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Protective role of *S*-methylmethionine-salicylate in maize plants infected with *Maize dwarf mosaic virus*

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Abstract

This study aimed to detect the harmful effects of *Maize dwarf mosaic virus* (MDMV) infection, and to demonstrate the potential benefits of S-methylmethionine-salicylate (MMS) pretreatment in infected maize (*Zea mays* L.) plants. The results of chlorophyll *a* fluorescence measurements showed that in MDMV-infected plants additional quenchers of fluorescence appear, probably as the result of associations between the virus coat protein and thylakoid membranes. It is important to note that when infected plants were pretreated with MMS, such associations were not formed. MDMV infection and MMS pretreatment resulted in a decrease in ascorbate peroxidase (APX) activity in maize leaves, while infection contributed to an increase in activity in the roots. Infection raised the guaiacol peroxidase (GPX) enzyme activity level, which was reduced by MMS pretreatment. MMS contributed to a decrease in both the RNA and coat protein content of MDMV, to an equal extent in maize leaves and roots. The results showed that MMS pretreatment enhanced the stress response reactions against MDMV infection in maize plants and retarded the spreading of infection.

Key words:

S-methylmethionine-salicylate, *Maize dwarf mosaic virus*, ascorbate peroxidase, guaiacol peroxidase, qRT-PCR, chlorophyll *a* fluorescence induction

Introduction

Maize (*Zea mays* L.) is an important, widely cultivated crop, which also plays a major role in industry, so the maintenance of plant health and crop production is of great importance. *Maize dwarf mosaic virus* (MDMV) is one of the most important microbial stressors of sweet corn varieties. The infection often causes a crop loss of 10–45%, but the damage may reach up to 100% (Oertel et al. 1997; Tóbiás et al. 2007). MDMV preferentially colonizes members of the Poaceae family and is spread via aphids, pollen, and seed transmission (Urcuqui-Inchima et al. 2001; Gell et al. 2010; Stewart et al. 2012).

The application of biologically active compounds seems to be a feasible way of improving the stress tolerance of plants. Previous research has shown the beneficial effects of two protective compounds, *S*-methylmethionine (SMM) and salicylic acid (SA) (Raskin 1992; Bi et al. 1995; Conrath et al. 1995; Rao et al. 1997; Ranocha et al. 2001; Ko et al. 2004; Rácz et al. 2008; Páldi et al. 2014; Ludmerszki et al. 2015). SMM is involved in the methylation processes inside plant cells. It contributes to plant resistance, being a direct precursor of the osmoprotectant sulfopropionates, and it also influences the biosynthesis of other regulatory and defence compounds (such as polyamines and ethylene) (Ranocha et al. 2001; Ko et al. 2004; Rácz et al. 2008). SA is an important signalling molecule in flowering, plant growth, ethylene production, and even stomatal movement (Raskin 1992). It also contributes to pathogenesis-related resistance (Bi et al. 1995; Conrath et al. 1995). The aim of the present work was to combine these two defence-related compounds and to test the effects of the combined molecule, known as S-methylmethionine-salicylate (MMS).

During MDMV infection, the viral particles accumulate in the cytoplasm of the leaf mesophyll cells where they are generally associated with cytoplasmic inclusions and may use chloroplasts for their replication (Mayhew and Ford 1974; Chen et al. 1994; Hammond 1998; Wei et al. 2010). MDMV causes the breakdown of the thylakoid membranes in infected mesophyll cells, resulting in a reduction in the size and number of chloroplasts and in the chlorophyll content (Tu et al. 1968; Gates and Gudauskas 1969; Musetti et al. 2002; Williams and Pataky 2012; Ludmerszki et al. 2015). The disintegration of the thylakoid membranes negatively affects the photosynthetic electron transport, resulting in a disruption of the photosynthetic electron transport chain in photosystem II (PSII). As a result, the relative surplus of excitation energy damages the PSII reaction centres due to singlet oxygen formation. In order to avoid extensive damage, non-photochemical quenching (NPQ) processes eliminate the excess light energy by heat dissipation. These thermal dissipation pathways have three basic mechanisms (D'Ambrosio et al. 2008). Some of the antennae-based excitation energy quenching processes depend on a high energy transmembrane ΔpH across the thylakoid membranes, which induces zeaxanthin formation in the light harvesting complex of PSII (LHCII) antennae, which is followed by the formation of quenching centres in cooperation with the protonated PsbS proteins (Kiss et al. 2008; Horton 2012). As a result, LHCII kinase becomes active, resulting in the phosphorylation of LHCII complexes and the migration of the antennae to the PSI complexes.

Any damage in the thylakoid membranes leads to the formation of highly reactive oxygen species (ROS). Compared to other plant species, maize bundle sheet cells are unusually sensitive to

oxidative damage (Asada 1996; Kingston-Smith and Foyer 2000). This is overcome by the presence of antioxidant enzymes in the plant cells, which effectively scavenge these reactive molecules (Fryer et al. 1998). One of these enzymes is ascorbate peroxidase (APX), two main isoforms of which are found in chloroplasts and in the cytosol (Amako et al. 1994). One of the most important functions of ascorbate is to protect plant cells from oxidative damage by scavenging hydrogen peroxide (Asada 1996). Another important antioxidant enzyme is guaiacol peroxidase (GPX), which is localized in vacuoles, cell walls and cytosol, but is absent from chloroplasts (Nakano and Asada 1981).

