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PEROXIDASE ACTIVITY IN MAIZE INBRED LINES RESISTANT OR SUSCEPTIBLE TO MAIZE DWARF MOSAIC VIRUS[1]

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ABSTRACT - Peroxidase (EC 1.11.1.7) activity levels were analyzed in maize seedling inbred lines contrasting with their resistance to maize dwarf mosaic virus (MDMV). Groups of non-inoculated resistant and susceptible inbred lines showed different levels of guaiacol peroxidase activity at the seedling stage, with 78 % of susceptible inbred lines presenting enzyme activity values below 91.46 A_{470} min^{-1}g FW^{-1}. However, the zymogram patterns of peroxidase did not allow the differentiation of non-inoculated resistant or susceptible inbred lines. The virus complex induced a general enhancement of enzyme activity, without qualitative changes in the isoenzymes, when inoculated into resistant or susceptible inbred lines. However, a quantitative change, with 19% OD average increase in a moderately anionic isoform was observed in some resistant inbred lines in response to virus inoculation. Our results could suggest that peroxidase activity prior to infection, and the increase in activity of an specific anionic isoform in some resistant inbred lines, due to virus inoculation, could be related to a defense mechanism against MDMV.

Key words*: Zea mays* L., potyvirus, isoforms.

ATIVIDADE DA PEROXIDASE EM LINHAGENS DE MILHO RESISTANTES OU SUSCEPTÍVEIS AO MOSAICO COMUM DO MILHO

RESUMO - Níveis de atividade da peroxidase (EC 1.11.1.7) foram analisados em plântulas de linhagens de milho contrastantes quanto à resistência ao mosaico comum do milho (MDMV). Grupos de linhagens não-inoculadas, resistentes e susceptíveis apresentaram diferentes níveis de atividade da guaiacol peroxidase no estádio de plântulas, com 78 % das linhagens susceptíveis apresentando valores de atividade da enzima abaixo de 91.46 A_{470} min^{-1}g FW^{-1}. Entretanto, os padrões dos zimogramas da peroxidase não permitiram a diferenciação de linhagens resistentes ou susceptíveis. O complexo viral, quando inoculado nas linhagens resistentes ou susceptíveis, induziu um aumento geral na atividade da enzima, sem alterações qualitativas nas isoformas. Entretanto, uma alteração quantitativa, com aumento médio de 19 % OD em uma isoforma moderadamente aniônica foi observada em algumas linhagens resistentes, em resposta à inoculação com vírus. Os resultados parecem indicar que, em algumas linhagens resistentes, a atividade da peroxidase antes da infecção e o aumento, devido à inoculação com vírus, na atividade de uma isoforma aniônica específica, podem estar relacionados ao mecanismo de defesa contra MDMV.

Palavras-chave: *Zea mays* L., potyvirus, isoformas.

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Resistance to some plant diseases is associated with increased peroxidase activity and expression of specific isoenzymes (Ye *et al.*, 1990; Goy *et al.*, 1992). Peroxidase activity as a preliminar biochemical marker may predict resistance of uninfected muskmelon to *Pseudoperonospora cubensis* (Reuveni *et al.*, 1992) and resistance of maize to gray leaf spot (Garraway and Beltran, 1997). However, studies designed to verify these associations using maize (*Zea mays* L.) genotypes contrasting their resistance to potyvirus-induced-mosaic are rare. The potyvirus-induced-mosaic is one of the most important diseases in maize due to its ubiquity and imposed yield losses. The common maize viruses can be caused by four distinct potyviruses: Maize dwarf mosaic virus (MDMV), Sugarcane mosaic virus (SCMV), Johnsongrass mosaic virus (JGMV) and Sorghum mosaic virus (SrMV) (Shukla *et al.*, 1994). In Brazil MDMV-B is the most common virus (Melo, 2000; Melo *et al.*,2000). Most commercial maize cultivars are susceptible to MDMV and yield losses can be as great as 50% (Waquil *et al.*, 1996). The objectives of this study were: (i) to determine the activity and expression of peroxidase isoenzymes in seedlings of maize inbred lines contrasting with their resistance to MDMV, and (ii) to determine the effect of MDMV infection on the activity and expression of peroxidase isoenzymes in maize leaves.

