The alleviation of manganese toxicity by ammonium in sugarcane is related to pectin content, pectin methyl esterification, and nitric oxide

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Abstract

Coexistence of ammonium (NH4+) with manganese (Mn) in acid soils may facilitate the alleviation of Mn toxicity to plants. However, the effect of NH4+ on Mn toxicity and the corresponding mechanisms are unclear. In this study, the effects of NH4+ and nitrate (NO3-) on Mn toxicity, cell wall properties, and nitric oxide (NO) signaling in sugarcane were compared. NH4+ alleviated Mn-induced chlorosis in sugarcane seedlings and increased seedling biomass compared with NO3-. Exogenous application of NH4+ decreased the root cell wall pectin content and methyl esterase (PME) activity, but increased the degree of root pectin esterification (PMD). These changes were accompanied by reductions in the Mn content in roots, leaves, root cell wall, and cell wall pectin. An analysis of adsorption kinetic revealed less Mn-adsorption capacity in cell walls extracted from NH4+-fed than from NO3+-fed sugarcane. Mn induced NO accumulation in sugarcane roots, but NH4+-fed seedlings accumulated less NO. Exogenous application of the NO donor sodium nitroprusside increased the Mn content of root cell wall pectin in NH4+-fed sugarcane, while the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid decreased the Mn content in NO3+-fed sugarcane. These treatments eliminated the difference in the pectin Mn content between NH4+-fed and NO3+-fed sugarcane, as did a similar treatment with the nitrate reductase inhibitor tungstate, which decreased root cell wall pectin content and NO accumulation. These results suggest that (i) NH4+ alleviates Mn toxicity in sugarcane by reducing root pectin accumulation and root cell wall PME activity, thereby increasing cell wall PMD and decreasing both the Mn-binding capacity of cell wall and Mn accumulation, and (ii) NO mediates the accumulation of both pectin and Mn in response to different forms of nitrogen. The physiological mechanisms underlying the alleviation of ammonium on Mn phytotoxicity were...
Manganese (Mn) is an important nutrient for plant growth and development but in excess it is toxic to most plants (Millaloe et al., 2010). In fact, Mn toxicity is one of the major factors limiting plant growth in acidic soils (Marschner, 1995), which cover >30% of ice-free land globally. Mn toxicity is particularly favored when the soil pH is <5.5, which results in a strong increase in the dissolved Mn$^{2+}$ concentration, and thus in Mn toxicity to plants.

Nitrogen (N) is an essential macronutrient for plants and a key determinant for crop growth and development. Ammonium (NH$_4^+$) and nitrate (NO$_3^-$) are the two main forms of N taken up by roots. In acidic soils, NH$_4^+$ is the predominant inorganic N form because of weak nitrification and the application of NH$_4^+$-based fertilizers (Che et al., 2015; De Boer & Kowalchuk, 2001). Several studies have shown that NH$_4^+$ but not NO$_3^-$ mitigates Mn toxicity in rice (Oryza sativa L.) and other plant species (Hu et al., 2019). By contrast, NH$_4^+$-fed cowpea (Vigna unguiculata (L.) Walp) is more sensitive to Mn toxicity than is the NO$_3^-$-fed plant (Horst et al., 1999). Thus, the effect of a particular N source on Mn toxicity in plants is complex and requires further study.

The highly negatively charged cell wall is a major binding site for cations, and accumulating evidence indicates that it plays an important role in heavy metal toxicity (Colzi et al., 2012; Horst et al., 2010; Sun et al., 2016; Zhu et al., 2016). In cucumber (Cucumis sativus L.), a Mn-sensitive genotype fixes more Mn in its root cell walls than does a Mn-tolerant genotype (Wang et al., 1992). However, since the cell wall is the first barrier to cellular cation uptake, its binding of toxic metals may also mediate metal tolerance (Le Gall et al., 2015; Yang et al., 2011). Whether the cell wall is also involved in the effects of different N sources on Mn toxicity is unknown.

Nitric oxide (NO) is a key signaling molecule in many physiological processes in higher plants, but it has also been implicated in the response to toxic metal stress. For example, exogenous aluminum (Al) inhibited No synthase activity in swamp rose mallow (Hibiscus moscheutos L.), thereby reducing NO accumulation in its root cells (Tian et al., 2007). Similarly, Al treatment completely abolished NO production in Arabidopsis thaliana (L.) Heynh. roots (Illes et al., 2006). By contrast, exogenous iron induced NO accumulation in Arabidopsis cell suspensions (Arnaud et al., 2006). However, changes in endogenous NO accumulation in plants subjected to Mn toxic stress have yet to be demonstrated experimentally (Wei et al., 2020), although the ability of an exogenous NO donor to alleviate Mn-induced oxidative stress was reported (Srivastava & Dubey, 2012). Therefore, whether NO mediates different effects of N sources on Mn retention in the cell wall remains to be determined.