The precise molecular details of maize responses to MDMV infection are largely unknown. RNA silencing is a well-known plant defence mechanism (Marathe et al. 2000; Pradeep et al. 2012; Zhang et al. 2013), which helps the plant to restrict viral replication and spreading, and to decrease the number of viral particles. One of the most widely used techniques to monitor changes in the amount of viral particles in infected plant samples is enzyme-linked immunoabsorbent assay (ELISA) (Clark and Adams 1977). Antibodies uniquely designed to interact with certain regions of the viral coat proteins make precise detection possible. The coat protein is important in the transmission of the virus through both aphids and plants, and also takes an important part in the regulation of the replication process (Shaw et al. 1986; Osbourn et al. 1990; Murry et al. 1993; Gell et al. 2010). When the virus particles enter the plant cells, they induce the formation of special inclusion bodies, where replication will take place. In a later step, the viral coat proteins are resynthesized and encapsidate the newly replicated virus RNAs (Cassone et al. 2014). Therefore, measuring the coat protein content of infected plant samples only gives information on the amount of infectious, self-assembled viral particles, not on the exact amount of RNA. Currently, polymerase chain reaction (PCR) - based techniques are the most popular method for detecting viruses in plant samples (Balaji et al. 2003; López-Fabuel et al. 2013). With this technique, the viral RNA load of the plants can also be determined, supplementing the results of the ELISA technique.

This paper presents the possible advantages of MMS against MDMV infection in maize plants by examining the different thermal dissipating pathways, the level of activity of APX and GPX enzymes, and the amount of viral coat protein and viral RNA content in infected plants.

Materials and Methods

Plant material

Sweet corn (*Zea mays* var. *saccharata* (Sturt.) Bailey cv. 'Honey') plants were grown on ¼ strength Hoagland solution (80 µM $Fe^{(III)}$-EDTA as iron form) in a SANYO MLR-350 HT growth chamber (SANYO Electric Co., Ltd., Japan). The environmental parameters were: 14 h light, 10 h dark periods, 300 µmol photon m^{-2} s^{-1} photosynthetic photon flux density, 25 °C day/ 22 °C night temperatures, and 70% relative air humidity. Plants without further treatment are referred to as control plants. To test the effects of MMS, 10-day-old plants were treated with 0.5 mM MMS for 24 h (code: *mms*). MDMV infection was carried out on 11 and 13-day-old plants (code: *mdmv*). Leaves from infected plants showing macroscopic symptoms were homogenized in Sörensen phosphate buffer (pH 7.2, 0.067 M KH_2PO_4 and Na_2HPO_4•$2H_2O$), and were used for inoculation. Carborundum was added as abrasive. The first and second leaves of the maize plants were inoculated mechanically with Dallas-A strain MDMV. To study the effects of MMS treatment on MDMV-infected plants, MMS-treated plants were infected with MDMV (code: *mms+mdmv*). Mock inoculation was performed on leaves mechanically injured as for MDMV infection, but with no virus inoculum. The 3rd, 4th and 5th leaves were investigated 1, 2 and 3 weeks after the first inoculation (1 *wpi*; 2 *wpi* and 3 *wpi*, respectively).

Chlorophyll *a* fluorescence induction

Measurements were carried out on intact leaves using a PAM 101-102-103 Chlorophyll *a* Fluorometer (Walz, Effeltrich, Germany). After dark adaptation for 12 min, a 3 s illumination with far-red light was applied in order to eliminate reduced electron carriers (Belkhodja et al. 1998). The F_0 level of fluorescence was determined by switching on the measuring light (1.6 kHz modulation frequency and less than 1 µmol m^{-2} s^{-1} photosynthetic photon flux density). The maximum and minimum fluorescence yields in the dark-adapted state (F_m and F_0, respectively) were measured by applying a 0.7 slight pulse (3500 µmol m^{-2} s^{-1} photosynthetic photon flux density; PPFD). Actinic light (100 µmol m^{-2} s^{-1} PPFD) was provided for the quenching analysis. Simultaneously with the onset of actinic light, the modulation frequency was switched to 100 kHz. Light-adapted maximal fluorescence (F_m') was determined using a 3500 µmol m^{-2} s^{-1} PPFD flash after 10 min light adaptation; then the actinic light was switched off and a 0.7 s light flash (3500 µmol m^{-2} s^{-1}) was applied 20 s (F_{md} *20"*), 5 min (F_{md} *5'*) and 15 min (F_{md} *15'*) after the start of dark adaptation. The following equations were used to calculate the NPQ components (Baker 2008):

$$qE = [(F_m – F_{md}\ 20") – (F_m – F_{md}\ 5')] / (F_m – F_0),$$

$$qT = [(F_m – F_{md}\ 5') – (F_m – F_{md}\ 15')] / (F_m – F_0),$$

$$qI = (F_m – F_{md}\ 15') / (F_m – F_0),$$

where qE is the ΔpH-dependent process of the high energy state; qT includes state transition processes, by which the excitation energy of PSII and I can be reversibly balanced with the phosphorylation-related migration of the LHCII pool between PSI and PSII; and qI is known as the photoinhibition of photosynthesis (Dodd et al. 1998).