Material and Methods

Eighteen MDMV susceptible and 15 resistant maize inbred lines were previous selected from one-thousand maize inbred lines from the breeding program at the National Maize and Sorghum Research Center/Brazilian Agricultural Corporation. These lines had been screened by inoculating MDMV onto seedlings, 7 days after sowing. Evaluations for the presence of mosaic symptoms were made 15 days post-inoculation. Three seedlings of each genotype were evaluated three times throughout the year under different climatic conditions. Only inbred lines completely free of MDMV disease symptoms in all three plants at each of the three evaluations were considered resistant to MDMV.

Inoculum was obtained from the leaves of 30 days old field-grown maize hybrid plants expressing strong symptoms of MDMV. Inoculum was prepared by macerating infected leaves in 10 mM phosphate buffer at pH 7.0 to a ratio 1:5 (w/v). Plants were inoculated by gently rubbing, approximately 1 ml of inoculum over the seedling leaf surface, after youngest upper leaves had been gently wounded with 600 mesh carborundum, and subsequently rinsed with sterile water (Almeida *et al.*, 2000).

Peroxidase activity and isoenzyme expression was measured in non-inoculated maize inbred lines resistant and susceptible to MDMV. This experiment was carried out in an greenhouse under controlled conditions (radiance of 500 $\mu mol\ m^{-2}\ s^{-1}$ supplied by 100 W incandescent lamps, diurnal temperature 28 ^{o}C, night temperature 18 ^{o}C, relative humidity 60 %). Fifteen resistant and 18 susceptible maize inbred lines were used. Six seeds of each genotype were sowed per pot and thinned to three seedlings after germination. The treatment was completely randomized with three replicates per treatment. Enzyme activity and expression of isoenzymes were determined in the third leaf of each genotype in 17 day-old seedlings.

Effect of MDMV infection on peroxidase activity and expression of peroxidase isoenzymes in maize inbred lines resistant or susceptible to potyvirus-induced-mosaic. This experiment was carried out under the greenhouse conditions described above. Four resistant (R) maize inbred lines: 161, 505, 516, 575, and five susceptible (S): 8, 22, 38, 40, 43 were used, and six seeds of each genotype were sowed per pot and thinned to three

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seedlings after germination. The inoculation treatments were imposed to the youngest leaf on the 7th day after sowing. The treatments were completely randomized, with 3 replicate pots per treatment, and included: non-inoculation, buffer after gentle wounding with carborundum, and inoculation after gentle wounding with carborundum. Eight days after inoculation the susceptible inbred lines showed symptoms of viral infection. The third leaf from each seedling, on the 17th day after sowing, was collected for extraction of total soluble and ionically bound protein.

Soluble plus ionically bound protein fractions were extracted from 0.3 g of the third leaf, ground in liquid N_2 and suspended in two volumes of 50 mM phosphate buffer, pH 6.0, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 M NaCl and 1.5% polyvinylpolypyrrolidone (PVPP) (w/v). The homogenate was centrifuged at 14000 g at 4 °C for 30 min, and the supernatant containing the total soluble and ionically bound protein fraction retained for analysis. Seedlings were preserved by using the third leaf for analyses, based on the possibility of peroxidase activity being a biochemical marker for resistance.

For isoelectric focusing 150 µl of the protein extracts were desalted and concentrated to 36 µl through microcon-10 filters (Millipore Corporation, Bedford, MA) before being subjected to electrophoresis. Fifteen µg of protein of the leaf extract, approximately 5 µl, from each sample was loaded onto a 1% agarose native gel prepared with 12% sorbitol (w/v) and 6.7% ampholytes (v/v) in the 3 to 10 pH range (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Gels were cast onto Gelbond using a mold 125 x 260 x 1 mm (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and were run in Multiphor II (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Gel prefocusing was done for 20 min at 1200 V/1 W and focusing for 2 hours at 1200 V/4 W at 10°C. After electrophoresis the gel was stained for peroxidase activity using the PPD-PC protocol (Imberty *et al.*, 1984). The quantification of peroxidase isoenzymes in the gel was carried out by determining the optical density (OD) of the peak height using the personal densitometer SI (Amersham Pharmacia, NJ, USA).