In southern China, NH$_4^+$-N is the main fertilizer applied to dryland crops, and total N applied has more than doubled in the past 40 years. Sugarcane (Saccharum officinarum L.) is an important crop, including for bioenergy, with high economic value worldwide. We previously showed that sugarcane grown in acidic soils in southern China suffers from severe Mn toxicity (Huang et al., 2016). However, the effects of different N sources on Mn toxicity in sugarcane and the corresponding mechanisms were not determined. Thus, in this study, we investigated the effects of NH$_4^+$-N and NO$_3^-$-N on pectin accumulation and methyl esterification, Mn accumulation, and NO accumulation in sugarcane seedlings. The results suggest that (i) NH$_4^+$ alleviates Mn toxicity and reduces both pectin accumulation and PME activity in sugarcane, in turn reducing Mn binding to the root cell walls, and (ii) NO mediates the effects of NH$_4^+$-N and NO$_3^-$-N on Mn toxicity.

## 2 MATERIALS AND METHODS

### 2.1 Plant cultures

Seedlings of sugarcane cultivar G32 were prepared and cultured as described by Yang et al. (2019). Briefly, the seedlings were cultured in a solar greenhouse in 5-L pots (four seedlings per pot) in an aerated solution initially containing 0.5 mM CaCl$_2$ and then 1/5 strength Hoagland nutrient solution (pH 5.5). After 7–14 days, the seedlings were used in the experiments. The culture solution was renewed every 2 days.

**KEYWORDS**

cell wall, manganese toxicity, nitric oxide, nitrogen source, pectin, sugarcane

**INTRODUCTION**

Manganese (Mn) is an important nutrient for plant growth and development but in excess it is toxic to most plants (Millaloe et al., 2010). In fact, Mn toxicity is one of the major factors limiting plant growth in acidic soils (Marschner, 1995), which cover >30% of ice-free land globally. Mn toxicity is particularly favored when the soil pH is <5.5, which results in a strong increase in the dissolved Mn$^{2+}$ concentration, and thus in Mn toxicity to plants.

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The highly negatively charged cell wall is a major binding site for cations, and accumulating evidence indicates that it plays an important role in heavy metal toxicity (Colzi et al., 2012; Horst et al., 2010; Sun et al., 2016; Zhu et al., 2016). In cucumber (Cucumis sativus L.), a Mn-sensitive genotype fixes more Mn in its root cell walls than does a Mn-tolerant genotype (Wang et al., 1992). However, since the cell wall is the first barrier to cellular cation uptake, its binding of toxic metals may also mediate metal tolerance (Le Gall et al., 2015; Yang et al., 2011). Whether the cell wall is also involved in the effects of different N sources on Mn toxicity is unknown.

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2.2 | Treatments

The effects of N sources (NH$_4^+$-N and NO$_3^-$-N) on chlorosis and biomass in sugarcane seedlings were investigated as follows: Roots of the seedlings prepared as described above were exposed to a N-free basal solution consisting of 1/5 strength Hoagland solution (pH 5.5) to which 0.5 mM MnCl$_2$ and 1.0 mM NH$_4$Cl (NH$_4^+$) or NaNO$_3$ (NO$_3^-$) had been added. After 17 days, the seedlings were imaged (EOS 5D IV; Canon Co.). A dose–response experiment was also carried out in which sugarcane seedling roots were exposed to 0, 0.1, 0.2, or 0.5 mM MnCl$_2$ for 17 days, after which biomass was measured.

The effects of NH$_4^+$-N and NO$_3^-$-N on plant Mn accumulation, cell wall polysaccharide content, and PME activity were assessed by exposing the roots of the seedlings to basal solution containing 0.5 mM MnCl$_2$ and 1.0 mM NH$_4$Cl (NH$_4^+$) or NaNO$_3$ (NO$_3^-$) for 0, 4, 8, or 12 days. The first expanded leaf and a portion of the roots were collected, washed three times with deionized water, oven-dried first at 105°C for 1 h and then at 65°C for 3 days, and digested for Mn determination. The remaining portion of the roots was frozen in liquid N$_2$ and stored at −20°C until further use.

