Zhao et al., 2004, 2007). This signaling role of NO has led to the general perception that elevation of NO enhances plant tolerance to salinity stress. Such salinity tolerance is thought to be a result of the scavenging of ROS that otherwise accumulate in plant tissue during salinity stress (Shi et al., 2007; Xie et al., 2008). The involvement of NO in ROS scavenging is associated with the activation antioxidant enzymes such as SOD and ascorbate peroxidase (EC 1.11.1.11) in sweet potato (Lin et al., 2011), which suggests that conditions that trigger accumulation of NO are likely to lead to reduction of salinity-induced oxidative stress.

Signaling by NO is transduced in part by stimulation of guanylate cyclase (EC 4.6.1.2) activity, which converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP); leading to elevated cGMP levels in plants (Durner et al., 1998). Although the involvement of cGMP in antioxidant enzyme activity is not well established in plants, there is evidence that cGMP modulates ROS production by increasing nicotinamide adenine dinucleotide phosphate (NADPH) levels during salinity stress, cascading towards ROS elevation by stimulating NADPH oxidase activity (Li et al., 2011). This implicates cGMP signaling in plant responses to salinity via a pathway that may involve modulation of ROS accumulation in plants. In fact, a role for cGMP in plant responses to salinity has been described (Maathuis and Sanders, 2001; Rubio et al., 2003; Donaldson et al., 2004) as involving changes in the uptake of

Abbreviations: CA, caffeic acid; cGMP, cyclic guanosine monophosphate; DAF-FM DA, 4-amino-5-methylamino-2,7-difluorofluorescein diacetate; MDA, malondialdehyde; NO, nitric oxide; ROS, reactive oxygen species; TCA, trichloroacetic acid

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sodium ions via regulation of voltage-independent channels (Maathuis and Sanders, 2001).

Given the role of CA as an antioxidant with a capacity to inhibit ROS production (Jayanthi and Subash, 2010) and the link between CA and plant salinity tolerance (Klein et al., 2013), together with NO-mediated salinity tolerance that operates via ROS scavenging by antioxidant enzymes (Keyster et al., 2012) and transduction of the NO signal by cGMP (Mur et al., 2013); we investigated if CA influences NO and cGMP content as well as oxidative stress caused by NaCl-induced salinity stress in soybean. We propose that the exogenous application of CA alleviates salinity stress by enhancing NO biosynthesis in plant tissue and that the elevated NO amplifies cGMP biosynthesis, together acting to enhance the scavenging of salinity-induced ROS and reduce salinity-induced oxidative damage and the detrimental effects of salinity stress.

2. Materials and methods

2.1. Plant growth and treatments

Soybean (*Glycine max* L. merr.) was grown and treated as described in Klein et al. (2013). The treatments consisted of control plants (untreated, without any added NaCl), CA-treated plants (CA, 100 μM CA over a period of 12 days), salinity-treated plants (NaCl, 70 mM NaCl over a period of 12 days) alongside plants treated with a combination of 70 mM NaCl and 100 μM CA over a period of 12 days (CA + NaCl). The plants were scored for trifoliolate leaf number, shoot length and trifoliolate leaf area (using the AM350 Portable Leaf Area Meter, ADC BioScientific Ltd.). The rest of the plants were used for the various measurements as described below. For measurements involving absorbance readings using spectrophotometry, the POLARstar® Omega microplate reader (BMG LABTECH) was used.

2.2. Determination of leaf chlorophyll content

Estimation of chlorophyll content was based on a method modified from Hiscox and Israelstam (1979). Freshly harvested leaves were cut into pieces of approximately 0.5 cm^{-2}. Leaf tissue (200 mg per plant) was mixed with 10 ml of dimethylsulfoxide (DMSO) and incubated at 65 °C for 3 h. The absorbance of an aliquot of the leaf-DMSO extract (200 μl) was read at 645 nm and 663 nm, with DMSO being used as a blank.

2.3. Measurement of nitric oxide and cyclic guanosine monophosphate content

NO was detected in soybean nodule sections (generated with a Vibratome to be 150 μm thick) using 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA) as described by Ludidi (2013). The resulting fluorescence images were analyzed using AlphaEaseFC™ 4.0 imaging software (Alpha Innotech Corporation) to quantify the level of NO based on the pixel intensities generated from the image analysis. To confirm the fluorescence analysis, the oxyhemoglobin-based spectrophotometric assay described by Ludidi (2013) was used to determine the content of NO in the soybean root nodules. Root nodule cGMP content was determined in freshly harvested nodule tissue (approximately 200 mg) using the acetylation procedure of the cGMP Enzyme Immunoassay Kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO).