Peroxidase activity was assayed as described by Souza and MacAdam (1998) at 30 °C in a 1 ml reaction mixture containing 96 mM potassium phosphate buffer pH 6.0, 3.2 mM guaiacol, and 0.38 mM hydrogen peroxide. The reaction was initiated by adding 10 µl protein extract to the reaction mixture, and the increase in absorption of guaiacol min^{-1} was measured at A_{470} nm.

The presence of potyviruses associated with mosaic symptoms was confirmed through transmission electron microscope analysis using "leaf dip" preparations stained with 2 % phosphotungistic acid and by dot Enzyme-linked immunosorbent assay (ELISA), using specific IgG against these viruses, as described by Almeida *et al.* (2000).

The variance analysis was realized by using the GLM procedure (SAS INSTITUTE, 1993). The Scott & Knott (1974) test, that allows comparisons of the treatment means to form independent and homogeneous clusters, was applied to peroxidase activity to verify the separation of the maize inbred lines in clusters of the susceptible and resistant to MDMV.

Results and Discussion

Peroxidase activity and expression of its isoenzymes in non-inoculated maize inbred lines resistant or susceptible to MDMV.

All data were analyzed by ANOVA. Where ANOVA indicated that peroxidase activity differed significantly ($P<0.01$) without inoculation between maize inbred lines resistant or susceptible to MDMV. The highly significant ($P<0.01$) interaction between

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the susceptible and resistant inbred lines (S vs R), indicates that the difference in peroxidase activity was larger between the resistant and susceptible groups than within each one of these groups. The separation of clusters by the Scott and Knott test resulted in a large number of susceptible inbred lines (78 %) with peroxidase activity values below 91.46 A_{470} min^{-1} g^{-1} FW and resistant inbred lines (60%) with peroxidase activity above this value (Table 1). This difference suggests that in some inbred lines, peroxidase could be involved as one of the resistance mechanisms to this maize virus. Peroxidase activity could be related to a fast production of physical barriers involved in avoiding virus translocation through the plant restricting it to the localized region of infection. Lignin synthesis (Whitmore 1978; Siegel, 1953), oxidative coupling reactions involving phenolics that are esterified to wall polysaccharides (Geissman and Neukon, 1971; Hartley 1973; Fry 1982a), and the formation of isodityrosine bridges that are believed to crosslink extensin molecules (Fry 1982b) are among the functions proposed for peroxidase that could be responsible for reinforcements of the cell wall as a barrier to the viruses. However, it is likely that other mechanisms of resistance are also involved in conferring resistance in the resistant maize inbred lines, since 40 % of these had peroxidase activities lower than 91.46 A_{470} min^{-1} g^{-1} FW (Table 1). The mean peroxidase activity for the group of resistant genotypes was 97.359 A_{470} min^{-1} g^{-1} FW whereas for the group of susceptible genotypes it was 75.301 A_{470} min^{-1} g^{-1} FW. Therefore, these results indicate that the resistant genotypes on average had higher (29.30%) peroxidase activity than the group of susceptible genotypes. Apparent susceptibility could be related to a low level of peroxidase activity prior to infection (Table 1). Similar results were obtained when peroxidase activities were calculated between groups of muskmelon genotypes varying in their resistance to *Pseudoperonospora cubensis*

TABLE 1. Peroxidase activity levels in non-inoculated maize seedling inbred lines susceptible (S) or resistant (R) to MDMV.

Inbred line	Peroxidase activity (A_{470} min^{-1} g FW^{-1})	
520(R)	173.70	a
134(R)	130.20	a
505(R)	117.20	a
18(R)	113.90	a
26(S)	110.70	a
402(R)	103.80	a
215(R)	102.30	a
39(S)	99.38	a
406(R)	99.03	a
454(R)	98.40	a
40(S)	98.19	a
20(R)	94.03	a
16(S)	91.46	a
L20(S)	81.39	b
33(S)	78.61	b
38(S)	79.46	b
1(S)	76.18	b
258(R)	75.52	b
431(R)	73.82	b
30(S)	72.64	b
22(S)	72.01	b
20-50(R)	71.81	b
8(S)	71.53	b
2(S)	70.90	b
516(R)	70.83	b
28(S)	69.31	b
29(S)	68.96	b
257(R)	68.13	b
161(R)	67.71	b
19(S)	62.50	b
17(S)	59.17	b
15(S)	57.29	b
25(S)	35.73	b

a, b Means within column followed by the same letter belongs to the same cluster according to Scott & Knott test. Data are the means of three replications.