The effect of Mn on NO accumulation was investigated by exposing the roots of the seedlings to 0.5 mM CaCl$_2$ supplemented with 0 (control) or 0.5 mM MnCl$_2$ for 24 h. Root apices were washed in 20 mM HEPEs/NaOH buffer (pH 7.4) for 15 min, loaded with 10 μM 4-amino-5-aminomethyl-2',7'-difluorescein diacetate for 30 min in the dark, washed three times in HEPEs-KOH (pH 7.4) buffer, and then observed and imaged under an epifluorescence microscope (TCS SP8MP; Leica). The signal intensity of the green fluorescence in the epifluorescence images of the roots was quantified according to the method of Guo and Crawford (2005). The results are presented as the mean fluorescence intensity relative to that of the control.

The effects of N sources on NO accumulation under Mn stress were further investigated by exposing the roots to 1.0 mM NH$_4^+$ or NO$_3^-$ and 0.5 mM MnCl$_2$ for 24 h and then determining NO accumulation as described above. The results are presented as the mean fluorescence intensity relative to that of NO$_3^-$-fed seedlings.

The effects of a NO donor, NO scavenger, and nitrate reductase inhibitor on NO accumulation were also investigated in roots exposed for 24 h to nutrient solution (control), or the solution with 10 μM sodium nitroprusside (SNP), 0.1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), or 0.15 mM tungstate, respectively. The nutrient solution contained 0.5 Mn and 1.0 mM NH$_4^+$ or NO$_3^-$. NO accumulation was determined as described above and is presented as the mean fluorescence intensity relative to that of the control. Mn accumulation in the cell wall pectin extracted from plants treated as above for 8 days was also determined.

The effects of a NO donor and nitrate reductase inhibitor on pectin accumulation were investigated in roots exposed for 0, 4, 8, or 12 days to nutrient solution containing 1.0 mM NH$_4^+$ and 0.5 mM Mn with or without 10 μM SNP. Cell wall pectin was extracted after each treatment. The pectin content of roots exposed for 0, 4, 8, or 12 days to nutrient solution containing 1.0 mM NO$_3^-$ and 0.5 mM Mn with or without 0.15 mM SNP was also determined.

2.3 | Mn measurement

The Mn concentration was measured using flame atomic absorption spectrometry (PinAAcle 900 T; PerkinElmer). Before the analysis, plant tissue samples were oven-dried and ground into a powder and then digested with concentrated HNO$_3$ at 140°C.

2.4 | Cell wall extraction and cell wall polysaccharide measurement

The cell wall was extracted using ethanol, methanol–chloroform (1:1, v/v), while cell wall pectin, hemicellulose I (HClI), and hemicellulose II (HCII) were extracted using boiling Milli-Q water, 4% (w/v) KOH containing 0.1% (w/v) KBH$_4$, and 24% (w/v) KOH containing 0.1% (w/v) KBH$_4$, respectively, according to the methods of Yang et al. (2019). The total polysaccharide content in the HClI and HCII fractions and the uronic acid content in the pectin fraction were measured spectrophotometrically at 490 and 520 nm, respectively (Yang et al., 2019).

2.5 | Estimation of the degree of pectin methyl esterification

The degree of pectin methyl esterification (PMD) was calculated as the ratio of the molar amount of methoxy esters to the molar amount of galacturonic acid residues, expressed as a percent. The pectin solution (900 μl) was mixed with 10 M NaOH (100 ml), incubated at 25°C for 1 h, neutralized with 1 M HCl (1 ml), and then centrifuged for 5 min at 12,000 × g at 4°C. The amount of methanol released was determined spectrophotometrically according to Yang et al. (2019). The galacturonic acid concentration in the pectin fraction was also determined as described previously (Yang et al., 2019).
2.6 | Pectin methylesterase activity assay

Pectin methylesterase (PME) was extract with 5 mM phosphate buffer containing 1 M NaCl (pH 7.5). Enzyme activity was determined spectrophotometrically at 525 nm (UV2600; Shimadzu) as described previously (Oikawa et al., 2011; Ye et al., 2015).