2.4. Assessment of leghaemoglobin content, nitrogenase activity, H_2O_2 content and lipid peroxidation

Leghaemoglobin content was measured as described by Chouhan et al. (2008). Soybean root nodules (200 mg) were homogenized in 4 ml of 50 mM phosphate buffer (pH 6.5), followed by filtration through two layers of Miracloth. The filtrate was centrifuged at 20,000 ×*g* for 20 min and the supernatant was transferred to a clean tube. An equal volume of alkaline pyridine reagent was mixed with the cleared filtrate and split into two equal portions. A few crystals of sodium dithionate were added to one portion (for reduction of the hemechrome in the filtrate) whereas an equal volume of 5 mM of potassium hexacynoferric acid was added to the other portion (for oxidation of the hemechrome). Absorbance readings for both tubes were taken at 556 nm and 539 nm, respectively, and leghaemoglobin concentration was calculated as described by Chouhan et al. (2008). Nitrogenase activity was measured as the rate of reduction of acetylene to ethylene, as described by Leach et al. (2010).

The H_2O_2 content was measured in the root nodules based on a method described by Velikova et al. (2000); whereas lipid peroxidation in root nodules was assessed by determining malondialdehyde (MDA) content to reflect the extent of oxidative stress-induced lipid peroxidation, using a method modified from Buege and Aust (1978). Nodules (800 mg) were ground into a fine powder in liquid nitrogen and homogenized in 2 ml of 10% trichloroacetic acid (TCA). The resulting homogenate was subjected to centrifugation at 12,000 ×*g* for 15 min. The resulting supernatants were used for MDA and H_2O_2 content measurements.

To measure H_2O_2 content, 75 μl of the TCA extract was mixed with an assay buffer (containing a final concentration of 50 mM K_2HPO_4 and 0.5 M KI, pH 5.0). Samples were incubated at 25 °C for 20 min and absorbance readings of the samples were taken at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance ($A_{390\ nm}$) of H_2O_2 standards. For the malondialdehyde assay, 1 ml of the TCA extract was mixed with 4 ml 0.5% 2-thiobarbituric acid that had been made up in 20% TCA. The mixture was heated at 95 °C for 30 min, followed by cooling on ice for 10 min. Thiobarbituric acid-reactive substances (indicative of MDA) were determined by reading absorbance at 532 nm and nonspecific background absorbance was read at 600 nm. The concentration of MDA was calculated using a molar extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

2.5. Statistical analyses

Data for trifoliolate leaf numbers, shoot lengths, NO, cGMP, MDA and H_2O_2 contents were analyzed using two-way analysis of variance (ANOVA). The results were tested for significance by the Tukey–Kramer test at 5% level of significance, using GraphPad Prism 5.03 software. All results are the mean of three replicates ($n = 3$), with twelve plants used in each replicate per treatment for measuring growth parameters (trifoliolate leaf number, shoot length and trifoliolate leaf area) whereas three plants were used in each replicate per treatment for cellular or biochemical assays.

3. Results

3.1. Effect of caffeic acid and salinity on plant growth and leaf chlorophyll content during salinity stress

The number of trifoliolate leaves was reduced by approximately 20% compared to untreated plants in response to treatment with CA (Fig. 1A). The reduction in trifoliolate leaf number in response to salinity was more pronounced (approximately 40% less than the trifoliolate leaf number of untreated plants) in salinity-treated plants than the reduction seen in trifoliolate leaf number of CA-treated plants (Fig. 1A). Supplementation of CA to the salinity treatment did not alter the effect of salinity on trifoliolate leaf number (Fig. 1A).