Enzyme activity measurements

0.5 g plant material was ground in liquid N_2, after which 5 ml of extraction buffer (0.5 mM Tris pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA) was added to the powdered material. Soluble enzymes were purified by centrifugation for 20 min at 15 000 x g. The measurements were carried out using a LAMBDA 25 UV-VIS spectrophotometer (PerkinElmer Ltd., Chalfont Road, Seer Green, Beaconsfield, United Kingdom). To measure APX activity, 534 µl distilled water, 150 µl 1 M pH 7.8 TRIS buffer, and 7.5 µl 0.05 M ascorbate was used for blank measurements. After blanking, 0.75 µl 0.1 mM EDTA, 7.5 µl 0.1 M H_2O_2, and finally 50 µl extracted plant sample was added to the cuvette. Enzyme kinetics was measured for 3 min at 290 nm, and the results are given as ΔA_{290} min^{-1} g^{-1} total protein. To measure GPX enzyme activity, 375 µl reaction buffer (0.2 M sodium phosphate buffer pH 7 and 0.01 M guaiacol) and 243 µl distilled water was added for blank measurements. After blanking, 0.75 µl 0.1 mM EDTA, 112.5 µl 0.1 M H_2O_2, and 18.75 µl extracted plant sample was added to the cuvette. Enzyme kinetics was measured for 3 min at 470 nm, and the results are given as ΔA_{470} min^{-1} g^{-1} total protein. For total protein content measurements, the plant extracts were measured at 260 and 280 nm (100 x diluted with distilled water) and the protein content was calculated using the following equation: $1.55\ A_{280} - 0.76\ A_{260}$.

Expression analysis

A ZR Plant RNA MiniPrep™ 2024 kit (Zymo Research, Irvine, CA, USA) was used for RNA extraction, and a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA) for cDNA synthesis following the manufacturers' instructions.

Quantitative real-time PCR (qRT-PCR) measurements were carried out using the GoTaq® Probe qPCR Master Mix (Promega, Madison, WI, USA). All experiments were run on an ABI StepOnePlus™ Real-Time PCR System (Life Technologies, Foster, CA, USA). The final volume of the reaction was 20 µl, containing: 2 µl 2.5 µM PrimeTime probe (IDT Integrated DNA Technologies, Coralville, IA, USA), 10 µl GoTaq 2xMM (containing ROX), 6 µl cDNA, 1 µl 500 nmol forward and reverse primers. The primers were designed for the MDMV genome (Accession number in Uniprot database: CAA04929.1). The forward and reverse primers were: CACCAAGGCTTAGATTCCAC and ACCAAAGCATCAGTAGACCG, respectively, and the length of the amplicon was 124 bp. The sequence of the PrimeTime probe was: 5'-/56-FAM/GCTCAAAGG/ZEN/AAGGTGGAACGGAGA/3IABkFQ/-3'. The thermal cycling conditions for qRT-PCR consisted of 40 cycles of 95 °C 15 s, and 60 °C 1 min, previously heating up to 95 °C for 2 min. A 500 bp fragment of the MDMV genome (CAA04929.1) containing the given primer sequences was synthesized (IDT, Coralville, IA, USA) and was used as a reference for absolute quantification. Since a synthesized fragment was available for absolute quantification, no further reference primers were used in this investigation. The results are expressed as viral RNA concentration (attomol/µl).

ELISA test

The concentration of MDMV coat protein was determined with ELISA, using an MDMV antiserum kit (Bioreba A.G., Reinach, Switzerland) for detection. The virus coat protein content was measured at 405 nm with a Labsystem Multiscan MS spectrophotometer.

Statistical analysis

The results were evaluated by ANOVA, using the Tukey-Kramer multiple comparison *post-hoc* test (GraphPad InStat statistical software) to assess for significant differences between the different sets of data. Three biological and six technical repeats were performed for each experiment.

Results

The first symptoms were detected as early as 4 days after the first inoculation. The first measurements were taken a week after inoculation*1 wpi*, and were repeated *2* and *3 wpi*. There was no significant difference between the control and mock-inoculated plants (results not shown) (Ludmerszki et al. 2014, 2015).

Analysis of NPQ parameters

Fig. 1 Changes in the NPQ parameters of *mms*, *mdmv* and *mms+mdmv* plants 1, 2 and 3 *wpi* (n=18). *mms*: MMS-treated plants, *mdmv*: MDMV-infected plants, *mms+mdmv*: MDMV-infected plants pretreated with MMS, *wpi*: weeks post infection. Values recorded for control plants (all shown in arbitrary units): 1 *wpi* qE 0.035 ± 0.008; 1 *wpi* qT 0.062 ± 0.008; 1 *wpi* qI 0.006 ± 0.005; 2 *wpi* qE 0.038 ± 0.005; 2 *wpi* qT 0.059 ± 0.005; 2 *wpi* qI 0.045 ± 0.005; 3 *wpi* qE 0.005 ± 0.002; 3 *wpi* qT 0.054 ± 0.006; 3 *wpi* qI 0.009 ± 0.003.The results of ANOVA are shown at the right of each column, using letters to indicate significance. Other significant relationships are given in the text. Total NPQ values are the sum of qI + qT + qE.

