



Research article

The modifications of cell wall composition and water status of cucumber leaves induced by powdery mildew and manganese nutrition

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ABSTRACT

Powdery mildew caused by *Podosphaera fuliginea*, is one of the most important diseases, damaging cucumber's yield worldwide. Among the different controlling ways, management of plant nutrition is an effective method. The present experiment was aimed to study the effects of foliar application of manganese (Mn) on disease suppression and to elucidate the possible mechanisms by which Mn-treated plants resist to fungal disease. Cucumber plants were hydroponically grown and sprayed by MnSO₄ alone and in combination with lysine (Lys) and methionine (Met) four days before pathogen inoculation. The results showed that the foliar application of Mn reduced the fungal disease severity by increasing of lignin, cellulose and pectin contents of cell wall (CW) and improving of leaf water status. The reduction of disease severity by application of MnSO₄, Mn + Lys and Mn + Met, were 40, 33, and 40% compared to control, respectively. The increases of lignin contents at the same treatments were 33, 27, and 36%, respectively. The leaf water potential (LWP) enhanced by foliar spray of above-mentioned fertilizers up to 15, 18, and 17%, respectively. The results of present study revealed that the Mn nutrition could control the cucumber powdery mildew by reinforcing of CW structure and reducing of water loss from infected leaves. The current findings are the first reports elucidating the CW-related defense mechanisms in which Mn has important role. The obtained results have a practical importance either in studies of plant physiology and biochemistry or in agricultural sciences for cost-effective control of powdery mildew in cucumber plants.

1. Introduction

Powdery mildew is one of the most important diseases limiting the growth and yield of cucumber in greenhouses and fields of Iran. The causing fungus, *Podosphaera fuliginea*, is a bio-trophic pathogen that does not kill the host, but decreases the crop yield about 20–40% mainly due to the decrease of nutrient utilization and photosynthesis rate, growth impairment, and increase of respiration and transpiration (Sitterly, 1978). Controlling powdery mildew is generally achieved by using fungicides, but it's rarely attained by applying resistant cultivars (Kuzuya et al., 2003; Pitrat and Besombes, 2008). However, the frequent use of fungicides over time has led to the serious problems such as development of resistant pathogens and increased levels of fungicide residues in cucumber (O' Brien, 1993). Therefore, it is economically important to find safer approaches for powdery mildew control

including plant nutrition management.

The positive effects of nutrients particularly micronutrients on suppression of plant diseases have been known in recent years (Dordas, 2008). The effect of micronutrients in this case could be attributed to their functions in physiology and biochemistry of plant (Marschner, 1995). Manganese (Mn) is one of the most effective micronutrients with well-known roles in suppression of plant diseases (Dordas, 2008). Many biochemical reactions in plants are affected by Mn (Thompson and Huber, 2007). Peroxidases, involved in production of phenolic compounds and flavonoids, are dependent on the Mn (Hamond-Kosack and Jones, 2000). Moreover, peroxidases are glycoproteins that catalyze several reactions in plant including lignin and suberin production in root and decrease the pathogen colonization in the host tissues (Goodman et al., 1986).

Although there is not any report regarding the effect of Mn on

Abbreviations: AA, amino acid; AcBr, acetyl bromide; AcHO, acetic acid; CW, cell wall; SAS, Statistical Analysis System; SAR, systemic acquired resistance; HClO, per chloride acid; LWP, leaf water potential; RWC, relative water content; HA, Hemicellulose A; HB, Hemicellulose B; HR, hypersensitive response; Ctrl, control; Lys, lysine; Met, methionine

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composition of cell wall (CW) polysaccharides, it seems that Mn can induce some changes in CW chemistry of plants by activation of certain enzymes involved in lignin biosynthesis and carbohydrate metabolism (Sharma and Uttam, 2016). Over the years, researchers have observed a close relationship between the primary CWs and micronutrient nutrition so that CW could actively be modified by trace metal exposure (Krzesłowska, 2011; Parrotta et al., 2015). Remodeling of primary CW can affect plant resistance to pathogen as it has been illustrated by the increased resistance to some pathogens of mutants defective in cellulose synthase (CESA) subunits that are needed for cellulose biosynthesis of primary CW. Consistently, modification of pectin structure (e.g., degree of methyl esterification or acetylation) can affect pathogen resistance. The CW polysaccharides, including xylan, mannan and xyloglucan may be acetylated. The acetylation degree of certain polymers in secondary CW may determine its susceptibility to specific pathogen (Miedes et al., 2014).

The bio-trophic fungi such as *Podosphaera fuliginea* destroy the CW in a localized and controlled manner to keep the host alive and to use the cell structures (Bellincampi et al., 2014). Such pathogens routinely apply astute strategies to interact with the CW. Haustorium making pathogens, like various fungal mildews, have to enter CWs to create haustorial feeding structure in close connection with the lying beneath host cell (Szabo and Bushnell, 2001).

Lignification and cross-linkage of proteins in the papillary CW can disrupt the penetration peg of bio-trophic fungi and make the CW more resistant to mechanical pressure of fungal appressorium. It is also known that the lignified CWs help to maintain the cell turgor pressure and reduction of water loss from plant tissues under stressful conditions (Wang et al., 2016). On the other hand, it has been proven that the fungal infection could induce the water stress in plant. The water loss from infected leaf areas could enhance due to destruction of leaf cuticle (Bassanezi et al., 2002), elevated permeability of leaf cell membranes (Chaerle et al., 2001), or prevention of stomata closure (Felle et al., 2004). Therefore, leaf water status may have a determining effect on sequence of plant-pathogen interactions. Leaf hydration exhibits a balance between water stream into tissues via the xylem and water evaporation through stomata, and both of these phenomena could be changed due to plant defense strategies (Freeman, 2009). So, Mn nutrition may have an important role in disease suppression by affecting lignification, mechanical properties of CW and water status of plants.

The present work continues our earlier work (Eskandari et al., 2018) and investigates the Mn-induced changes in CW polysaccharides, lignin and leaf water status of cucumber plants. The major topic of this work has been allocated to the plant CW as a dynamic and essential component of the plant cell which can promptly respond to the environmental changes. It was hypothesized that the improved tolerance to fungal disease by Mn nutrition is associated with the physiological roles of this nutrient in stiffening the CW and improving the water status of cucumber plants. Here, Mn was used in combination with single AAs as nutrient sources and chelating agents of Mn to study their potential effects on plant resistance to fungal disease.