2.7 | Analysis of Mn adsorption kinetics

The Mn adsorption kinetics of cell walls extracted from the roots of seedlings exposed for 8 days to basal solution containing 1.0 mM NH₄⁺ or NO₃⁻ and 0.5 mM MnCl₂ were analyzed according to the method of Yang et al. (2019). After their reactivation with 2 M HCl, followed by a wash with Milli-Q water and freeze-drying, the cell walls were transferred to a column. The eluent was composed of 1 mM KNO₃ and 40 μM MnCl₂ (pH 5.5) and its flow rate was 0.2 ml min⁻¹. Eluates were collected every 10 min and their Mn concentration was determined by atomic absorption spectrometry.

2.8 | Statistical analysis

The data were analyzed using analysis of variance. The treatment means were compared using Student’s t-test or Duncan’s multiple range test.

3 | RESULTS

3.1 | Effect of N sources on Mn toxicity

Leaf chlorosis is the first visible symptom of Mn toxicity in sugarcane seedlings (Huang et al., 2016). In this study, the young leaves of NO₃⁻-fed, but not NH₄⁺-fed, seedlings exhibited noticeable chlorosis (Figure 1a). The latter result suggests that NH₄⁺ supplementation reduces Mn toxicity in sugarcane.

Growth inhibition is also a typical symptom of Mn toxicity in crops. The biomass of the NH₄⁺-fed sugarcane seedlings tended to increase compared to that of NO₃⁻-fed seedlings in parallel with increasing Mn concentration. Relative biomass was <100% at moderate Mn concentrations (0 and 0.1 mM) but >100% at higher Mn concentrations (0.2 and 0.5 mM). These results provide further evidence that NH₄⁺ supplementation lowers Mn toxicity in sugarcane.

3.2 | Effect of N sources on Mn accumulation

Because Mn accumulation is a determinant of Mn toxicity in plants, it was examined in the roots of NH₄⁺-fed or NO₃⁻-fed sugarcane under excess Mn conditions. Less Mn accumulated in the leaves and roots of NH₄⁺-fed seedlings than in those of NO₃⁻-fed seedlings (Figure 2a,b). Thus, under Mn stress, NH₄⁺ supplementation reduces Mn accumulation in sugarcane.

Because most of the Mn in sugarcane accumulates in the cell wall, especially in pectin, Mn accumulation in both was compared in NH₄⁺-fed and NO₃⁻-fed seedlings. Less Mn accumulated in the root cell walls of NH₄⁺-fed than in NO₃⁻-fed sugarcane (Figure 2c). Specifically, the Mn content in the cell walls of NH₄⁺-fed plants was only 43%–48% of that in the cell walls of NO₃⁻-fed plants. Mn accumulation in cell wall pectin was also significantly lower in NH₄⁺-fed seedlings (Figure 2d). These results suggest that NH₄⁺ supplementation reduces Mn binding to cell wall pectin.

FIGURE 1 Effect of N source on plant growth and chlorosis. Seedlings exposed to N-free nutrient solution containing 0.5 mM MnCl₂ (Mn) and 1.0 mM NH₄⁺ or (NO₃⁻) for 17 days (a). Relative biomass of NH₄⁺-fed sugarcane compared with NO₃⁻-fed sugarcane (b). The sugarcane seedlings were exposed to the nutrient solution with 1.0 mM NH₄⁺ or NO₃⁻ and 0, 0.1, 0.2, or 0.5 mM MnCl₂ for 17 days. The biomass is presented as the ratio of NH₄⁺-fed seedlings biomass to NO₃⁻-fed seedlings biomass. Data are means ± SE. Different letters indicate that the values are significantly different at p < 0.05, according to Duncan’s multiple-range test.
3.3 | Effect of N sources on the cell wall polysaccharide content

Although the plant cell wall mainly consists of cellulose, hemicellulose, and pectin, only pectin polysaccharides significantly bind Mn in sugarcane (Yang et al., 2019). Under excess Mn conditions, the cell wall pectin content in NO$_3^-$-fed seedlings tended to increase with increasing treatment time, whereas there was no change in the pectin content in NH$_4^+$-fed seedlings (Figure 3a). Specifically, NH$_4^+$ supplementation significantly decreased the pectin content compared with the content of NO$_3^-$-fed seedlings between 4 and 12 days of treatment. Since cell wall hemicellulose is a major polysaccharide capable of binding metals such as cadmium (Cd$^{2+}$) and Al$^{3+}$ (Zheng, 2014), we also extracted HCI and HCII and compared their concentrations in the cell walls of NH$_4^+$-fed and NO$_3^-$-fed seedlings, but the difference in either case was not significant (Figure 3b,c). These results indicate that the reduced accumulation of Mn in the roots and cell walls of NH$_4^+$-fed sugarcane are related to the decrease in cell wall pectin.