qI increased significantly ($p < 0.001$) in *mms* and *mms+mdmv* plants 1 *wpi* (making up 73.06 ± 9.05% and 84.65 ± 9.03% of total NPQ, respectively, **Fig. 1**). 2 and 3 *wpi* the rate of qI decreased in the *mms* and *mms+mdmv* groups, but in *mms* plants still played an important role in total NPQ 3 *wpi* (56.80 ± 7.42%). The contribution of qI to total NPQ decreased 2 and 3 *wpi* in *mms+mdmv* plants, and reached the level of *mdmv* plants 3 *wpi* (*ns*).

qE significantly ($p < 0.001$) increased in *mdmv* plants 3 *wpi*, making up 79.03 ± 8.64% of total NPQ (as shown in **Fig. 1**). On the other hand, in *mms+mdmv* plants considerably lower qE values were measured 3 *wpi* compared to *mdmv*, with a smaller percentage contribution to total NPQ (24.42 ± 1.22%, **Fig. 1**).

The values of qT in *mms+mdmv* were significantly ($p < 0.001$) smaller than those of *mms* and *mdmv* 1 *wpi* (**Fig. 1**), as was the contribution of qT to total NPQ (8.46 ± 1.30%). Smaller values were also recorded in *mms+mdmv* plants 2 *wpi* (*ns* compared to 1 *wpi mms+mdmv*, but $p< 0.001$ compared to 2 *wpi mms* and *mdmv*, **Fig. 1**). On the other hand, it made a greater contribution to

NPQ (41.18 ± 4.59%). The qT values of *mms*+*mdmv* did not differ significantly from those of *mms* and *mdmv* 3 *wpi*, while they made a larger contribution to total NPQ (61.85 ± 6.76%, **Fig. 1**). In *mdmv* plants the values of qT rose slightly from 1 *wpi* to 2 *wpi* ($p < 0.05$), but dropped again from 2 *wpi* to 3 *wpi* ($p < 0.001$), when they were not significantly different from 1 *wpi*. The values of qT increased significantly in *mms* plants ($p < 0.001$) 2 *wpi* compared to both 1 *wpi mms* and to 2 *wpi mdmv* and *mms*+*mdmv*, resulting in a greater contribution to total NPQ (78.15 ± 7.75 %, **Fig. 1**). At 3 *wpi* there was no significant difference between, the qT values of *mms*, *mdmv* and *mms*+*mdmv* plants.

Enzyme activity measurements

Significantly higher levels of APX enzyme activity were measured in the leaves of *mms* plants ($p < 0.001$) 1 *wpi* than in *mdmv* and *mms*+*mdmv* (**Table 1**). A week later (2 *wpi*) the APX activity in *mdmv* leaves was significantly ($p < 0.001$) higher than in the other treated groups, but was still significantly lower than in the control (**Table 1**). The APX values of *mms* increased 3 *wpi*, approaching the control levels, while *mdmv* and *mms*+*mdmv* were significantly lower ($p < 0.001$) (**Table 1**). In the roots, the APX values of *mdmv* were considerably higher 1 and 2 *wpi* than those of the other treated groups, which did not differ significantly from each other or from the control plants. The values of *mdmv* decreased significantly ($p < 0.001$) 3 *wpi* compared to the 1 and 2 *wpi* *mdmv* values, reaching the control levels and not differing significantly from the *mms* plants. The values of *mms*+*mdmv* decreased ($p < 0.001$) compared to the 2 *wpi* data. The enzyme activities measured in the roots always exceeded those measured in the leaves (**Table 1**).

Table 1 APX and GPX enzyme activity in the leaves and roots of maize plants, expressed as ΔA_{290} * min^{-1}*g^{-1} total protein and ΔA_{470} * min^{-1}*g^{-1} total protein, respectively. Error values represent standard deviation, n = 18. The results of ANOVA, performed separately for leaves and roots, are shown in subscript, where different letters indicate significant differences.