To eliminate the negative effects of fungicides on environmental pollution and human health, application of nutrients having a fungicide role such as Mn on control of plant diseases seems helpful. Successful CW-related defenses can impede the invading pathogens at the initiate stage (before disease development) and can dispense with the necessity for more excessive defense responses including hypersensitive response (HR) cell death. Therefore, it is important to identify the mechanisms by which CW-related defenses are developed. Such studies can expand our knowledge based on the effect of metals on host-parasite interactions. Therefore, the aim of present study was to evaluate the effect of Mn combined with AAs for controlling powdery mildew of the cucumber plants and to elucidate the CW-related defense mechanisms involved in plant resistance to fungal disease. The outcome of current study can be considered as a practical strategy by farmers to control of powdery mildew via a safe method and by researchers to identify the

defense mechanisms thereby Mn-nourished plants resist to fungal disease.

2. Materials and methods

2.1. Plant culture and Mn treatment

After rinsing with distilled water, cucumber (*Cucumis sativus* L. cv. Espadana RZ) seeds were germinated on moist filter paper in an incubator at 28 °C. The germinated seeds were sown in sterilized quartz sand and grown up to the cotyledon stage. The irrigation of seedlings was done using a diluted Johnson nutrient solution (0.1 strength) every day. After a week, cucumber seedlings were transplanted to pots (2 L) containing full nutrient solution. The nutrient solution contained the following composition: 1.0 mM KNO₃, 1.0 mM Ca(NO₃)₂, 1.0 mM NH₄H₂PO₄, 1.0 mM MgSO₄, 50 μM FeEDTA, 50 μM KCl, 25 μM H₃BO₃, 2.0 μM MnSO₄, 2.0 μM ZnSO₄, 0.5 μM CuSO₄, 0.5 μM H₂Mo₇O₄. The pH of the nutrient solution was adjusted in the range of 5.8–6 by adding 0.1 N HCl or KOH when needed. The solution inside the pots was permanently aerated with an air pump and renewed weekly. Pots were kept in a greenhouse with 14 h light as well as average daily and nightly temperatures of 30 and 18 °C, respectively.

Manganese solutions containing pure MnSO₄ and MnSO₄ in combination with the lysine (Lys) and methionine (Met) in a molar ratio of 2:1 were supplied by foliar spraying. Concentration of Mn in all of the foliar-applied treatments was 1% (w/v). To characterize the effects of Mn and AAs, a group of plants were supplied with only Lys or Met. The control plants were also sprayed with distilled water. At the 6-leaf stage, the foliar spray was carried out on the upper surface of the cucumber leaves. The time of foliar Mn application was selected based on the results of previous study i.e., four days before inoculation (Eskandari et al., 2018).

2.2. Pathogen inoculation

The inoculum of *P. fuliginea* was obtained from infected cucumber plants in a local greenhouse of Isfahan province, Iran and maintained on the cucumber plants by intervallic transfer to the new plants when necessary. The fresh inoculum was taken from recently sporulating colonies on the infected leaves of cucumber about 9–12 days after inoculation. Conidia were gently brushed into 100 mL distilled water containing 20 μL Tween-20 and counted with the aid of a haemocytometer to give a concentration of 10⁶ conidia/mL. Four days after foliar Mn application, the upper surfaces of cucumber leaves were sprayed with a conidial suspension supplied by a hand sprayer. After inoculation, plants were incubated in a dew chamber at 20 °C for 24 h in darkness. Plants were then returned back to the greenhouse bench (28–32 °C during the day and 16–20 °C at night, 14 h of light per day) for disease development. The non-inoculated plants were kept in an individual greenhouse with the same condition to avoid contamination by fungal disease.

2.3. Sample collection

Fourteen days after pathogen inoculation, three fully recently expanded leaves of plants at the same position of each treatment were sampled, frozen in liquid nitrogen and kept in a –80 °C freezer for biochemical analysis.

2.4. Evaluating of disease severity

Disease severity was evaluated when disease symptoms were fully observed (14 days after inoculation with *P. fuliginea*). The percentage of the infected leaf area to the healthy leaf area of each tested plants was determined using the leaf area meter (Winarea-UT-11) device.

2.5. Isolation of CW

The frozen fresh leaves were ground to powder using liquid nitrogen. A sample (10 g) of the powdered leaf material was homogenized in distilled water. The suspension was then centrifuged for 10 min at $10,000 \times g$. The supernatant discarded and the precipitated materials were re-suspended in distilled water and centrifuged again. The precipitation was washed twice with 10 vol of absolute ethanol, rinsed in chloroform: methanol (1:2, v/v) for 12 h, and washed by acetone. The CW pellet was re-suspended and filtered after each wash. The samples were finally dried overnight with air stream at 35 °C. The residue, considered as CW material, was weighed and kept in a desiccator at room temperature until use.

2.6. Quantification of lignin

Isolated leaf CWs were used for determining of lignin contents. Lignin contents were measured in samples by using the acetyl bromide method with a little modification (Iiyama and Wallis, 1990). Briefly, 6 mg of fine-powdered wall preparation was treated with a mixture (total of 2.5 mL) of 25% (w/w) AcBr in AcHO and 0.1 mL of 70% HClO₄ at 70 °C for 30 min with shaking at 10 min intervals. After cooling, the digestion mixture was transferred to a 50 mL volumetric flask containing 10 mL of 2 M NaOH and 12 mL AcHO and made up to 50 mL. The lignin contents were determined by quantifying the absorbance at 280 nm using a specific absorption coefficient of 20.0 g⁻¹.L.cm⁻¹.

2.7. Fractionation of CW polysaccharides

Extraction of major CW polysaccharides was done according to the method of Sakurai and Nevins (1997). The pectin component was extracted with EDTA solution (50 mM EDTA in 50 mM Na-phosphate buffer, pH 6.8, 95 °C). The extraction process was repeated three times and the CW material was filtered after each time on the mesh nylon (42 μm). The obtained solution was dialyzed, freeze-dried and weighted. To perform dialysis, the solution belonging to each sample was transferred to the dialysis bags by means of a pipette and the ends of bags were blocked by knitting. After immersing the filled dialysis bags in distilled water, the containers were placed on a stirrer for 24 h. The distilled water was replaced with the fresh one when it appeared opaque in the containers. The Hemicellulose was extracted by an alkaline solution (0.02% NaBH₄ in 17.5% NaOH) from residual mass of the previous step (after pectin extraction) and extraction was repeated three times. The obtained solution was neutralized by adding half volume of absolute glacial acetic acid and purified by dialyzing as mentioned before. The solution was centrifuged at $14,000 \times g$ for 20 min to dissociate Hemicellulose A and B (HA and HB). The supernatant containing HB was transferred to the plastic falcon, frozen in liquid nitrogen, dried and weighted. The precipitation containing HA was dried in the small petri dish under a fuming hood and weighted. The final residue, cellulose fraction, was washed two times with a mixture of ethanol and diethyl ether (1:1, v/v), filtered by the glass filter, dried under a fuming hood, and weighed. All fractions were reported based on the total weight of CW.