3.4 | Effect of N sources on PME activity and PMD

Since the overall negative charge of the cell wall is due to the demethylation of pectin by PME, we measured PME activity in seedlings supplied with NH$_4^+$-N and NO$_3^-$-N. PME activity was significantly lower in NH$_4^+$-fed than in NO$_3^-$-fed plants under Mn stress (Figure 4a). Consequently, PMD was significantly higher in NH$_4^+$-fed than in NO$_3^-$-fed seedlings (Figure 4b). Thus, compared with NO$_3^-$ supplementation, NH$_4^+$ supplementation results in a less negatively charged cell wall.

3.5 | Effect of N sources on Mn binding to pectin

To determine whether the cell wall alterations induced by NH$_4^+$ and NO$_3^-$ affect Mn binding, cell walls from the roots of the respective seedlings were extracted and their Mn adsorption kinetics were analyzed. As expected, significantly less Mn was adsorbed by cell walls in NH$_4^+$-fed seedlings than in NO$_3^-$-fed seedlings (Figure 5). These findings indicate that the cell wall alterations induced by NH$_4^+$ and NO$_3^-$ result in different levels of Mn binding.

3.6 | Effect of N sources on root NO accumulation

Since exposure to different N sources can impact the endogenous NO content and NO is related to Mn toxicity, we hypothesized a direct relationship between Mn stress, NH$_4^+$ or NO$_3^-$, and NO production. NO-associated fluorescence was increased in roots exposed to 0.5 mM Mn (Figure 6a,c). Under Mn stress, less NO accumulated in root apices in NH$_4^+$-fed sugarcane than in NO$_3^-$-fed sugarcane (Figure 6b,d). These results were in line with the difference in root Mn accumulation by seedlings supplied with NH$_4^+$ and NO$_3^-$ and indicate that NO mediates Mn accumulation.
To determine whether NO accumulation was related to the alterations in cell wall Mn accumulation induced by NH$_4^+$ and NO$_3^-$, seedlings were exposed to the NO scavenger cPTIO or to the nitrate reductase inhibitor tungstate by their addition to the culture solution. As expected, less NO accumulated in the cPTIO-treated roots than in the control roots (Figure 7a). Moreover, the presence of either cPTIO or tungstate blocked the NO$_3^-$-induced increase in Mn accumulation in root cell wall pectin and eliminated the difference in pectin Mn accumulation between NH$_4^+$-fed and NO$_3^-$-fed plants under Mn stress (Figure 7b). To determine whether the NO level was directly related to Mn accumulation by cell wall pectin, a NO donor (SNP) was added to the nutrient solution. The increase in NO accumulation resulted in an increase in pectin Mn accumulation in roots supplied with NH$_4^+$ (Figure 7a,b). By contrast, SNP application did not cause a further increase in root NO accumulation in roots supplied with NO$_3^-$; however, it eliminated the difference in pectin Mn accumulation in NH$_4^+$-fed versus NO$_3^-$-fed seedlings under Mn stress (Figure 7b). This finding suggests that NO plays an important role in N resource-induced alterations in the cell wall of sugarcane seedlings exposed to excess Mn.

3.8 | Effect of a NO donor and nitrate reductase inhibitor on the cell wall pectin content

To investigate whether changes in the amount of cell wall pectin are involved in NO-induced Mn accumulation, the uronic acid content of the cell wall was determined in roots exposed to nutrient solution with or without the NO donor SNP and the nitrate reductase inhibitor tungstate. The pectin content increased significantly with increasing treatment time in SNP-treated seedlings (Figure 8a) but decreased significantly in the seedlings exposed to tungstate (Figure 8b). These results were in line with NO-induced pectin Mn accumulation and suggest that the NO-induced accumulation of Mn results from pectin accumulation.

