Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

Analysis of ribonuclease and peroxidase activities during maize (*Zea mays*) response to *Meloidogyne arenaria* infection

Arnika Przybylska*

Department of Molecular Biology and Biotechnology, Institute of Plant Protection – National Research Institute, Poznań, Poland

Vol. 61, No. 4: 371–376, 2021

DOI: 10.24425/jppr.2021.139245

Received: April 30, 2021
Accepted: July 19, 2021
Online publication: December 20, 2021

*Corresponding address:
a.przybylska@iorpib.poznan.pl

Responsible Editor:
Natasza Borodynko-Filas

Abstract

Meloidogyne arenaria belongs to root-knot nematodes (RKNs) which constitute a group of highly polyphagous nematodes causing serious damages to many crop varieties. Maize (*Zea mays*) is one of its main hosts. During plant response to RKN infection, many mechanisms are involved. Pathogenesis-related proteins (PRs), which present many functions and enzymatic activities, such as ribonucleases (RNases), antioxidative enzymes, or proteases are involved in these processes. The aim of this study was to describe changes in peroxidase and RNase activities induced in *Z. mays* during its response to *M. arenaria* infection. Moreover, proteins potentially responsible for peroxidase activity were indicated. RNase and peroxidase activities were tested on proteins extracted from roots of healthy plants, *M. arenaria* infected plants, and healthy plants mixed with *M. arenaria* juveniles, in native polyacrylamide (PAA) gels. Samples were collected from two varieties of maize at four time points. A selected fraction showing peroxidase activity was excised from the gel and analyzed using mass spectrometry (MS) to determine protein factors responsible for enzymatic activity. As a result, the analyzed varieties showed slight differences in their RNase and peroxidase activities. Higher activity was observed in the Tasty Sweet variety than in the Waza variety. There were no significant differences between healthy and infected plants in RNase activities at all time points. This was in contrast to peroxidase activity, which was the highest in *M. arenaria*-infected plants 15 days after inoculation. On the basis of protein identification in excised gel fractions using MS it can be assumed that mainly peroxidase 12 is responsible for the observed peroxidase activity. Moreover, peroxidase activity may be presented by glutathione-S-transferase as well.

Keywords: glutathione-S-transferase, *Meloidogyne arenaria,* peroxidase activity, plant-RKN interactions, ribonuclease activity, root-knot nematodes

Introduction

Plant-parasitic nematodes from the genus *Meloidogyne* spp. constitute a group of the most serious plant pests causing huge damages in crop production. They have a very wide host range, including maize (*Zea mays* L.), an important nutritional and economical crop. Root-knot nematodes (RKNs) can infect over 3,000 plant species (Elling 2013). The losses caused by nematodes result mainly from the damage of roots, root galls, and general underdevelopment of the host. The peanut root-knot nematode (*Meloidogyne arenaria*) is a pest of major food crops and is widely distributed throughout the world in tropical, subtropical, and temperate climates (Eisenback and Triantaphyllou 1991).

Root-knot nematode infection results in the wounding of plant tissue, particularly during the migration phase but also during the expansion of feeding sites (Holbein *et al.* 2016). Changes in gene expression associated with wound and defense responses have been observed in many plant-nematode interactions (Gheysen and Fenoll 2002; Przybylska and

Obrępalska-Stęplowska 2020). One of the protein groups involved in plant response to nematode infection is the pathogenesis-related protein family (PRs). These proteins play an important role in plant defense against pathogenic infection and also in general adaptation to stressful environments (Jain and Khurana 2018). Pathogenesis related proteins belong to one of the biggest families of proteins in plants and have been categorized into 17 families, based on molecular mass, isoelectric point, localization, and biological activity (Jain and Khurana 2018). They have many different functions in organisms, for example, acting as ribonucleases (RNases), antioxidative enzymes, or proteases (Edreva 2005). Their gene expression levels usually change in the early stage of pathogen infection (Przybylska *et al.* 2018). Moreover, Kyndt *et al.* (2012) reported that in the case of root-knot nematode infection, these changes are temporary and silenced immediately after they occur. Among other proteins with enzymatic functions in plant responses, peroxidases (POX) belong to an important enzyme system generating H_2O_2, which is required for structural defense. Moreover, H_2O_2 production may lead to the development of an antimicrobial environment within the apoplast (MlÝčkovß *et al.* 2004). On the other hand, ribonucleases (RNases), RNA-degrading enzymes, play a role in various cellular processes, primarily during RNA metabolism (Singh *et al.* 2020). It has also been suggested that the ribonuclease activity of PR10 proteins may be important for their fungicidal properties and the induction of apoptosis in the hypersensitivity response (Filipenko *et al.* 2013).