2.8. Measurement of leaf water potential and relative water content

Leaf water potential (LWP) was measured based on the pressure balance method (Kirkham, 2005) by using a pressure chamber (Model 3115, Soil Moisture Equipment Corporation). Immediately after cutting, the leaf sample was put in a pressure chamber bomb as tail-end of the cut surface was placed just above the hole of chamber's cap. After tightening the chamber's cap, the air influx tap was opened thereby the pressure inside the chamber was slowly increased. Meanwhile, the cross-cut of leaf (outside the chamber) was observed by using a

magnifying glass. Upon observing the glossy surface of cross-cut section (due to the efflux of xylem sap), the air tap was closed and the gas pressure (equal to the LWP with a minus sign) was recorded.

In order to determine the relative water contents (RWC), one leaf of each plant was picked up and immediately weighted (FW). For recording the inflation weight, the fresh leaves were placed in distilled water at 4 °C for 6 h in darkness. After measuring the inflation weight (TW), leaf samples were put in the oven at 65 °C for 48 h. After that, dry weights of the leaves (DW) were also measured. The RWC were determined by using the following equation (Smart and Bingham, 1974):

$$RWC(\%) = \frac{(FW - DW)}{(TW - DW)} \times 100 \quad (1)$$

2.9. Elemental analysis

At the end of experiment, the cucumber seedlings were harvested by cutting with a sterilized razor blade at the stem point leveled to the upper surface of plant supporting plates and separated into leaves and roots. Roots were discarded and leaves were first rinsed in tap water and then washed with distilled water. The leaves were oven dried at 70 °C for 48 h, weighed and ground. The powdered samples were dryashed at 500 °C, dissolved in 2 N HCl and made to volume with hot distilled water. Leaf Mn concentration was determined by atomic absorption spectrophotometer (PerkinElmer, Model 3030, US).

2.10. Statistical analysis

Treatments were arranged in a factorial arrangement in a completely randomized design with three replications. The main effects of individual treatments and their interaction effects were evaluated by analysis of variance (ANOVA) with the multiple comparison post hoc test, using SAS software (version 9.1), and significant differences among the means were determined by using LSD test, at the 5% significance level. All assays were run with three replicates.

3. Results

3.1. Lignin

Infection with *P. fuliginea* increased the CW lignin contents (Fig. 1) and this increase was dependent on the kind of applied fertilizer. For the non-inoculated plants, application of all of the Mn sources led to the enhancement of lignin contents. The highest increase in this parameter was obtained by application of Mn + Met. Foliar spray of MnSO₄, Mn + Lys and Mn + Met solutions led to the increase of lignin contents up to 31, 26 and 40% in comparison with control, respectively. The free AAs had also an increasing effect on leaf CW lignin contents, but their effects was less than Mn fertilizers. The effects of both AAs were similar on increasing of lignin contents.

For the inoculated plants, there was also an increasing trend in lignin contents of leaf CW by Mn application (Fig. 1) and there was not any difference among the Mn sources in this regard. Foliar application of Mn in the form of MnSO₄, Mn + Lys and Mn + Met increased the lignin contents up to 33, 27, and 36% compared to control, respectively. Met also increased the CW lignin contents, however, the effect of Lys was not significant on this parameter.

3.2. Cell wall polysaccharides

3.2.1. Cellulose

Inoculation with *P. fuliginea* decreased the leaf CW cellulose contents, regardless of the type of applied fertilizer (Fig. 2). Foliar application of all of the Mn fertilizers increased the leaf CW cellulose contents either in non-inoculated or in inoculated plants, however, the magnitude of increase varied dependent on the source of Mn. For the

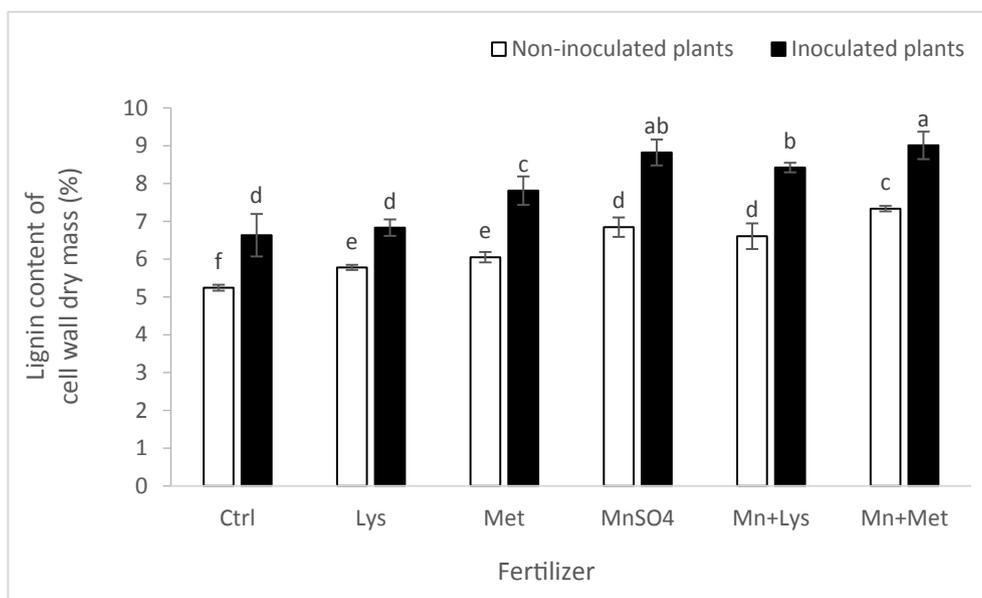


Fig. 1. The effect of inoculation with *P. fuliginosa* and foliar application of MnSO_4 alone and in combination with lysine (Lys) and methionine (Met) on the cell wall lignin content of cucumber leaves. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

non-inoculated plants, the maximum amount of cellulose was related to the MnSO_4 treatment. The effects of Mn + AA solutions and corresponding AAs were similar on the cellulose contents. The percentages of increase in this parameter by application of MnSO_4 , Mn + Lys and Mn + Met were 41, 20 and 16% in comparison with control, respectively (Fig. 2). For the inoculated plants, Mn + Lys was the most effective treatment in enhancing the CW cellulose contents. The free AAs also increased the CW cellulose contents, but this increase was less than that when AAs were applied with Mn. For example, foliar spray of MnSO_4 , Mn + Lys, Mn + Met, Lys and Met increased the cellulose contents of leaf CW up to 57, 80, 74, 39 and 51% compared to control, respectively.