APX (ΔA_{290} * min^{-1}*g^{-1} total protein)	1 wpi	2 wpi	3 wpi
control leaves	5.49 ± 1.67^{fi}	29.84 ± 2.32^{a}	9.30 ± 1.12^{c}
mms leaves	8.69 ± 1.52^{c}	8.21 ± 0.94^{e}	8.55 ± 1.13^{a}
mdmv leaves	6.37 ± 1.90^{i}	18.08 ± 1.60^{b}	5.05 ± 0.79^{fi}
mms+*mdmv* leaves	6.22 ± 1.21^{i}	4.23 ± 1.44^{i}	6.11 ± 0.69^{g}
control roots	22.94 ± 4.41^{i}	65.38 ± 2.87^{c}	78.88 ± 7.08^{b}
mms roots	25.09 ± 3.55^{i}	76.21 ± 1.84^{b}	72.28 ± 5.15^{bc}
mdmv roots	36.56 ±4.36^{e}	101.62 ± 6.27^{a}	78.80 ± 5.19^{b}
mms+*mdmv* roots	21.97 ± 2.18^{i}	71.50 ± 4.78^{bc}	55.70 ±4.14^{a}
GPX (ΔA_{470} * min^{-1}*g^{-1} total protein)			
control leaves	11.57 ± 0.93^{i}	11.93 ± 2.47^{i}	22.51 ± 1.97^{b}
mms leaves	10.82 ± 1.93^{ig}	9.05 ± 0.82^{ig}	16.86 ± 2.57^{a}
mdmv leaves	17.79 ± 1.27^{c}	8.64 ± 2.33^{ig}	31.06 ± 1.11^{a}
mms+*mdmv* leaves	15.35 ± 2.23^{e}	7.58 ± 1.02^{g}	12.42 ±2.01^{i}
control roots	116.1 ± 20.32^{ca}	143.71 ± 13.36^{c}	215.98 ± 22.46^{a}
mms roots	182.83 ±8.16^{b}	134.44 ± 11.36^{ca}	181.69 ±5.04^{b}
mdmv roots	108.08 ±19.14^{ae}	201.19 ±18.98^{ab}	202.27 ± 7.35^{a}
mms+*mdmv* roots	141.19 ±19.42^{c}	85.36 ± 6.14^{e}	142.01 ±15.28^{c}

In maize leaves, the activity of GPX increased significantly ($p < 0.001$) 1 *wpi* in *mdmv* and *mms*+*mdmv* compared to *mms*. Similarly high levels were measured in *mdmv* 3 *wpi*, while the activity levels dropped in the leaves of *mms* and *mms*+*mdmv* 2 *wpi*, and were significantly lower in *mms*+*mdmv* than in *mms* ($p < 0.01$) and 3 *wpi mdmv* ($p < 0.001$, **Table 1**). High GPX activity was measured in *mms* maize roots 1 *wpi* ($p < 0.001$), but this value decreased 2 *wpi*. After MDMV infection the enzyme activity of GPX was similar to that of the control plants 1 and3*wpi*, and higher 2 *wpi*. *mms*+*mdmv* plants showed a slight increase in enzyme activity 1 *wpi* ($p < 0.001$), but had lower values 2 and 3 *wpi,* when the level was significantly different ($p < 0.001$) from the other treatments (**Table 1**).

Results of expression analysis

Fig. 2 Results of PrimeTime-based qRT-PCR reactions. The virus concentration is given in *attomol/µl*. n=18. *mdmv*: MDMV-infected plants, *mms+mdmv*: MDMV-infected plants pretreated with MMS, *wpi*: weeks post infection. Different letters indicate significant differences in the ANOVA results.

At 1 *wpi* the highest viral RNA concentration was measured in *mdmv* leaves, with a significantly lower RNA concentration in *mms+mdmv* leaves (**Fig.2**). In the roots significantly higher RNA concentration was measured in *mms+mdmv*, than in *mdmv* 1 *wpi*. From the 2nd week onwards, both the leaves and roots of *mms+mdmv* plants contained less viral RNA than the *mdmv* plants. In *mdmv* leaves, higher RNA concentrations were measured 1 and 3 *wpi* than 2 *wpi*. By contrast, in *mdmv* roots, lower RNA contents were found 1 and 3 *wpi* than 2 *wpi*, showing a dynamic oscillation of viral RNA content between the roots and shoots.

ELISA measurements

Fig. 3 Changes in ELISA absorbance values, indicating the amount of viral coat protein present in the samples. n=18. *mdmv*: MDMV-infected plants, *mms+mdmv*: MDMV-infected plants pretreated with MMS, *wpi*: weeks post infection. Different letters indicate significant differences in the ANOVA results.

The highest absorbance levels were measured in *mdmv* leaves 1, 2 and 3 *wpi* (**Fig. 3**), but in the first two weeks similar values were measured in *mms+mdmv* leaves. The values measured in *mdmv* roots were always significantly lower than in the leaves ($p < 0.001$). Apart from the 2nd week, the coat protein content was significantly lower in *mms+mdmv* roots than in *mdmv* roots ($p < 0.001$).

Discussion

Alterations in NPQ under MMS treatment and MDMV infection

This study aimed to detect the harmful effects of MDMV infection, and to demonstrate the potential beneficial effects of MMS treatment in previously infected plants. Since xanthophylls not only take part in the quenching of excess light, but also contribute to the antioxidative stress defence, their concentration increases under various biotic and abiotic stresses. According to Moharekar et al. (2003) increased concentrations of SA significantly increased the size of the xanthophyll pool, and contributed to a slight degree of oxidative stress in some plant species. Similarly, Mateo et al. (2006) investigated the effects of low and high levels of SA on photosynthesis in Arabidopsis plants, and found that the larger part of the population suffered under photoinhibition. The increasing values of qI in MMS-treated plants can be explained by the characteristics of SA; hence high concentrations of this molecule contribute to slight photooxidative damage in plants (Moharekar et al. 2003; Mateo et al. 2006), which diminishes in time (**Fig. 1**). Moreover, MMS treatment contributed to increased state-transition, where phosphorylated LHCII trimers bind to PSI, leading to the elimination of the over-excited state of PSII reaction centres. When plants were exposed to MDMV infection, lower levels of qI were measured, but when MMS treatment was applied before infection, the initially high levels of qI diminished to those of the *mdmv* plants, coupled with a drop in qT (**Fig. 1**).