4 | DISCUSSION

4.1 | NH$_4^+$ alleviates Mn toxicity in sugarcane

Studies have shown that Al toxicity in rice can be alleviated by NH$_4^+$, which impacts Al accumulation (Chen et al., 2010; Zhao et al., 2009). Although Mn tends to accumulate and induce toxicity in the plant shoots, regulating uptake and avoiding over-accumulation of Mn in roots are the first steps in dealing with Mn toxicity (Tsunemitsu et al., 2018; Yang et al., 2019). The present study showed that NH$_4^+$ similarly alleviates Mn toxicity in sugarcane seedlings. The reduction of Mn toxicity in NH$_4^+$-fed plants
compared to NO\textsuperscript{−}-fed plants was demonstrated by the observation that Mn-induced chlorosis did not occur in the presence of exogenously applied NH\textsuperscript{4}\textsuperscript{+} (Figure 1a), and that in sugarcane under Mn stress, NH\textsuperscript{4}\textsuperscript{+}-fed plants had a higher biomass (Figure 1b) and accumulated less Mn (Figure 2) than NO\textsuperscript{−}-fed plants. These findings are in line with studies in other plant species (Elamin & Wilcox, 1986; Hu et al., 2019). For example, less Mn accumulated in the leaves and roots of NH\textsuperscript{4}\textsuperscript{+}-fed than in NO\textsuperscript{−}-fed rice plants (Hu et al., 2019). In muskmelon (Cucumis melo L.), Mn adsorption was lower in NH\textsuperscript{4}\textsuperscript{+}-fed than in NO\textsuperscript{−}-fed plants, such that Mn toxicity in the former was alleviated, as evidenced by improved growth and a reduction in Mn toxicity symptoms (Elamin & Wilcox, 1986). The reduced uptake of Mn was partly explained by NH\textsuperscript{4}\textsuperscript{+}-induced rhizosphere acidification, which decreased the expression of Mn transporter genes and therefore Mn influx (Hu et al., 2019).

In acidic soils, because Mn\textsuperscript{2+} dissolves in the soil solution, Mn toxicity can limit crop production. In sugarcane, Mn-induced chlorosis occurs only in strongly acidic soils (Huang et al., 2016). Another consequence of a low soil pH is an inhibition of the microbiological oxidation of NH\textsuperscript{4}\textsuperscript{+} to NO\textsuperscript{−} (i.e., nitrification) in soil. Therefore, in acidic soils, NH\textsuperscript{4}\textsuperscript{+} is the predominant N source, the dissolved Mn content is high, and so is the NH\textsuperscript{4}\textsuperscript{+}/NO\textsuperscript{−} ratio. The interaction of NH\textsuperscript{4}\textsuperscript{+} and Mn to reduce Mn toxicity in plants growing in acidic soils has important ecological implications and points to a strategy for obtaining superior plant growth in acidic soils, where NH\textsuperscript{4}\textsuperscript{+} and toxic levels of Mn often coexist (Zhao et al., 2014).

4.2 | NH\textsuperscript{4}\textsuperscript{+} decreases pectin accumulation, PEM activity and PMD

The cell wall plays an important role in sensing and responding to metal toxicity, and in metal-exposed plant roots it is the first site of contact (Krzesłowska, 2011). The cell wall is the major site of toxic metal accumulation. For example, >85% of the Al in barley (Hordeum vulgare L.) roots was found in root cell walls (Clarkson, 1967). In a previous study, we found that ~60% of sugarcane Mn was located in the cell walls (Yang et al., 2019). In the present study, the majority of root Mn was also in the cell walls; however, Mn accumulation in the cell wall was effectively reduced in plants treated with a NH\textsuperscript{4}\textsuperscript{+}-containing nutrient solution; this was not the case in NO\textsuperscript{−}-treated plants (Figure 2c). Moreover, the Mn adsorption capacity of the cell wall was weaker in NH\textsuperscript{4}\textsuperscript{+}-fed than in NO\textsuperscript{−}-fed plants (Figure 4). According to these findings, the NH\textsuperscript{4}\textsuperscript{+}-induced decrease in Mn accumulation in sugarcane roots was the result of less Mn adsorption to the cell walls, which, in turn, alleviated Mn toxicity in the NH\textsuperscript{4}\textsuperscript{+}-fed plants.

Pectin and hemicellulose are the major sites of cation binding in the cell wall because of their negative charges.
**FIGURE 6** Mn-induced accumulation of NO in the roots of sugarcane. NO green fluorescence (a, b) and relative fluorescence intensity (c, d) of the roots exposed to 0.5 mM CaCl$_2$ solution containing 0 (Control), or 0.5 mM MnCl$_2$ (Mn) (a, c), and 0.5 mM MnCl$_2$ combined with 1.0 mM NH$_4$Cl (NH$_4$+) or NaNO$_3$ (NO$_3$-) (b, d) for 1d. Data are means ± SE. Asterisks (**) indicate significant difference between the treatments at $p < 0.01$, according to Student’s $t$-test.