3.2.2. Hemicellulose

Fungal disease increased the leaf CW HA contents of non-Mn-treated plants, but it had no significant effect on this parameter in Mn-treated

plants (Fig. 3a). For the non-inoculated plants, application of free AAs and MnSO_4 had no significant effect on HA contents, however, the amount of this parameter decreased by foliar spray of Mn + AA solutions. The rate of HA reduction by foliar spray of Mn + Lys and Mn + Met was 23% compared to control. With respect to the inoculated plants, the effects of free AAs were non-significant on HA contents, while all of the Mn fertilizers significantly reduced the amount of this polysaccharide with the stronger effect related to the Mn + AA solutions. For instance, with application of MnSO_4 , Mn + Lys and Mn + Met, the contents of HA reduced up to 28, 39, and 43% compared to control, respectively. The magnitudes of the decrease in HA contents by application of Mn + AA solutions were similar in non-inoculated and inoculated plants (Fig. 3a).

Pathogen inoculation increased the HB contents of non-Mn-treated and MnSO_4 -treated plants, but it showed no significant effect on the amount of this polysaccharide in Mn + AA treatments (Fig. 3b). For the

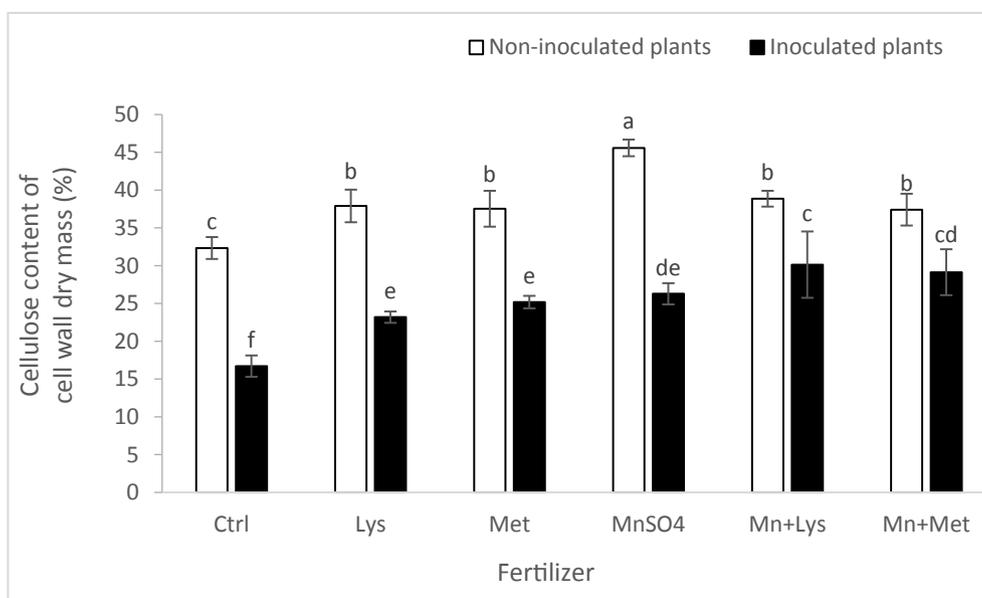


Fig. 2. The effect of inoculation with *P. fuliginosa* and foliar application of MnSO_4 alone and in combination with lysine (Lys) and methionine (Met) on the cell wall cellulose content of cucumber leaves. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

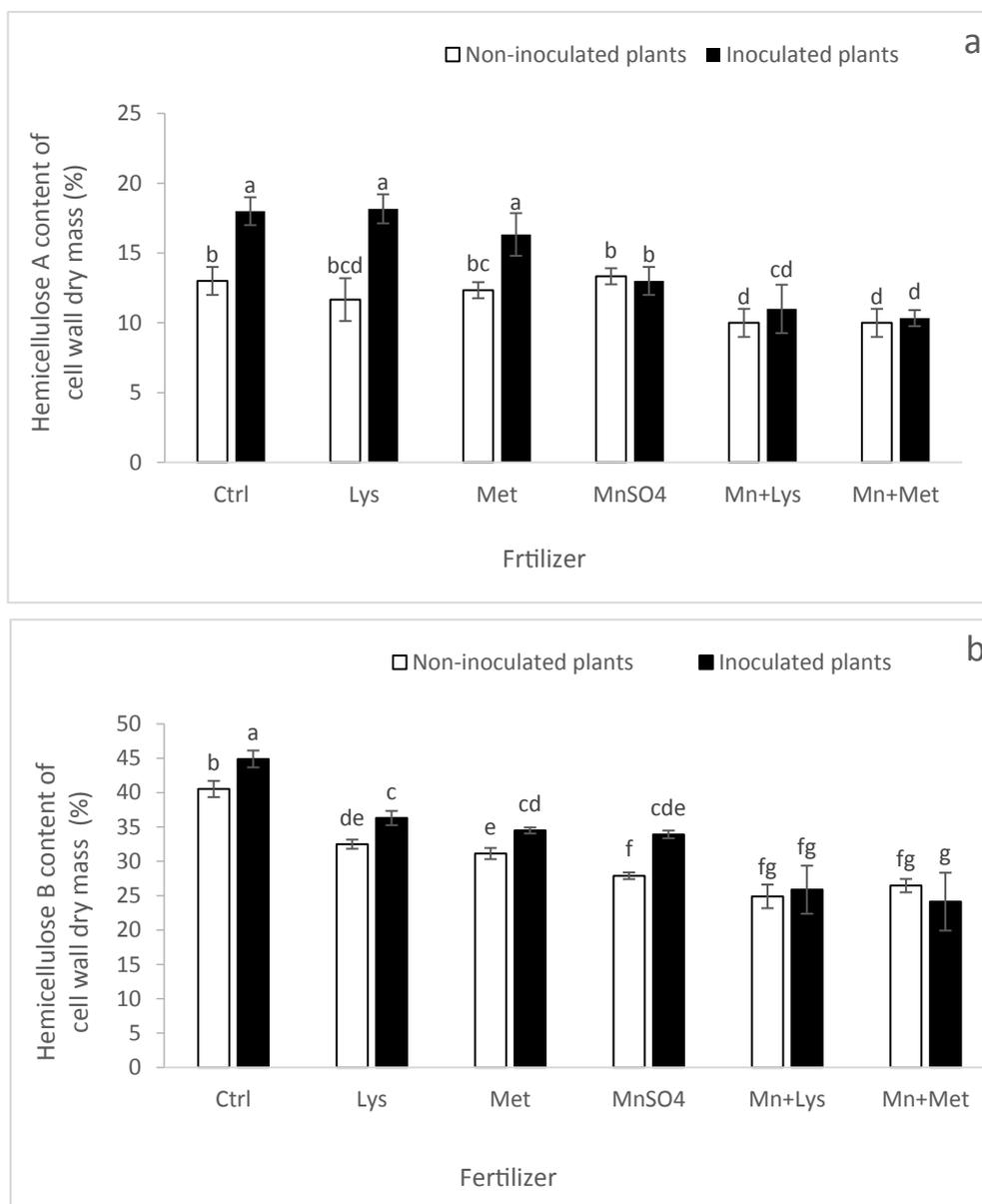


Fig. 3. The effect of inoculation with *P. fuliginea* and foliar application of MnSO₄ alone and in combination with lysine (Lys) and methionine (Met) on the cell wall hemicellulose A (a) and hemicellulose B (b) contents of cucumber leaves. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