Shoot length was reduced by exogenously applied CA by approximately 20% compared to the shoot length of untreated plants (Fig. 1B). Salinity stress reduced shoot length by approximately 30%. Application of CA to salinity-stressed plants did not reverse the

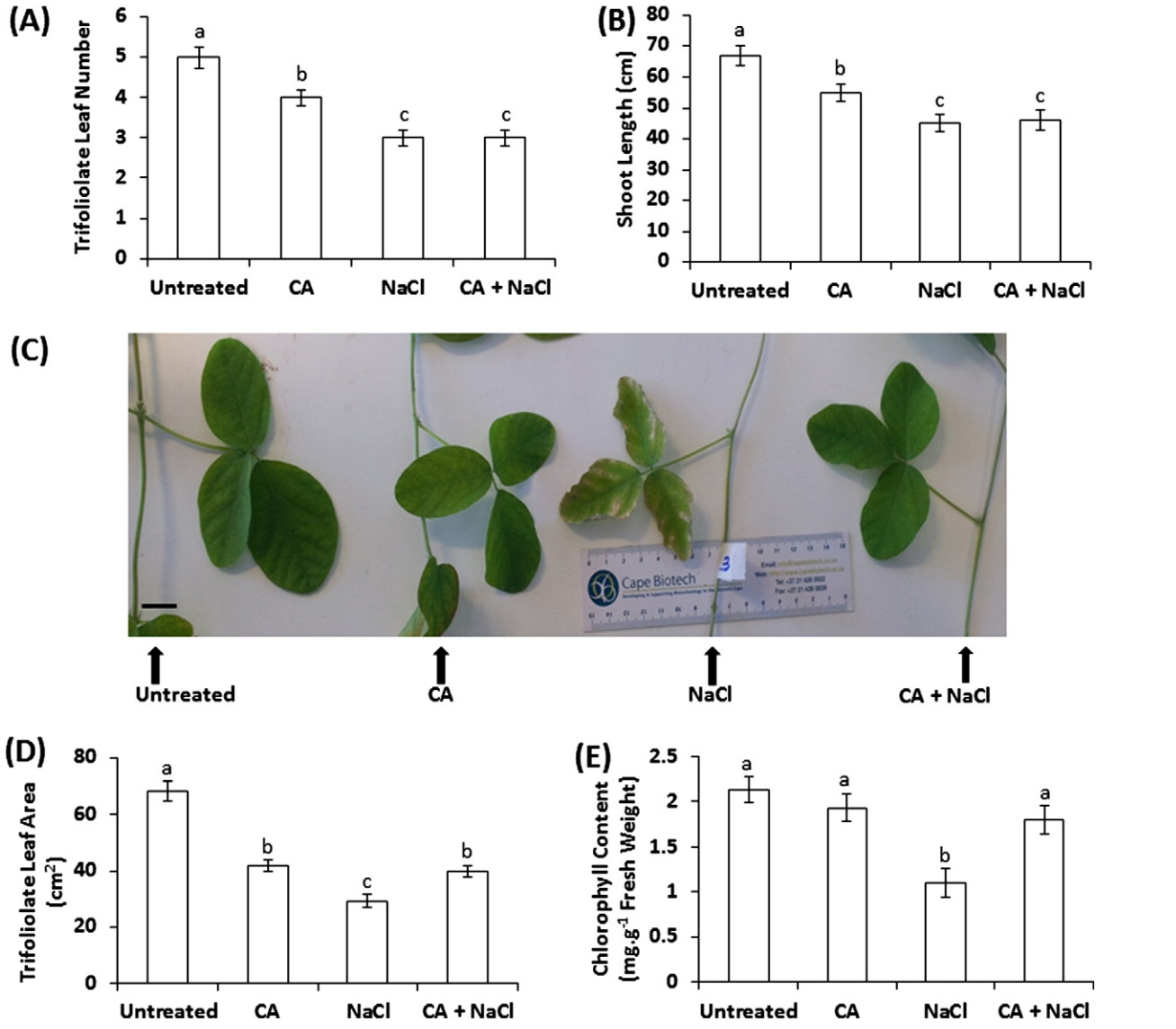

Fig. 1. Exogenous application of caffeic acid, salinity or a combination of caffeic acid and salinity alters soybean trifoliolate leaf growth (A, C and D), shoot length (B), and chlorophyll content (E). The data are representative of mean ± SE obtained from 3 independent experiments with 12 plants used for each replicate in each treatment. Different letters above the error bars indicate means that are statistically significantly different at 5% level of significance.

reduction of shoot length that resulted from salinity stress (Fig. 1B). Visual examination of the leaves showed that CA-treated plants had smaller leaves than untreated plants, whereas salinity-treated leaves were even smaller than the CA-treated plants and suffered chlorosis (Fig. 1C). Supplementation of CA to the salinity-treated plants resulted in alleviation of the salinity-induced chlorosis and improved leaf size that appeared similar to plants treated with CA (Fig. 1C).