This study aimed to describe changes in peroxidase and RNase activities induced in *Zea mays* in the early stages of pathogenesis by *M. arenaria* and to determine when their activation occurs. Two varieties of maize were compared.

Materials and Methods

Materials

Biological material used in this study included two susceptible maize varieties, Tasty Sweet and Waza, as well as a population of *M. arenaria* species. Nematode larvae were propagated on tobacco (*Nicotiana tabacum* L.) plants and extracted from roots using NaOCl assay (Hussey 1973).

Growing conditions and sample collection

Plants were grown from seeds under greenhouse conditions at 25°C day/20°C night. The 3–4-week old seedlings of maize plants at the 4–5 leaf stage were inoculated with J2 larvae of *M. arenaria*. For each plant, around 1,500 individuals suspended in water were placed in holes in the soil near the root system. Root samples were collected at four time points: 24 h, 3 days, 7 days, and 15 days after inoculation. For each time point, samples from four plants were taken. Galls were observed and counted 2 months after nematode inoculation.

Enzymatic activity assays

All assays were conducted in four replicates on *M. arenaria* infected plants as well as four replicates on healthy plants, and four replicates on healthy plants mixed with *M. arenaria* juveniles as a control, to exclude the possibility of wounding affecting enzymatic activities. Total proteins were isolated from root samples under native conditions as follows: roots (or cut parts of roots mixed with juveniles immediately before protein extraction) were ground in liquid nitrogen, suspended in extraction buffer (20% glycerol in 1 M Tris-Cl pH 7.5), and centrifuged at 15,000 rpm at 4°C for 15 min. Then, the supernatant containing the protein extract was used in enzymatic activity assays. The protein concentration in the supernatant was determined using the Bradford assay (Bradford 1976). To allow for a quantitative comparison, the same amount of proteins were loaded on each line of a gel – 10 µg.

The RNase and peroxidase activities were tested in native polyacrylamide (PAA) gels according to the protocol described by Blank *et al.* (1982), and Christensen *et al.* (1998), respectively. Selected fractions showing peroxidase activity were excised from gels and analyzed using mass spectrometry (MS) to determine protein factors responsible for enzymatic activity. In the first step, protein hydrolyzate was separated by liquid chromatography (LC) and subsequently peptide masses were determined with a mass spectrometer (Orbitrap, Thermo Fisher Scientific). The MS assay was conducted in the Mass Spectrometry Lab in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Obtained results were analyzed using MASCOT software against *Viridiplantae* database.

Results

Enzymatic activity assays were performed on root samples from two varieties of maize, Waza and Tasty Sweet. In the two analyzed varieties, a similar number of root galls (Fig. 1) was found which suggests their similar susceptibility to *M. arenaria* infection.

In the RNase activity (Fig. 2) as well as peroxidase activity assays (Fig. 3) we observed slight differences between analyzed varieties in the plant response to

Fig. 1. Morphological symptoms of *Meloidogyne arenaria* infection of two varieties of maize: Waza and Tasty Sweet