Balachandran et al. (1994) demonstrated that in the thylakoid membranes, the association of *Tobacco mosaic virus* coat protein with PSII reaction centres inhibits the photosynthetic electron transport, leading to severe photoinhibition and to the destruction of chloroplasts. The association of viral coat proteins to PSII reaction centres results in the formation of quenching centres (Reneiro and Beachy 1986; Hodgson et al. 1989). Beddard and Porter (1976) stated that the relatively close association of chlorophyll molecules can form a non-fluorescent trap for the excitational energy. Therefore, the aggregation of chlorophyll molecules coupled with their association with viral coat proteins may result in an increase in non-photochemical quenching. In the course of MDMV infection high qE values were measured 3 *wpi* (making up 79.03 ± 8.64 % of total NPQ, **Fig. 1**). This high qE value can be explained by the appearance of additional quenchers of fluorescence, possibly as a result of virus coat protein- thylakoid membrane associations. When infected plants were pretreated with MMS, low qE values were measured 3 *wpi*, indicating that such associations were not formed. This statement is supported by the ELISA and PrimeTime-based qRT-PCR results, which showed a decrease in the amount of MDMV coat protein and viral RNA in the leaves of *mms+mdmv* plants (**Figs. 2, 3**), providing an explanation for the lower qE values.

Alterations in the activity of antioxidant enzymes after MMS treatment and MDMV infection

Effects of MMS treatment and viral infection on APX activity

MMS treatment modified the enzymatic activity of APX in maize leaves, causing a great increase 1 *wpi*, followed by a considerable decrease 2 *wpi*, then returning to the level of the control plants 3 *wpi* (**Table 1**). Different results were reported by Kang and Saltveit (2002), who investigated

the effects of SA (0.5 mM) treatment and chilling tolerance on the activity of antioxidant enzymes, including APX and GPX, in maize plants. According to their results, SA treatment had no significant effect on the activity of APX. On the other hand, Durner and Klessig (1995), who investigated the effects of SA treatment (0.1 and 1 mM) on tobacco plants and measured changes in antioxidant enzyme activities, obtained results in contradiction to those of Kang and Saltveit (2002). They found that SA treatment inhibited APX activity (0.1 mM by 59 % and 1 mM by 95%), thus contributing to an increase in H_2O_2 (Fodor et al. 1997; Rao et al. 1997). The present results are in agreement with those of Durner and Klessig (1995), since MMS treatment was shown to have an inhibiting effect on APX enzyme activity (27% of control). One explanation could be the different concentrations used in the different experiments. Higher concentrations of SA have negative effects on enzyme activity, whereas smaller concentrations tend to have no effect. Since different species were used in the various experiments, the discrepancy could be caused by differences in plant responses. In tobacco plants 0.1 mM SA concentrations are high enough to cause an inhibition in enzyme activity, while in maize plants, higher concentrations have no effect. Great differences exist not only between different plant species, but also between different cultivars of the same species. Kang and Saltveit (2002) used 'Golden Jubilee' for their experiments, while 'Honey' was tested in the present work. Since 'Honey' is much more sensitive to viral infection, smaller concentrations of SA may be required for inhibition than in 'Golden Jubilee' (Ludmerszki et al. unpublished).

It is interesting to note that MMS treatment did not appear to affect the enzyme activity in maize roots, since no substantial changes in APX activity were detected in the roots of MMS-treated plants (**Table 1**). This could be due to the different metabolic pathways in leaves and roots. Another explanation could be that after the plant takes up MMS from the growing medium, it is transported to the shoot and leaves, so that its effects diminish in the root area.

MDMV infection resulted in a decrease in APX activity in maize leaves (**Table 1**). Similar results were acquired by Mittler et al. (1998), who investigated cytosolic APX activity in infected tobacco plants in areas where programmed cell death was induced. They found that the pathogen inhibited the expression and activity of APX, probably to enhance H_2O_2 production and increase the rate of cell death. Fodor et al. (1997) investigated the effects of *Tobacco mosaic virus* infection and also measured a decrease in the enzyme activity following infection.

Contrasting results were seen in maize roots, since a significant increase in APX activity was observed in infected plants 1 and 2 weeks after infection (**Table 1**). It thus seems that infection results in an increase in APX activity in the roots and a decrease in the leaves. One explanation could be that higher initial levels of APX enzyme activity are present in the roots, preventing the virus from restricting its activity (**Table 1**). The decrease measured in the 3rd week can be explained by the decrease in the viral coat protein content measured with ELISA and qRT-PCR, which resulted in fewer infectious virus particles being present in the roots (**Figs. 2, 3**).

MMS pretreatment had no effect on infected maize leaves1 and 3 *wpi*, however, a considerable decrease was detected2 *wpi* compared to infected plants.