The qualitative observations on leaf size and chlorosis were in agreement with quantitative measurements of leaf area (Fig. 1D) and leaf chlorophyll content (Fig. 1E). Measurement of the area of the first trifoliolate leaf from each plant showed that exogenously applied CA reduced the trifoliolate leaf area by approximately 38% compared to the trifoliolate leaf area of untreated plants (Fig. 1D). Salinity caused more drastic reduction (approximately 55% compared to the trifoliolate leaf area of untreated plants) in trifoliolate leaf area, whereas supplementation of CA to the salinity treatment reversed the salinity-induced reduction in trifoliolate leaf area back to levels similar to the CA treatment, i.e. approximately 38% reduction compared to the trifoliolate leaf area of untreated plants (Fig. 1D).

Determination of chlorophyll content revealed that exogenously applied CA did not have a statistically significant effect on leaf chlorophyll content; whereas salinity treatment reduced leaf chlorophyll content by approximately 50% compared to the chlorophyll content of leaves from untreated plants (Fig. 1E). On the other hand, supplementation of the salinity treatment with CA alleviated the salinity-induced reduction in chlorophyll content as the chlorophyll content in the combination treatment (CA + NaCl) was statistically similar to that seen in leaves from untreated or CA-treated plants.

3.2. Changes in nitric oxide content and cyclic guanosine monophosphate in nodules

Exogenously applied CA resulted in augmentation of NO content in root nodules when compared to root nodule NO content from untreated plants (NO content was increased by approximately 130% in nodules from CA-treated plants compared to nodules from untreated plants), on the basis of fluorescence intensity generated from the reaction of NO with DAF-FM DA to form triazolofluorescein signified by green fluorescence (Fig. 2A and B). A similar trend of CA-induced increase in NO content was observed for nodules when NO was measured with the oxyhemoglobin method (Fig. 2C). Salinity treatment led to nodule NO content that was approximately 30% higher than the NO content of