non-inoculated plants, foliar application of all of the Mn sources similarly reduced the amount of HB. The rates of decrease in HB contents by application of MnSO₄, Mn + Lys and Mn + Met were 31, 38 and 35% in comparison to control, respectively. The applied AAs had a similar effect on the decrease of this parameter. For the inoculated plants, the magnitude of decrease in HB contents was stronger in Mn + AA treatments than that in MnSO₄ treatment. By application of MnSO₄, Mn + Lys and Mn + Met, the HB contents decreased up to 24, 42, and 46% in comparison with control, respectively. The effects of free AAs on reduction of HB contents were equal and similar to the MnSO₄ treatment.

3.2.3. Pectin

Inoculation with *P. fuliginea* caused a significant increase in leaf CW pectin contents, although this increase was dependent on the fertilizer treatments (Fig. 4). For the non-inoculated plants, only Mn + AA solutions increased the pectin contents. Application of Mn + Lys and Mn + Met enhanced the leaf CW pectin contents up to 51 and 49%

compared to control, respectively. MnSO₄ and free AAs had no significant effect on the contents of this polysaccharide and their effects were similar but less than the effect of MnSO₄ treatment. For the inoculated plants, all of the Mn solutions increased the CW pectin contents with the stronger effect in the case of MnSO₄ treatment (Fig. 4). There was not any significant difference among the applied Mn solutions in this case with the exception of MnSO₄ and Mn + Lys. With foliar spray of MnSO₄, Mn + Lys and Mn + Met, the leaf CW pectin contents enhanced up to 31, 18 and 29% compared to control, respectively. Although Lys had no significant effect on pectin contents, but Met application increased the amount of pectin.

3.3. Leaf water potential and relative water content

Infection with *P. fuliginea* had no significant effect on LWP (Fig. 5). For the non-inoculated plants, the effects of Mn fertilizers and free AAs were insignificant on LWP. For the inoculated plants, application of all of the Mn solutions increased the LWP and there was not any difference

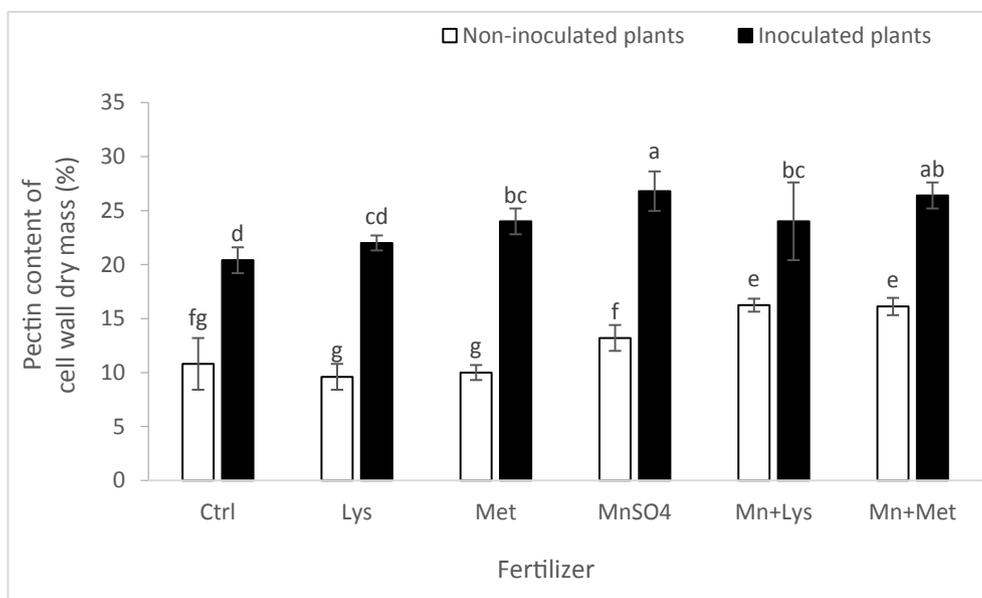


Fig. 4. The effect of inoculation with *P. fulginea* and foliar application of $MnSO_4$ alone and in combination with lysine (Lys) and methionine (Met) on the cell wall pectin content of cucumber leaves. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

among the Mn fertilizers in this regard. For instance, with application of $MnSO_4$, Mn + Lys and Mn + Met, LWP increased up to 15, 18, and 17% compared to control, respectively. Free AAs had no significant effect on LWP.

Inoculation with *P. fulginea* decreased the RWC of cucumber leaves at all of the fertilizer treatments (Fig. 6). For the non-inoculated plants, Mn application clearly increased the leaf RWC and all of the Mn fertilizers equally increased the amount of this parameter. By application of $MnSO_4$, Mn + Lys and Mn + Met, the leaf RWC enhanced up to 4, 5, and 5% in comparison with control, respectively. Met application also led to the increase of leaf RWC, but Lys had no significant effect on this parameter. For the inoculated plants, application of Mn treatments led to a significant increase in leaf RWC. Although there was not any difference between $MnSO_4$ and Mn + AA solutions in increase of leaf RWC, but the Mn + Met was more effective than Mn + Lys in this case. Foliar spray of $MnSO_4$, Mn + Lys and Mn + Met, increased the leaf

RWC up to 3, 2 and 4% in comparison with control, respectively. The free AAs had no significant effects on leaf RWC of inoculated plants, however, Met was more effective than Lys in increase of leaf RWC.

3.4. Disease severity

Foliar application of Mn caused a significant decrease in disease severity of cucumber leaves (Fig. 7) and no significant difference was found among the Mn fertilizers in this case. By application of $MnSO_4$, Mn + Lys and Mn + Met, the severity of fungal disease decreased up to 40, 33, and 40% in comparison with control, respectively. Although Lys had no effect on suppression of disease severity, Met application decreased the severity of disease on the cucumber leaves (Fig. 7). The reduction of fungal disease by foliar spray of all of the Mn fertilizers was stronger than that by application of Met.