**FIGURE 7** Effects of NO donor (sodium nitroprusside [SNP]), NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [cPTIO]) and nitrate reductase inhibitor (tungstate) on root NO content (a) and Mn accumulation (b) in cell wall pectin. The roots were exposed to nutrient solution containing 0.5 mM MnCl$_2$, 1.0 mM NH$_4$+ or NO$_3$-, and 10 $\mu$M SNP, 0.1 mM cPTIO, or 0.15 mM tungstate for 8 days. Data are means ± SE. Different letters indicate that the values are significantly different at $p < 0.05$, according to Duncan’s multiple-range test.

**FIGURE 8** Effects of NO donor (sodium nitroprusside [SNP]) and NR inhibitor (tungstate) on uronic acid content in cell wall pectin of roots. Roots were exposed to the nutrient solution containing 0.5 mM MnCl$_2$ and 0 (Control) or10 $\mu$M SNP (a), and 0 (Control), or 0.15 mM tungstate (b) for indicated days. Data are means ± SE. Asterisks (*) indicate significant difference between the treatments with NH$_4$+ and NO$_3$- at $p < 0.05$, according to Student’s $t$-test.
Consequently, both play a key role in the binding and accumulation of divalent metal cations such as Cd and Cu (Krzesłowska, 2011; Yang et al., 2011). However, in a previous study in rice neither hemicellulose nor pectin in the cell wall was related to the NH$_4^+$-mediated reduction in Mn accumulation (Hu et al., 2019). Our results also ruled out the involvement of hemicelluloses in Mn retention by the root cell walls of sugarcane, as the difference in the contents of these polysaccharides in NH$_4^+$-fed and NO$_3^-$-fed plants did not significantly differ (Figure 3b,c). By contrast, differences in the cell wall pectin content mediated by NH$_4^+$ and NO$_3^-$ accounted for the difference in cell wall Mn accumulation between NH$_4^+$-fed and NO$_3^-$-fed sugarcane. Mn accumulation increased with prolonged Mn treatment (Figure 2d) and was lower in NH$_4^+$-fed than in NO$_3^-$-fed plants (Figure 3a). These findings indicate that alterations in cell wall properties contribute to the reduced accumulation of Mn mediated by NH$_4^+$.

The PMD largely determines the negative charge carried by the pectin matrix and thus the cation binding capacity of pectin. PMD is negatively related to the concentration of cations in the cell wall (Li et al., 2017; Schmohl et al., 2000). PME, which catalyzes PMD, decreases the amount of free carboxylic acid groups on the galacturonic acid residues of pectin and thus the latter's cation binding capacity. Previous studies in plants demonstrated a negative effect of PME-regulated PMD of the cell wall with respect to both metal toxicity and cation accumulation. For example, the Al-induced accumulation of NO activated PME but reduced PMD, which increased the binding of Al to pectin in wheat (Triticum aestivum L.) (Sun et al., 2016). Previously, we showed that excess Mn induced a decrease in PMD in sugarcane (Yang et al., 2019). However, under excessive Mn stress, PME activity was lower in NH$_4^+$-fed than in NO$_3^-$-fed plants (Figures 3c and 4a). Thus, compared to NO$_3^-$ treatment, NH$_4^+$ treatment resulted in a higher PMD and less Mn adsorption in root cell walls (Figure 4b). The mechanism involved a reduction in the pectin content of the root cell wall, a reduction in PME activity, and an increased PMD, which together limited Mn adsorption to cell walls and decreased Mn accumulation in sugarcane roots. Together, these results suggest that the NH$_4^+$-mediated reduction in Mn accumulation in sugarcane is related to decreased in the pectin concentration and increase in PMD.

4.3 | NO contributes to Mn accumulation in sugarcane in response to different N sources

Metal stress has been shown to induce NO production in plants (Sun et al., 2016; Wei et al., 2020; Zhu et al., 2019), while experimental evidence that Mn toxic stress regulates NO accumulation in plants is lacking (Wei et al., 2020). Our study showed that Mn induced the accumulation of NO in sugarcane roots (Figure 6a,c), as evidenced by the inhibitory effect of the NO scavenger cPTIO (Figure 7). That NO production by many plant species increases in response to Al and Cd stresses is well established (Sun et al., 2014, 2016; Wei et al., 2020), whereas we demonstrated the Mn-induced accumulation of NO and its reduction in NH$_4^+$-fed versus NO$_3^-$-fed sugarcane (Figure 6b,d). This finding is in line with the different effects of different N compounds on NO accumulation in maize (Zea mays L.) under osmotic stress (Zhang et al., 2015).