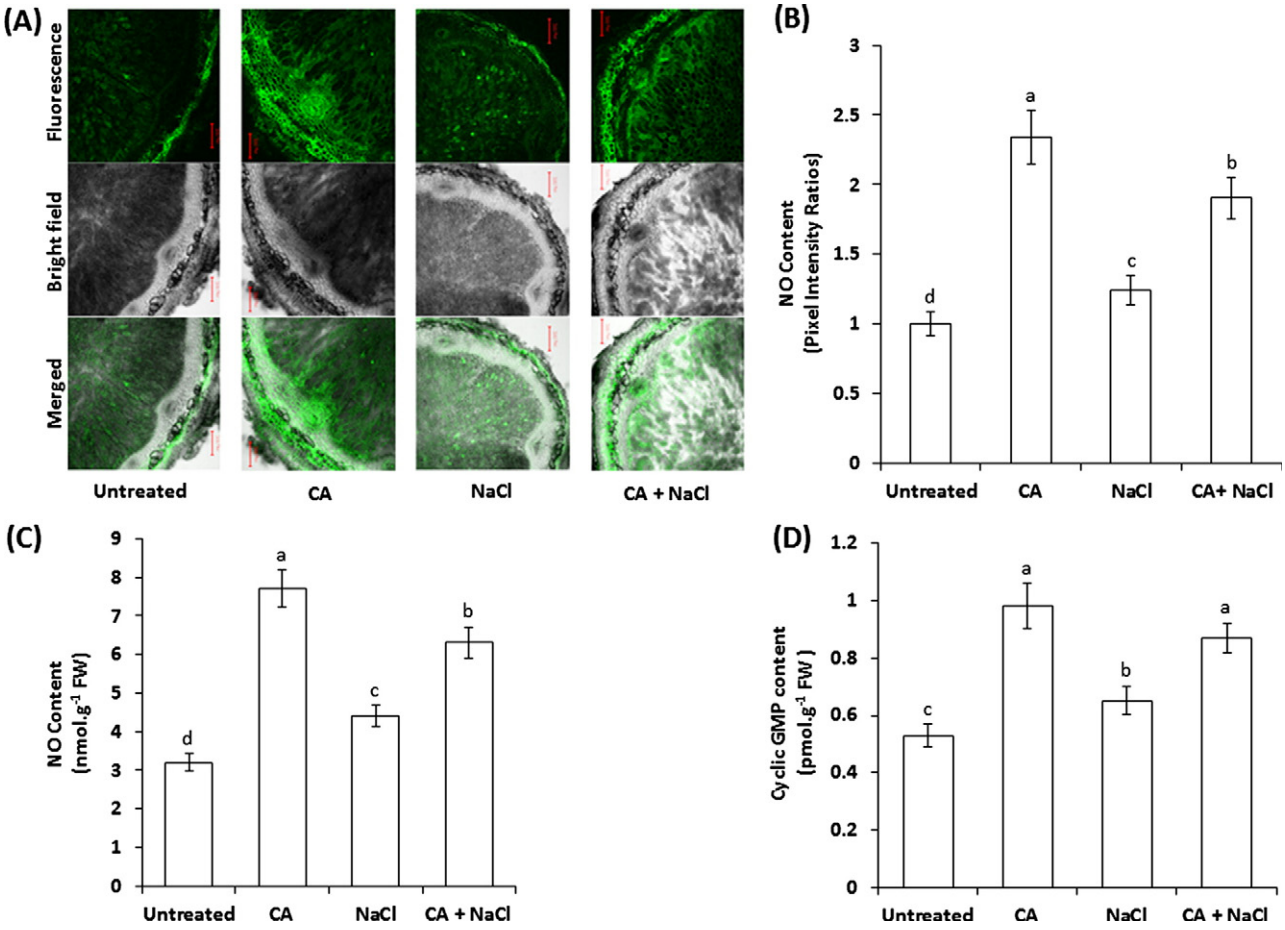

Fig. 2. Caffeic acid and salinity influence changes in soybean root nodule NO content (A, B and C) as measured using DAF-FM DA (A), AlphaEaseFC™ 4.0 software to analyze the triazolofluorescein-derived green fluorescence (B), and the oxyhemoglobin assay (C); together with alteration of cGMP content (D). Red bar (A) = 100 μm. The data represent the means ± SE obtained from 3 independent experiments with 3 plants used for each replicate in each treatment. Different letters signify means that are statistically different at 5% level of significance.

nodules from untreated plants, whereas a supplementation of CA in the salinity treatment resulted in nodule NO content that was approximately 85% higher than the NO content of nodules from untreated plants (Fig. 2A, B and C).

Application of CA caused an increase of approximately 75% in nodule cGMP content in comparison to cGMP content of nodules from untreated plants, whereas the increase in cGMP content in nodules from plants exposed to salinity stress was only approximately 20% higher than the cGMP content of nodules from untreated plants (Fig. 2D). On the other hand, the cGMP content in nodules from salinity-treated plants that were supplemented with CA was approximately 70% higher than the cGMP content of nodules from untreated plants and was statistically similar to the cGMP content of nodules from CA-treated plants (Fig. 2D).

3.3. Changes in leghaemoglobin content, nitrogenase activity, H_2O_2 content and lipid peroxidation in nodules

Exogenously applied CA had no statistically significant effect on root nodule leghaemoglobin content whereas salinity stress reduced leghaemoglobin content (by approximately 45%) in root nodules in comparison to nodules from untreated plants (Fig. 3A). On the other hand, the decrease in leghaemoglobin content in nodules from salinity-treated plants that were supplemented with CA (CA + NaCl) was alleviated so that it was only a reduction of approximately 25% compared to the leghaemoglobin content of nodules from untreated plants (Fig. 3A).

No statistically significant differences were detected in root nodule nitrogenase activity (reflective of nitrogen fixation) in response to exogenous application of CA when compared to the nitrogenase activity of nodules from untreated plants (Fig. 3B). Salinity stress caused a decline in nodule nitrogenase activity by approximately 55% compared to nitrogenase activity in nodules from untreated plants (Fig. 3B). The salinity-induced decrease nitrogenase activity was alleviated as the nitrogenase activity in the combination treatment (CA + NaCl) was reduced by approximately 28% compared to the nitrogenase activity of nodules from untreated plants (Fig. 3B).

Application of CA caused a decline in nodule H_2O_2 content by approximately 30% compared to nodules from untreated plants (Fig. 3C). However, salinity treatment elevated nodule H_2O_2 content by approximately 98% compared to nodules from untreated plants and the salinity-induced increase in nodule H_2O_2 content was partially reversed to levels that are approximately 35% more than H_2O_2 content in nodules from untreated plants (Fig. 3C).