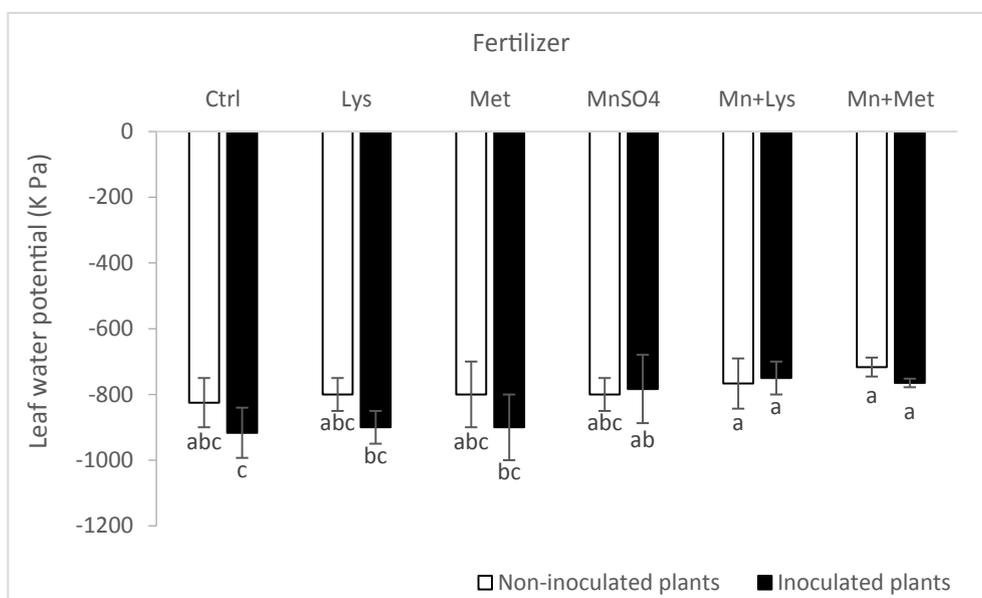


Fig. 5. The effect of inoculation with *P. fulginea* and foliar application of $MnSO_4$ alone and in combination with lysine (Lys) and methionine (Met) on the leaf water potential of cucumber leaves. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

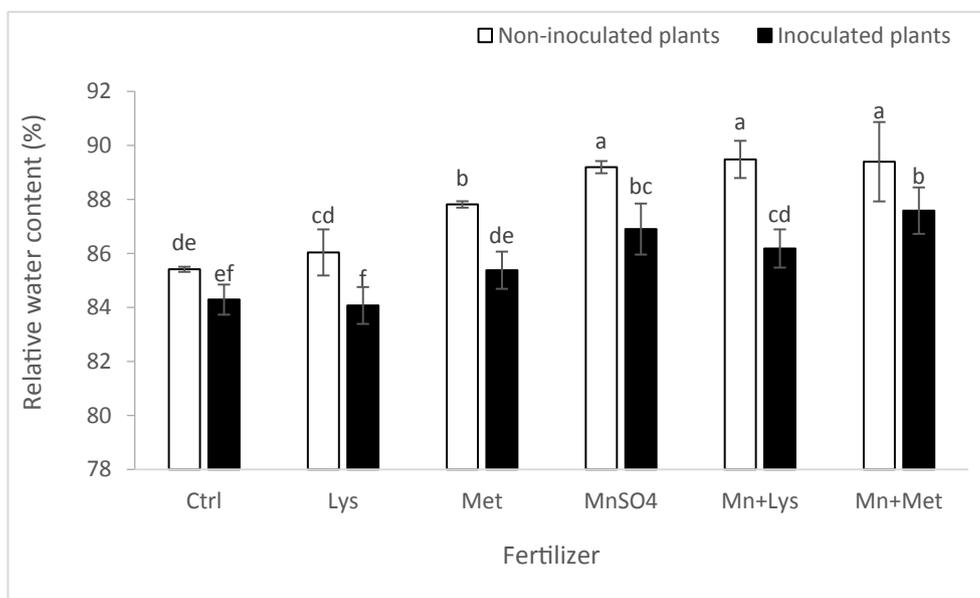


Fig. 6. The effect of inoculation with *P. fuliginea* and foliar application of MnSO₄ alone and in combination with lysine (Lys) and methionine (Met) on the relative water content of cucumber leaves. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

3.5. Leaf Mn concentration

The effect of *P. fuliginea* on leaf Mn concentration was dependent on the kind of applied fertilizer. The free AAs had no significant effect on this parameter. However, all of the Mn fertilizers increased the leaf Mn concentration (Table 1). For the non-inoculated plants, application of all of the Mn fertilizers led to the increase of leaf Mn contents with the stronger effect in the case of MnSO₄. The effect of Mn + Lys was greater than Mn + Met on enhancement of leaf Mn concentration. The percentages of increase in Mn contents of MnSO₄-treated leaves compared to the Mn + Lys- and Mn + Met-treated ones were 47 and 74%, respectively. For the inoculated plants, the leaf Mn concentration of MnSO₄ treatment was more than that of Mn + AA treatments. The Mn + AA solutions had a similar effect on leaf Mn concentration of inoculated plants (Table 1). The rate of increase in leaf Mn contents of MnSO₄-treated plants was mostly twofold compared to the Mn + AA-

treated ones.

4. Discussion

Based on the results of present study, foliar application of Mn solutions increased the cucumber resistance to powdery mildew and the induced resistance was related to the alteration of CW composition and improvement of water status in the cucumber leaves.

Comparison of the treatment effects indicated that the Mn application, regardless of the kind of Mn fertilizer, decreased the disease severity on the cucumber leaves. Therefore, it could be argued that the effect of Mn on disease suppression is more important than accompanying AAs and the physiological roles of Mn in plant has mainly been caused this resistance. Consistently, for the diseased plants, all of the Mn fertilizers similarly affect the lignin, cellulose and pectin contents of leaf CW which suggests the greater effect of Mn than AAs on leaf CW