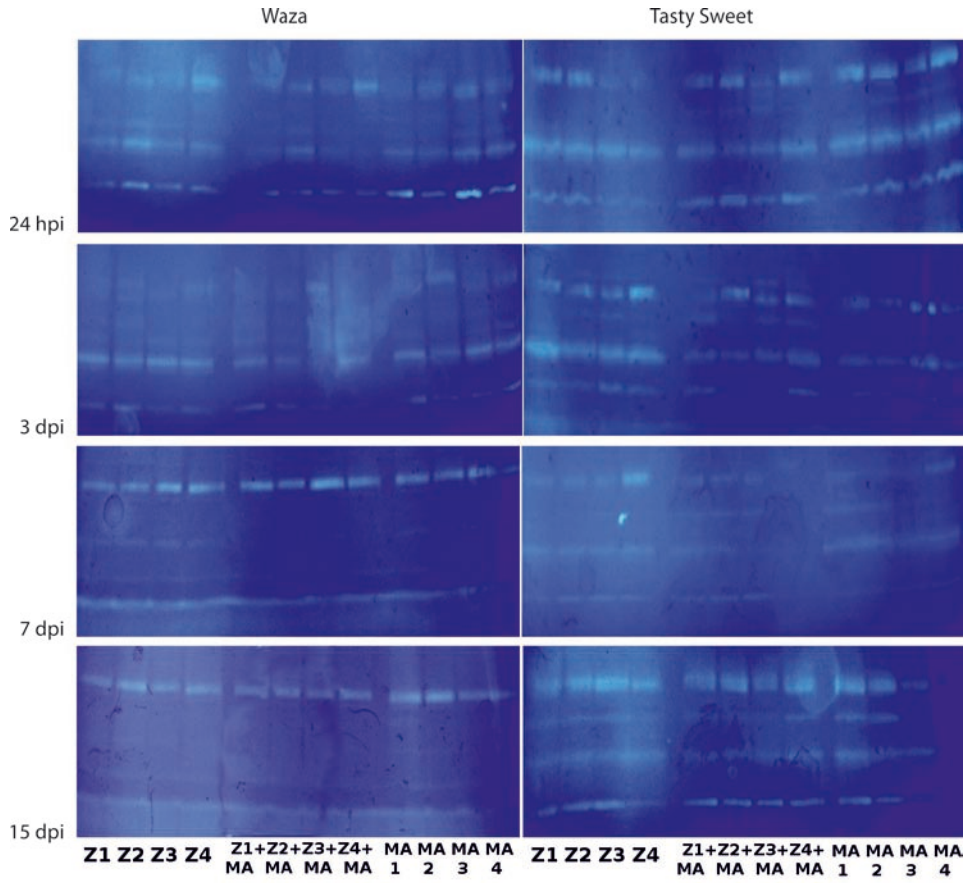

Fig. 2. RNA in-gel activity assay performed on root samples of two varieties of maize and at four time points: 24 hpi – 24 hours, 3 dpi – 3 days, 7 dpi – 7 days, and 15 dpi – 15 days after inoculation, Z1–Z4 – healthy plants, Z+MA–Z4+MA – healthy plants mixed with *Meloidogyne arenaria* juveniles, MA1–MA4 – *M. arenaria* infected plants

nematode infection. Higher activity was observed in Tasty Sweet than in Waza. There were no significant differences in RNase activity between healthy and infected plants at all time points analyzed. On the other hand, higher peroxidase activity was observed in *M. arenaria*-infected plants 15 days after inoculation than at other time points, in contrast to samples derived from healthy plants and healthy plants mixed with *M. arenaria* juveniles. Moreover, in the Tasty Sweet variety, some additional activity was observed. Therefore, for this time point, gel fractions containing proteins with peroxidase activity, significantly differentiating *M. arenaria*-infected plants from healthy ones from the Tasty Sweet variety were excised to MS analysis. The results are shown in the Figure 3.

As a result of protein identification for excised gel fractions using mass spectrometry, sequences of about 170 peptides were obtained. All proteins assigned to *Zea mays* species, with a score higher than 70 are listed in Table 1. From identified proteins, peroxidase 12