Effects of MMS treatment and MDMV infection on GPX activity

SA treatment was found to increase the activity of GPX in maize leaves (Kang and Saltveit 2002). In the present work MMS treatment slightly modified the GPX activity in maize leaves, resulting in a slight inhibition 2 and 3 *wpi*. However, in maize roots, an increase similar was observed in MMS-treated plants, to that reported by Kang and Saltveit (2002), which diminished with time (**Table 1**).

Ye et al. (1990) investigated the peroxidase activity in infected tobacco plants, and found that the peroxidase activity rose in all the tissues investigated after *Tobacco mosaic virus* infection. They assumed that increased levels of peroxidase activity induced systemic resistance. These authors measured GPX activity after adding guaiacol as substrate. It is therefore assumed that the high levels of GPX activity in infected maize plants are associated with increased plant defence as a response to MDMV infection. If MMS treatment was applied before infection, smaller levels of enzyme activity were measured, indicating a reduced level of resistance, which can be explained by the decrease in the amount of viral particles due to MMS pretreatment (**Figs. 2, 3, Table 1**).

Changes in the amount of MDMV following MMS pretreatment

MMS pretreatment contributed to a decrease in MDMV RNA and coat protein content, both in leaves and roots (**Figs. 2, 3**). The beneficial effects of SA on virus-infected plants has already been shown (White 1979; Malamy et al. 1990; Métraux et al. 1990; Delaney et al. 1994; Vernooij et al. 1994). According to White (1979), SA reduced the number of *Tobacco mosaic virus* particles in tobacco plants, and further increased the plants' defence against viral infection. It is well known that SA participates in the pathways of systemic acquired resistance (SAR), which is triggered by infection with certain pathogens. The accumulation of SA induces plant resistance and contributes to better plant defence (Malamy et al. 1990; Métraux et al. 1990; Delaney et al. 1994; Vernooij et al. 1994). Similarly, SMM has an important role in plant resistance pathways, since it is a direct precursor of the osmoprotectant sulphopropionates and also influences the biosynthesis of other regulatory and defence compounds (such as polyamines and ethylene) (Ranocha et al. 2001; Ko et al. 2004; Rácz et al. 2008). As MMS is built up from SMM and SA, their beneficial effects also appear when plants are treated with this new compound. By boosting their resistance and defence pathways, MMS-treated plants are more able to fight infections, and viral replication and movement are restricted.

Based on the present results, it can be stated MMS treatment has protective and beneficial effects in MDMV-infected maize plants. MMS is capable of protecting photochemical systems and reducing the oxidative stress caused by infection. It has also been proved to restrict viral RNA replication and MDMV coat protein formation.

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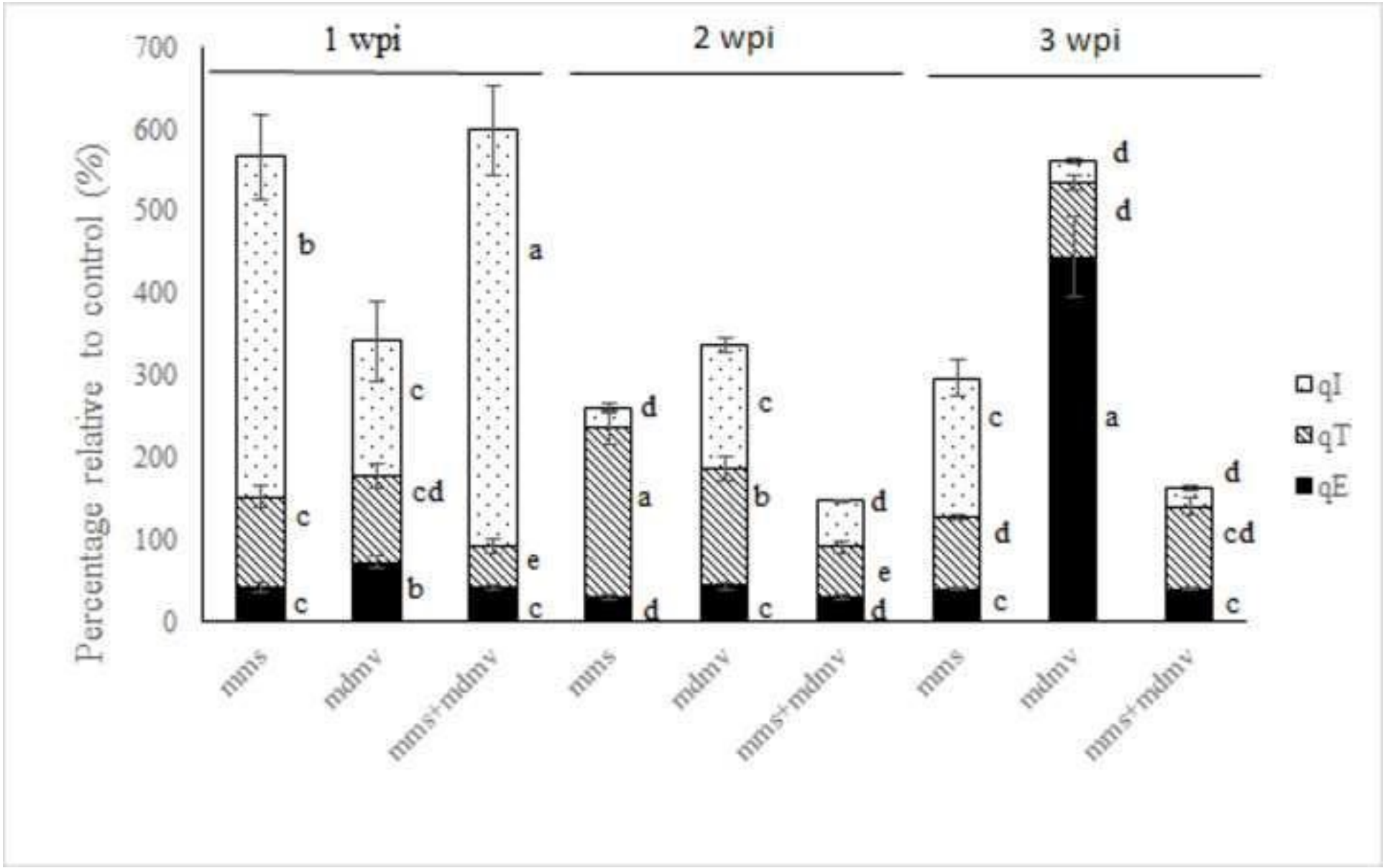