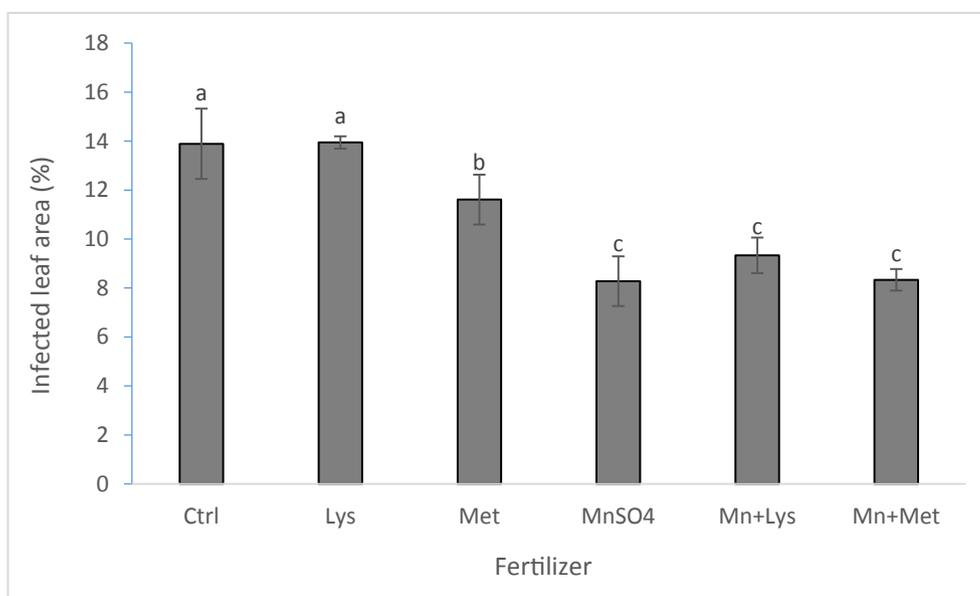


Fig. 7. The effect of foliar application of MnSO₄ alone and in combination with lysine (Lys) and methionine (Met) on percentage of infected leaf area of cucumber plants. Bars and vertical lines are means (\pm SD). Bars sharing the same letters are not significantly different at $P < 0.05$.

Table 1

Leaf Mn concentration (mg kg⁻¹ dry mass) as affected by the inoculation with *P. fuliginea* and foliar application of MnSO₄ alone and in combination with lysine (Lys) and methionine (Met).

	Fertilizer					
	Ctrl	Lys	Met	MnSO ₄	Mn + Lys	Mn + Met
Non-inoculated plants	35.5 ± 1 ^a f	22.5 ± 2.18 f	31.5 ± 3.04 f	563 ± 28.75 a	378 ± 32 c	323 ± 19 d
Inoculated plants	39.7 ± 2.25 f	34.5 ± 3 f	28.3 ± 1.6 f	461 ± 13.75 b	232 ± 28.5 e	217 ± 8.26 e

Means with similar letters in rows are not significantly different at P < 0.05.

^a Values in front of “±” represent the standard deviation (SD).

components.

Such relevance between Mn nutrition and CW composition could be associated with the role of Mn in synthesizing of CW polysaccharides and lignin polymer. The effect of Mn on CW structure including alteration in synthesis and composition of polysaccharides has been reported in several studies (Wang et al., 2003; Sharma and Uttam, 2016).

Since Mn plays a key role in carbohydrates and nitrogen metabolism and AAs synthesis, it is not surprising that the transformation of glucose into cellulose increases by Mn application. The induction of pectin production by Mn application may also be related to the formation of egg-box structure similar to what occurs by exposure to calcium. It has been suggested that the low-methylesterified homogalacturonan (HGA) can interact via calcium bridging between two free carboxyl groups and make a so-called egg-box structure (Grant et al., 1973). This reaction leads to forming the calcium gel and strengthening the CW (Caffall and Mohnen, 2009). The same structure and outcome could be expected in the case of Mn as reported for the other trace elements (Kartel et al., 1999). The increase in lignin contents of leaves by Mn nutrition is probably due to the role of Mn in lignin biosynthesis. The accumulation of Mn in the leaves may activate the NADH-peroxidases (Fecht-Christoffers et al., 2003), which are key enzymes involved in lignin biosynthesis.

All above-mentioned changes induced by Mn aid to fortify the CW for limiting the penetration by fungal pathogen and to promote the plant resistance against *P. fuliginea*. For example, the most important role of cellulose in plant CW is providing the strength and rigidity which prevents the swelling of CW and rupturing of plasma membrane. Pectin also provides an embedding matrix which integrates the cellulose-hemicellulose network and it is essential for incorporating and reinforcing of tissue (Zhao and Dixon, 2014). The increase of lignin level enhances the CW strength and promotes the cell defensive physical barrier against pathogens (Broadley et al., 2012). Sharma and Uttam (2016) in their study on wheat plants showed that the amounts of lignin and cellulose increased by Mn exposure up to 200 μM. These researchers claimed that such increase in CW components could be due to the changes in physico-chemical properties of CW which leads to the increase of binding capacity of CW and decrease of heavy metals entrance to the protoplast. The same phenomenon could be considered in the case of pathogen attack.

It was interesting that the LWP and RWC of cucumber leaves similarly increased by application of all of the Mn fertilizers that was in agreement with the obtained results regarding the CW polysaccharides and confirms the close relationship between CW composition and water contents in cucumber leaves. The significant increase in LWP and RWC of inoculated leaves by Mn treatments (Figs. 5 and 6) indicates the optimal water status of leaf tissues in these plants and could be related to the more lignin contents synthesized in such plants (Fig. 1). It has been proven that the increase of lignin produces the thicker and more rigid CWs (Radin and Ackerson, 1982) and these modifications in tissue structure may have important effects on the water relationships of plants (Morgan, 1986) as well as the reduction of water loss from tissues under stressful conditions (Wang et al., 2016). The effect of Mn on improvement of leaf water status could also be explained by the role of Mn in fatty acid production. Although the fatty acid contents were not

measured in the present study, it has been reported that the Mn deficiency in plant tissues can impair the fatty acid production, which adversely affect the cuticular wax deposition, as wax synthesis was triggered by the fatty acid synthesis in plastids. The wax layer is responsible for limiting the non-stomatal water loss and reducing the heat load on leaves (Hebberly et al., 2009). Therefore, Mn application indirectly improve the leaf water status.

Based on the results, it could be concluded that the effect of foliar Mn nutrition on CW modification is dependent on the plant conditions (normal or stressful conditions) and implies that the physiological functions of Mn in plant update according to the environmental conditions. The evidence for this claim is that in non-inoculated leaves, there was a significant difference among the Mn sources which suggests the effect of accompanying AAs is more important than Mn on CW components in non-stressful conditions. Although there is no experimental evidence to elucidate the role of AAs in biosynthesis of CW polysaccharides, it seems that the high levels of nitrogen supplied by AAs help to soften the leaf tissue by less production of cellulose. In addition, the role of AAs like Met in lignification has been reported by Campbell and Sederoff (1996). It has been proven that Met not only plays a key role in lignin synthesis, but also acts as a substrate for some secondary metabolites involved in host defense (Kagan and Clarke, 1994; Roje, 2006). Such role for Met can also explain the reduction of disease severity by Met application that is in agreement with the increase of lignin contents in Met-treated plants (Figs. 1 and 7).

In spite of more concentration of Mn in MnSO₄-treated plants compared to Mn + AA-treated ones, the reduction of disease severity was similar in different treatments of Mn (Fig. 7). This findings indicate that the reduction of fungal disease in cucumber plants by application of Mn is not simply due to the toxicity effect of Mn on pathogen, but because of Mn effect on inducing the CW-related defenses and improving the leaf water status as confirmed by our results.