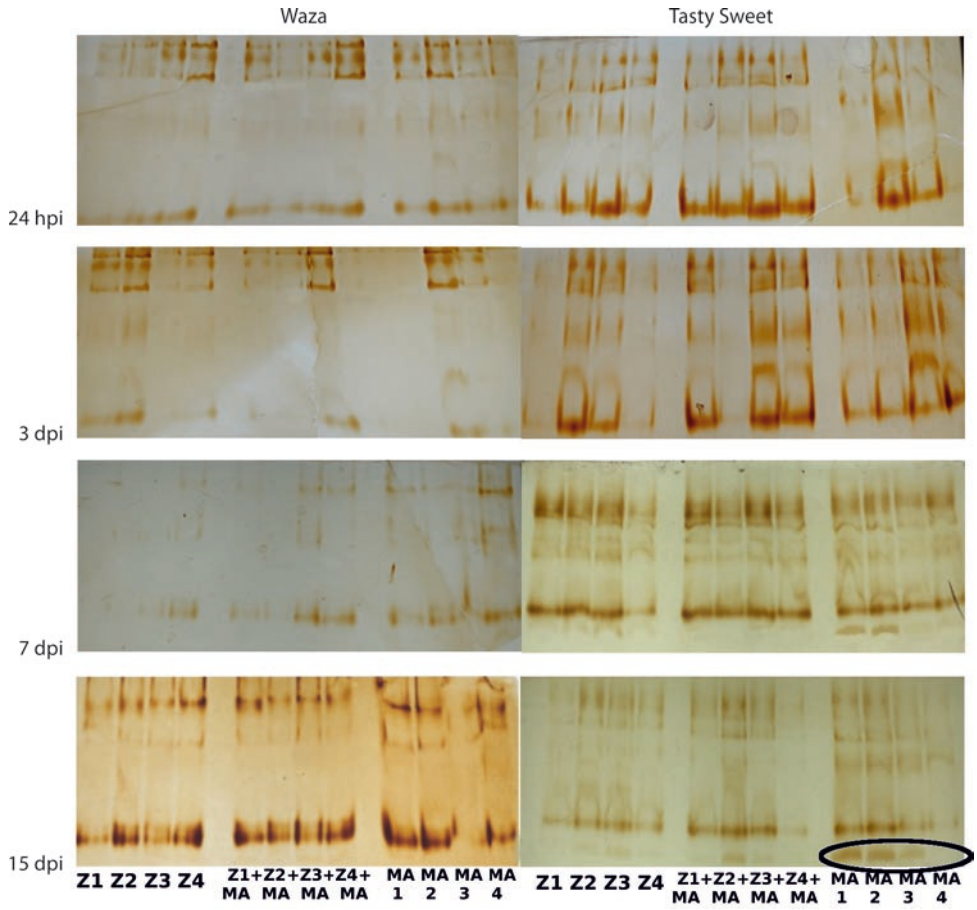

Fig. 3. Peroxidase in-gel activity assay on root samples for two varieties of maize and four time points; 24 hpi – 24 hours, 3 dpi – 3 days, 7 dpi – 7 days, and 15 dpi – 15 days after inoculation, Z1–Z4 – healthy plants, Z1+MA–Z4+MA – healthy plants mixed with *Meloidogyne arenaria* juveniles, MA1–MA4 – *M. arenaria* infected plants. Fractions selected for analysis using the mass spectrometry approach are marked

Table 1. Selected proteins identified for *Zea mays* species in mass spectrometry analysis in the sample excised from polyacrylamide (PAA) gel fraction expressing the peroxidase activity, and their accession numbers and score values

Protein name	Accession number	Score
14-3-3-like protein GF14-12	Q01526	233
hypothetical protein precursor	NP001168118	216
O-methyltransferase	AGS49192	193
putative actin family protein	NP001159156	178
40S ribosomal protein S27a	NP001148860	155
uncharacterized protein LOC100384761	NP001170690	109
peroxidase 12	PWZ38964	108
legumin-like protein	NP001105062	106
cytoplasmic malate dehydrogenase	ACD02021	99
fructokinase-2	NP001105211	99
methionine synthase	AAL33589	97
glutathione S-transferase, C-terminal-like	ACG32999	90
ATP synthase subunit beta, mitochondrial precursor	AFW79055	83
alpha-tubulin	AAF79147	83
hypothetical protein precursor	NP001142312	77
ATPase subunit 1	YP588408	75
fructokinase-1	ACG44849	75
proteasome subunit alpha type	AFW65669	73
12-oxo-phytodienoic acid reductase 2	ACG42962	71

(accession number: PWZ38964) with a score of 108 was selected as a potential protein factor responsible for peroxidase activity. Moreover, glutathione-S-transferase (accession number: ACG32999) with a score of 90 might be responsible for peroxidase activity as well.

Discussion

Many proteins are involved in plant response to RKN infection, with PR proteins as the most important group. Among proteins with peroxidase and RNase activity, some PRs can be found, but also many other plant proteins can exhibit those functions (Bariola and Green 1997; Hiraga *et al.* 2001). It was found that members of the PR9 class may be characterized by peroxidase activity while proteins from two families, PR4 and PR10, can exhibit RNase activity (Filipenko *et al.* 2013; Jain and Khurana 2018). Previously, some changes in RNase activity in dicotyledonous hosts during RKN pathogenesis, e.g., cowpea infected by *M. incognita* (Mahantheshwara *et al.* 2019), were reported. However, in contrast to results obtained during this study, no significant changes in RNase activity profiles between healthy and infected plants were observed. This result suggests that during compatible interactions between *M. arenaria* and its monocotyledonous host, namely maize, no additional ribonuclease activity is induced.