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(Reuveni *et al.*, 1992), between maize genotypes varying in their resistance to *Cercospora Zeae-maydis* (Garraway and Beltran, 1997), between lettuce genotypes contrasting in their resistance to downy mildew (Reuveni *et al.*, 1991), and in Nicotiana hybrid resistance to several diseases has been linked to high levels of peroxidase (Goy *et al.*, 1992). Some peroxidase isoforms have also been associated with disease resistance. In maize, resistance to northern leaf blight was associated to an specific isoform (Bar-Zur *et al.* 1998) and resistance to *Exesrohilium turcicum* was correlated to peroxidase banding patterns (Shimoni *et al.* 1996). Collectively these results indicate that increased peroxidase activity and expression of specific isoenzymes may be a constitutive mechanism used by plants against severe, pathogen infection. In our study the zymogram pattern of the peroxidase isoenzymes showed polymorphism but did not allow the group separation of non-inoculated susceptible and resistant inbred lines (data not shown).

Effect of MDMV infection on peroxidase activity and expression of its isoenzymes in maize inbred lines resistant or susceptible to MDMV

Peroxidase activity did not increase significantly ($P>0.05$) after the carborundum treatment (Table 2) but increased significantly ($P<0.01$) in the R and S inbred lines after inoculation with MDMV as indicated by ANOVA. These observations indicate that the presence of the virus was responsible for the increase in peroxidase activity in susceptible and resistant maize inbred lines. The significant ($P<0.01$) difference (S vs. R groups) showed that resistant and susceptible lines to MDMV gave different patterns in peroxidase activity related to treatments. In the virus-inoculated treatment, the resistant inbred lines on average exhibited more peroxidase activity than the susceptible lines, although this difference was not significant (Table 2). Although, in this study there was an increase in peroxidase activity in the susceptible inbred lines after inoculation, it would not be sufficient to stop the virus spreading. These results are in agreement with the inoculation of tobacco leaves with tobacco mosaic virus (TMV) leading to an increase in peroxidase activity (Ye *et al.* 1990), inoculation of okra with yellow vein mosaic virus (Ahmed *et al.* 1992), and inoculation of maize with *E. turcicum* (Shimoni *et al.* 1996). These studies all show that increased peroxidase activity occur in both resistant and susceptible genotypes. These observations suggest that this enzyme may play a role in plant defense against MDMV. Peroxidase is involved in the final steps of lignin biosynthesis and reinforcement of the cell wall, and is frequently mentioned as a mechanism of resistance (Vance *et al.* 1980). Peroxidase activity

TABLE 2. Mean peroxidase activity levels in virus-inoculated maize seedling inbred lines susceptible (S) or resistant (R) to MDMV.

Treatment	Peroxidase activity (A_{470} min^{-1} g FW^{-1})		
	S	R	Means
Non-inoculation	49.9	49.9	49.9 b
Carborundum	48.2	54.6	51.4 b
Inoculation	57.9	62.4	60.2 a

CV = 8.84%. Means within column followed by the same letter are not significantly different by the Tukey test at $P<05$. The treatmentes were: non-inoculation, carborundum and inoculation with MDMV. Data are the means of three replications.

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also increases locally and systematically in plants infected with various pathogens (Candela *et al.* 1994; Ye *et al.* 1990). In the present study, the peroxidase isoforms were not induced or repressed by the carborundum or inoculation treatments, as the zymograms in the susceptible (Fig. 1A) and resistant (Fig. 1B) lines, didn't show any qualitative changes in peroxidase isoenzyme patterns among treatments.