It must be noted that the alterations in biosynthesis of individual CW constituents can affect the synthesis and/or deposition of other CW polymers (Marga et al., 2003). As it is obvious from our findings, a significant decrease in CW hemicellulose contents of plants supplied by Mn counteracted the induced increase in the other CW components by application of Mn. Similarly, the increase of CW hemicellulose and pectin contents of inoculated plants may be for compensating the reduction of cellulose contents under fungal disease.

As it was expected, fungal disease diminished the content of most important polysaccharide in CW i.e., cellulose. It reveals that *P. fuliginea* has mainly targeted the cellulose component by activation of cellulase enzyme. In a similar study on bean plants infected by *Sclerotinia sclerotiorum*, a decrease in α-cellulose contents of CW was observed within two days after inoculation and a significant decrease was found nine days after inoculation (Lumsden, 1976). The fungal cellulolytic system (consists of endoglucanases, exoglucanases, and β-glucosidases) acting on cellulose destruction, has been characterized for some species of Ascomycetous and Basidiomycetous fungi (Kumar et al., 2008; Dashtban et al., 2009). A significant increase in CW pectin of inoculated plants as compared to the non-inoculated plants may suggest the role of pectin in signaling production under biotic stress. Vogel et al. (2004) indicated that a change in pectin modification probably leads to release

of active elicitor components, which can affect degradation of powdery mildew hydrolytic enzymes. The increase of hemicellulose through effect of xyloglucans on auxin can affect cell elongation, and, as a result, its growth (Fry, 1989). Obviously, the flexibility of CW is an essential factor for cell growth and improves the cell development under biotic stress.

The increase of lignification in inoculated plants compared to that in non-inoculated ones may be an adaptive response of plant to injury, rather than a resistance mechanism. Lignification (as one of the most important signals representing SAR) is usually induced in tolerant tissues. In cucumber plants, HR appears in tissues that exhibit SAR as CW is severely lignified under pathogen's germ tube (in order to restriction of sequence penetration by fungal pathogen) instead of appearance of necrotic spots. Lignified tissues make cucumber plants more tolerant to disease and this is one of the main reasons for less severity of fungal disease in Mn-treated plants compared to control untreated ones in our study.

A decrease in LWP and RWC of inoculated leaves compared to those in non-inoculated ones indicates the negative effect of fungal disease on leaf water availability as what occurs under water stress in plants. This phenomenon can be explained by “systemic toxin hypothesis”, discusses that the toxins produced by pathogens can disrupt the metabolism of host plant, leading to leaf wilt (Wu et al., 2008). In addition, toxins decrease the stem hydraulic conductance and LWP (Van Alfen and Turner, 1975a), regulate the stomatal opening (Lee et al., 1993), and damage the cell membrane which results in water leakage (Van Alfen and Turner, 1975b). The membrane injury of infected leaves may leads to the irregular water loss from damaged cells, and, as a result, disturbance of leaf water balance (Wang et al., 2012). Such changes in water status of infected plants can also affect the bioavailability, mobility and transport of nutrients in the infected plants. This claim can explain the reduction of Mn concentration of infected plants compared to that of healthy ones. One possible reason for this finding may be due to the effects of invading pathogen on oxidizing Mn (Mortvedt et al., 1961) and fungal oxidation of Mn in the infection court prior to invasion by pathogen which predispose plant to infection by disturbing defense mechanisms (Graham, 1983). However, the latter effect was not observed in our study revealing that the content of soluble Mn in the infected leaves is high enough to induce the plant resistance to *P. fuliginea*.

In addition to the major theme outlined above, it should be noted that the Mn nutrition may induce many other changes in plants under stressful conditions including activation of enzymes involved in biosynthesis of CW polysaccharides or gene expression associated with the CW composition alterations. However, there is still a major gap in our ability to relate such changes to the plant resistance against fungal pathogen. With respect to the numerous roles of Mn in plant physiology, it is recommended to study the other Mn-dependent parameters which may be important in plant resistance to biotic stress such as the role of Mn in salicylic acid signaling, expression of proteins involved in ethylene and jasmonic acid synthesis and induction of proteins related to pathogen resistance.

5. Conclusions

Based on the results, it could be concluded that the reduction of fungal infection in cucumber plants supplemented with Mn fertilizers either $MnSO_4$ alone or $MnSO_4$ in combination with AAs was mostly due to the role of Mn in physiological processes such as biosynthesis of CW polysaccharides and improvement of leaf water status. The increase of most abundant CW polysaccharides i.e., cellulose and lignin polymer by Mn application could impede the incidence of *p. fuliginea* pathogen. The improvement of leaf water status by Mn nutrition additionally aid to resistance of cucumber plants to powdery mildew that was probably associated with the increase of lignin contents in leaf CW. These effects of Mn was further than the toxic effect of this nutrient on fungal

pathogen. Such protective roles of Mn were mainly induced under biotic stress suggesting the importance of plant nutrition in such conditions. Although there was not any statistically significant difference among the Mn sources in suppression of fungal disease, the efficacy of $MnSO_4$ was higher than Mn + AA fertilizers on altering the CW composition. On the other hand, the Mn + AA fertilizers were partly more effective than $MnSO_4$ in improving leaf water status. It seems that many other factors are involved in disease reduction by application of different Mn sources that could be considered as a perspective for the further researches in this field. The database provided by current study could be expanded our knowledge based on the effect of metals on host-parasite interactions and suggested the practical importance of Mn nutrition in plants under stressful conditions.

Author contributions

Samane Eskandari conceived the original idea, carried out the experiment and performed the computations. Bahram Sharifnabi supervised the project and verified the analytical methods. Samane Eskandari wrote the manuscript with support from Bahram Sharifnabi. Bahram Sharifnabi encouraged Samane Eskandari to investigate [a specific aspect] and supervised the findings of this work. Both authors discussed the results and contributed to the final manuscript.

Contribution

The *Plant Physiology and Biochemistry* publishes original theoretical, experimental and technical contributions in the various fields of plant physiology (biochemistry, physiology, structure, genetics, plant-microbe interactions, etc.) at diverse levels of integration (molecular, subcellular, cellular, organ, whole plant, environmental). Thus, the present study which investigates the effect of foliar-applied Mn in the presence and absence of amino acids on cell wall composition and water status of cucumber leaves infected by powdery mildew would be suitable for publication in this journal. In this case, the present research has been performed to study the efficacy of aspartate-derived amino acids in combination with Mn on improvement of plant tolerance against fungal disease. So, this paper can be interesting for the readers of this journal.

Declaration of interest statement

There is not any potential conflicts of interest between authors.
This work is not against human and animal rights.
The manuscript is approved by both authors and tacitly or explicitly by the responsible.

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