Exogenously applied CA moderately reduced (by approximately 20%) MDA content in root nodules in comparison to nodules from untreated plants, whereas salinity treatment increased nodule MDA content by approximately 75% when compared to nodules from untreated plants (Fig. 3D). On the other hand, the increase in MDA content in nodules from salinity-treated plants that were supplemented with CA increased only by approximately 35% (Fig. 3D).

4. Discussion

We have used exogenous application of CA to study the mechanism by which CA confers salinity tolerance in soybean. Despite moderate reduction in plant growth as a result of exogenous application of CA, the

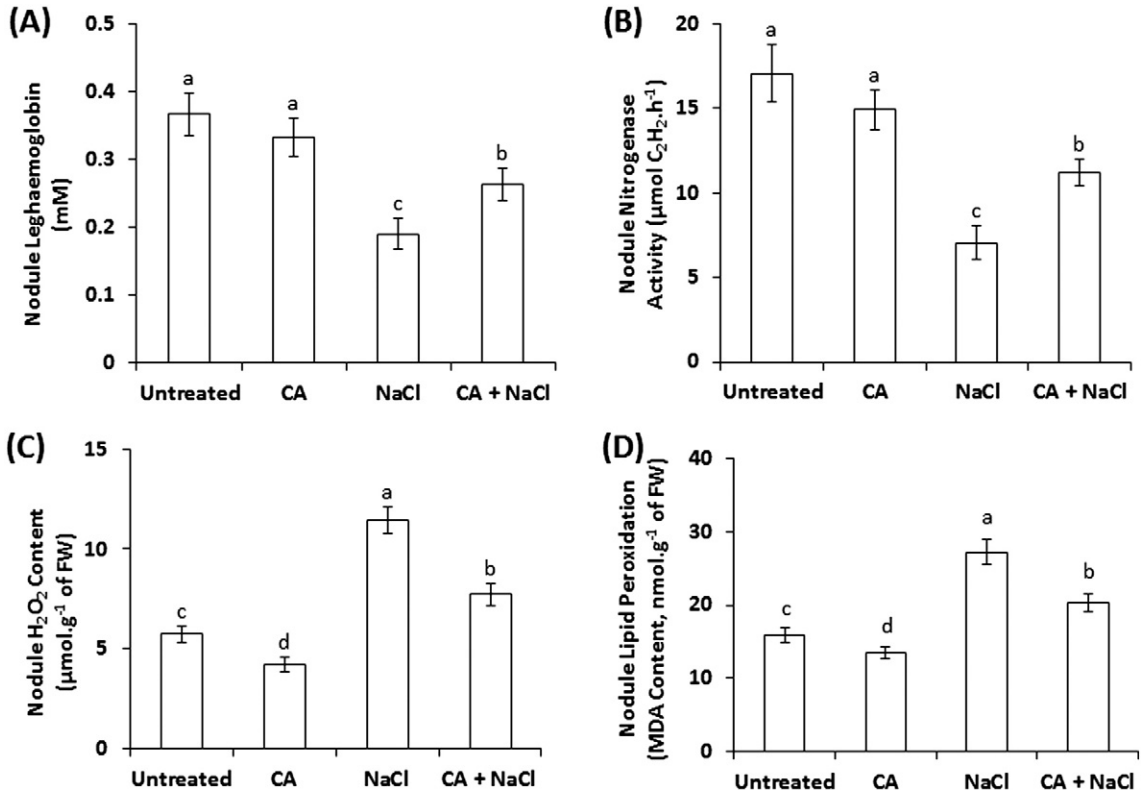

Fig. 3. Treatment with caffeic acid and salinity alters root nodule leghaemoglobin content (A), nitrogenase activity (B), and the extent of oxidative damage as illustrated by H_2O_2 content (C) and lipid peroxidation (D). The data represent mean ± SE obtained from 3 independent experiments with 3 plants used for each replicate in each treatment, $p < 0.05$.