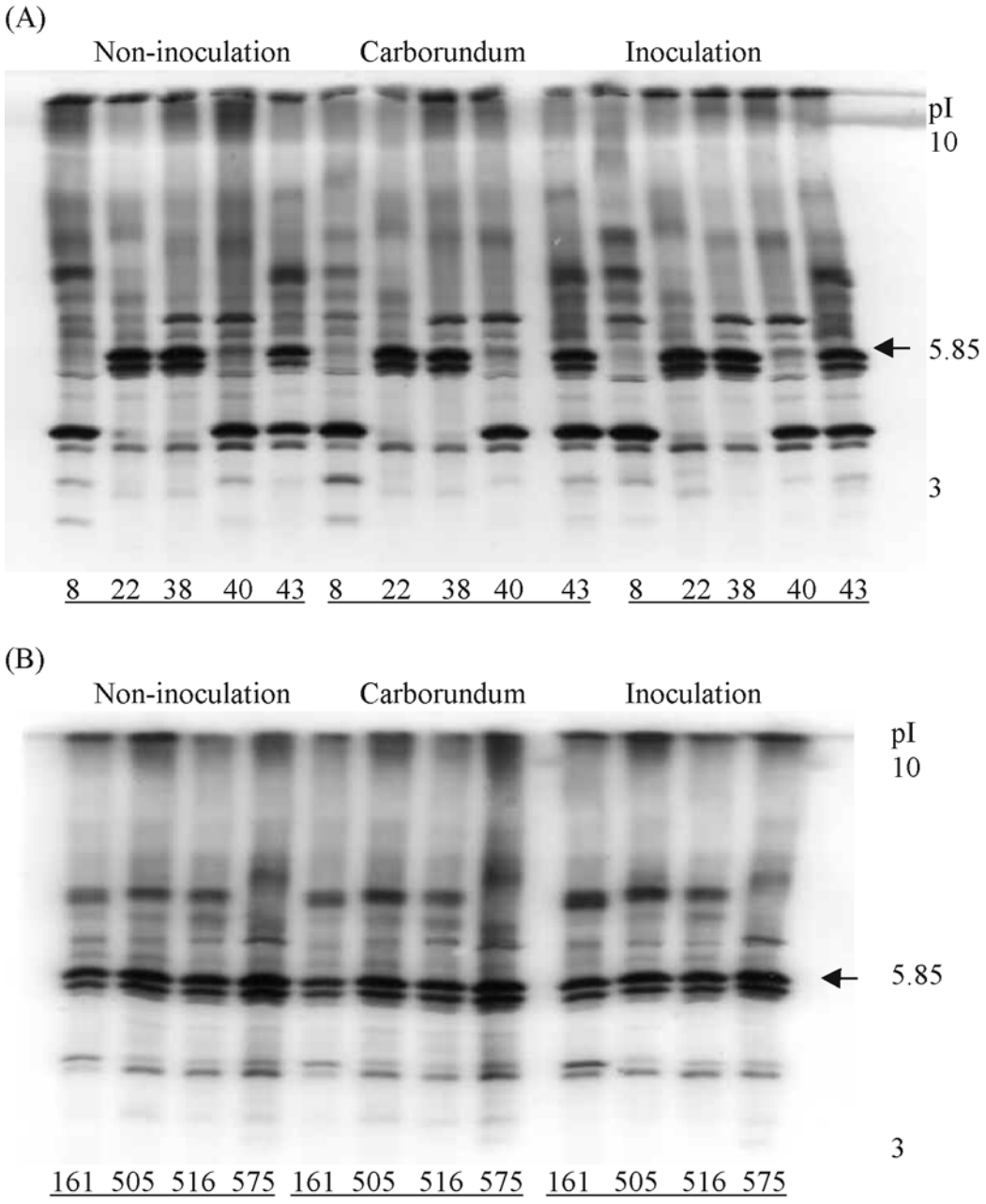

FIGURE 1. Zymogram of peroxidase isoenzymes from maize seedling inbred lines susceptible (A) or resistant (B) to MDMV submitted to three different treatments: non-inoculation, carborundum and inoculation with MDMV. Arrow shows an anionic isoform (pI 5.85) that in resistant inbred lines showed an 19% average increase in OD peak in response to virus inoculation.

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However, a constitutive anionic peroxidase isoform (pI 5.85) from some resistant inbred lines (Fig. 1B) showed an 19% average increase on OD peak due to virus inoculation. Moderately anionic isozymes has been shown to have high affinity for the cell wall, with both ionic and covalent interactions (Birecha and Miller, 1974). An enhanced peroxidase activity is probably important in the reinforcement of the cell wall to prevent the virus from spreading.

Conclusions

Our results seem to indicate that, in some resistant inbred lines, peroxidase activity prior to infection, and increase in activity of a specific anionic isoform, due to virus inoculation, could be related to a defense mechanism against to MDMV.

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