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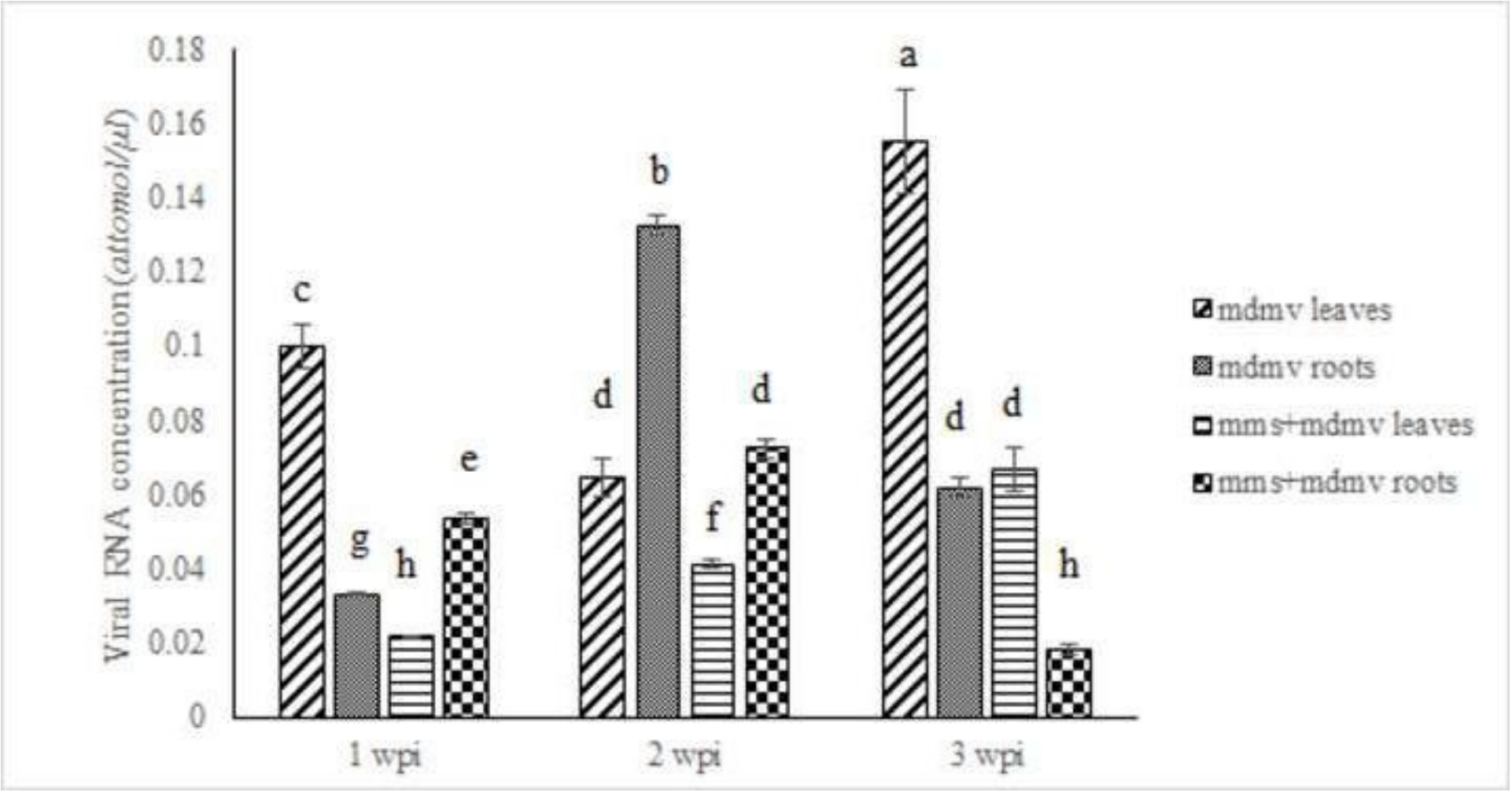

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