A relationship between NO signaling and the mobility of elements bound to the cell wall as a function of pectin or hemicellulose accumulation in the cell wall has also been reported (Zhu et al., 2016). In rice, the NH$_4^+$-induced accumulation of NO increased both pectin accumulation and PME activity in the cell wall, resulting in increased phosphorus reutilization (Zhu et al., 2016). In A. thaliana, the NH$_4^+$-induced production of NO accelerated the release of iron from cell wall hemicellulose (Zhu et al., 2019). In our study, an association of NO with the different effects of NH$_4^+$ and NO$_3^-$ in sugarcane was demonstrated. Specifically, the decrease in NO production in NH$_4^+$-fed versus NO$_3^-$-fed plants was related to the decreased accumulation of Mn in cell wall pectin (Figure 2c), which resulted in reduced adsorption of Mn to the cell walls (Figure 5). These findings link NO to the NH$_4^+$-mediated reduction in Mn accumulation in cell wall pectin. Support for this sequence of events was obtained by examining the effects of the NO donor SNP and the NO scavenger cPTIO. In the presence of cPTIO, the positive effect of NO$_3^-$ on cell wall Mn accumulation in sugarcane under Mn stress was reversed, such that the differences in root NO accumulation and root cell wall pectin Mn accumulation between NO$_3^-$-fed and NH$_4^+$-fed plants were abolished (Figure 7). However, in the presence of SNP, NO accumulation in roots increased in NH$_4^+$-fed sugarcane and was accompanied by an increase in Mn accumulation in cell wall pectin. By contrast, there was no increase in root NO or Mn accumulation in NO$_3^-$-fed sugarcane. Accordingly, Mn accumulation in root cell wall pectin did not differ between NH$_4^+$-fed and NO$_3^-$-fed sugarcane. These findings imply that NO signaling mediates the NH$_4^+$-induced reduction in Mn accumulation.

Further support for this conclusion came from observations of the effects of tungstate and SNP on cell wall pectin accumulation (Figure 8). Consistent with the increase in pectin Mn accumulation, after SNP was added to the nutrient solution containing NH$_4^+$, the accumulation of cell wall pectin in sugarcane increased in a time-dependent
manner (Figure 8a). However, when NO production in the roots of NO₃⁻-fed seedlings was inhibited using the nitrate reductase inhibitor tungstate, the accumulation of pectin in the cell walls and Mn in cell wall pectin decreased significantly (Figures 7b and 8b). These findings suggest that in sugarcane seedlings treated with NH₄⁺, the NO-mediated reduction in pectin accumulation reduces Mn accumulation in root cell walls and thus in the plants, thereby alleviating Mn toxicity.

Our results are inconsistent with those of previous studies in rice, which show that the NH₄⁺-mediated reduction in Mn accumulation is not related to cell wall properties but rather to NH₄⁺-induced rhizosphere acidification and the down-regulation of Mn influx transporter (Hu et al., 2019). It remains to be examined whether rhizosphere acidification and Mn transporter regulation are related to the NH₄⁺-mediated reduction in Mn accumulation in sugarcane. However, we first associate NO signaling with a reduction in cell wall pectin by NH₄⁺.

5 | CONCLUSIONS

We identified a physiological pathway for the alleviation of Mn toxicity by NH₄⁺ in sugarcane. In the presence of NH₄⁺, NO production was inhibited, in turn reducing root pectin accumulation but increasing PMD. As a result, root Mn adsorption capacity and Mn content of the plant reduced, thereby alleviating Mn toxicity (Figure 9).

ACKNOWLEDGMENTS

This study is supported by the National Natural Science Foundation of China (grant No. 31660593), Science and Technology Major Project of Guangxi (grant No. GK2018-266-Z01), and Guangxi Natural Science Foundation (grant No. 2016GXNSFDA380038, 2021GXNSFAA075017, 2021GXNSFAA22008).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ScienceDB at http://doi.org/10.11922/sciencedb.01548.

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How to cite this article: Ling, G. Z., Xiao, J. L., Yang, S., Li, D. L., Tang, X. L., Wang, X. X., Zhang, M. Q., & Li, X. F. (2022). The alleviation of manganese toxicity by ammonium in sugarcane is related to pectin content, pectin methyl esterification, and nitric oxide. GCB Bioenergy, 14, 585–596. https://doi.org/10.1111/gcbb.12936