detrimental effects of salinity stress on soybean are prevented by exogenously applied CA. This is clearly evident from the fact that salinity-induced leaf chlorosis is abolished by exogenous application of CA, suggesting that chlorophyll loss (and thus reduction of photosynthetic capacity) resulting from salinity stress (Wang et al., 2011) is prevented by the exogenous application of CA. Therefore, taken together with our previous observation that the biomass reduction caused by salinity is reversed if salinity-treated plants are supplemented with CA (Klein et al., 2013), the fact that salinity-induced reduction of both trifoliolate leaf number and shoot length is not reversed by exogenous application of CA does not mean that CA does not alleviate salinity-induced soybean growth reduction because the quality (reflected by lack of chlorosis and improved chlorophyll content) of the leaves in CA-supplemented plants under salinity is better than that in salinity-treated plants that are not supplemented with CA. This is supported by the significant improvement in trifoliolate leaf area upon supplementation of CA to the salinity treatment. Further support for a protective role of exogenous CA against salinity stress is the maintenance of the integrity (on the basis of legheamoglobin content and nitrogenase activity that reflect nodule functioning) of root nodules in plants that were supplemented with CA during salinity treatment. The fact that supplementation of CA to the salinity-treated plants results in better nitrogen fixation (estimated on the basis of nitrogenase activity) than in the absence of CA under salinity stress suggests that CA improves nodule functioning during salinity stress. This improved nitrogen fixation could also account for the improved growth of CA-supplemented plants grown under salinity. It is thus concluded that exogenous CA alleviates the negative effects of salinity stress on soybean growth and soybean nodule functioning.

Salinity tolerance in plants is in part mediated by NO (Xie et al., 2008). We thus investigated if the CA-induced tolerance in soybean may involve changes in NO content in soybean. For this, we evaluated changes in NO content in root nodules and consistently found that exogenously applied CA resulted in elevated NO content in soybean nodules both as a result of CA alone and CA in combination with NaCl-induced salinity. The fact that the level of increase in NO content in response to salinity alone is less than the NO content elevation in plants either treated with CA alone or CA in combination with salinity suggests that augmentation of CA levels in soybean can be used to maintain NO levels sufficiently high to offer NO-mediated salinity tolerance in soybean. It will be important to explore the link between CA and NO in more depth in the near future in order to understand the source of the CA-mediated increase in NO content. This can be achieved by using inhibitors of NO biosynthesis such as nitric oxide synthase inhibitors in conjunction with exogenously applied CA, since nitric oxide synthase activity exists in plants (Corpas et al., 2009). This would assist in determining if the protective effects of CA are a result of direct influence of CA on the plant's antioxidant system or a result of the CA-mediated increase in NO; which then positively affects the plant's antioxidant system. Such future studies are even more pertinent in light of the role of NO in scavenging ROS via regulation of the plant antioxidant system (Shi et al., 2007; Xie et al., 2008; Lin et al., 2011; Keyster et al., 2012). Very limited information is available on the role of cGMP in ROS homeostasis in plants (Li et al., 2011) but the fact that increased soybean nodule NO content upon exogenous application of CA corresponds with elevated cGMP content in the nodules may imply that cGMP also participates in the regulation of antioxidant enzyme activity, even though this may appear only as a coincidental event at this stage. It is thus imperative that further study to establish if the role of cGMP in salinity tolerance, known to involve stimulation of guanylate cyclase activity by NO and control of NaCl uptake by regulation of cGMP-gated/voltage-independent cation channels (Maathuis and Sanders, 2001; Rubio et al., 2003; Mur et al., 2013), also involves cGMP-dependent regulation of the plant's antioxidant system. Such studies can be achieved through the use of soluble guanylate cyclase inhibitors in conjunction with cell-permeable cGMP analogs and can be useful in dissecting the involvement of NO and CA in the salinity tolerance mechanism if coupled with the use of inhibitors of NO biosynthesis. Nonetheless, CA reduced MDA content in soybean nodules both in the absence and

presence of salinity. This reduction in MDA corresponds with decreased levels of H_2O_2 in the nodules, suggesting that part of the mechanism by which CA confers salinity stress tolerance involves scavenging of H_2O_2 to limit the extent of salinity-induced oxidative stress. Whether the CA-mediated reduction in salinity-induced H_2O_2 accumulation is a result of direct scavenging of H_2O_2 or a result of activation of H_2O_2-scavenging enzymes such as ascorbate peroxidase and catalase (EC 1.11.1.6) remains to be determined.

Our study thus shows that CA enhances salinity stress tolerance by reducing oxidative stress in soybean and this may potentially involve NO-mediated signaling, possibly via cGMP, to enhance the plant antioxidant system. The study opens further opportunities for the exploration of NO signaling in plants in relation to the phenylpropanoid pathway and its relevance to plant responses to salinity.

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