The role of enzymes with peroxidase activity in the RKN infection process and host resistance was described previously but most studies were conducted on dicotyledonous hosts e.g., tomato, cowpea, or chickpea infected by *M. incognita* (Bajaj *et al.* 1985; Mohanty *et al.* 1986; Siddiqui and Husain 1992). Results obtained for tomato and chickpea indicated a positive correlation between the degree of resistance to nematodes and an increase of peroxidase activity (Bajaj *et al.* 1985; Siddiqui and Husain 1992), while during cowpea response to *M. incognita* infection, an increase of peroxidase activity level was observed in both sensitive and tolerant varieties (Mohanty *et al.* 1986). In our study, we found that the highest peroxidase activity was observed at 15 days post inoculation (dpi) in plants infected with nematodes, in contrast to healthy plants and healthy plants mixed with *M. arenaria* juveniles. Moreover, we identified the presence of almost 170 peptides in gel fraction that showed peroxidase activity, from which it was assumed that mainly peroxidase 12 is responsible for this activity. Moreover, glutathione-S-transferase was also shown to possess peroxidase activity (Bartling *et al.* 1993). Glutathione-S-transferases constitute a diverse protein family playing a role in herbicide detoxification, signal transduction, or plant protection against ozone damages, heavy metals, and xenobiotics (Mohsenzadeh *et al.* 2011). In the compatible interaction between *M. incognita* and tomato analyzed previously (Veronico *et al.* 2018), the peroxidase activity was found to be increased after nematode penetration, especially at 7 dpi. However, later stages of infection were not analyzed (Veronico *et al.* 2018). This data partially corresponds to results from our previous study on the expression level of genes encoding proteins involved in maize response to *M. arenaria* infection, where an increase of the expression of gene encoding peroxidase in samples derived from roots of a susceptible maize variety at 7 dpi was described (Przybylska *et al.* 2018). On the other hand, the expression level of the gene encoding glutathione-S-transferase was at the highest level at 3 dpi during compatible interactions (Przybylska *et al.* 2018). Results obtained in this study may indicate the important role of peroxidase, glutathione-S--transferase, and other proteins with peroxidase activity in maize response to *M. arenaria* infection.

Acknowledgements

This study was supported by the Polish National Scientific Center grant: 2014/13/N/NZ9/00703.

I wish to thank Prof. Aleksandra Obrępalska-Stęplowska from IPP – INR for her valuable help and suggestions during preparation of the manuscript.

References

Bajaj K., Singh P., Mahajan R. 1985. Changes induced by *Meloidogyne incognita* in superoxide dismutase, peroxidase and polyphenol oxidase activity in tomato roots. Biochemie und Physiologie der Pflanzen 180: 543–546. DOI: https://doi.org/10.1016/S0015-3796(85)80102-5

Bariola P.A., Green P.J. 1997. Plant ribonucleases. p. 163–190. In: "Ribonucleases: Structures and Functions" (G. D'Alessio, J.F. Riordan, eds). Academic Press, USA. DOI: https://doi.org/10.1016/B978-012588945-2/50006-6

Bartling D., Radzio R., Steiner U., Weiler E.W. 1993. A glutathione S-transferase with glutathione-peroxidase activity from *Arabidopsis thaliana*: Molecular cloning and functional characterization. European Journal of Biochemistry 216: 579–586. DOI: https://doi.org/ 10.1111/j.1432-1033.1993.tb18177.x

Blank A., Sugiyama R., Dekker C.A. 1982. Activity staining of nucleolytic enzymes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis: use of aqueous isopropanol to remove detergent from gels. Analytical Biochemistry 120: 267–275. DOI: https://doi.org/10.1016/0003-2697-(82)90347-5

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254. DOI: https://doi.org/10.1016/0003-2697-(76)90527-3

Christensen J.H., Bauw G., Welinder K.G., Van Montagu M., Boerjan W. 1998. Purification and characterization of peroxidases correlated with lignification in poplar xylem. Plant Physiology 118: 125–135. DOI: https://doi.org/10.1104/pp.118.1.125

Edreva A. 2005. Pathogenesis-related proteins: research progress in the last 15 years. General and Applied Plant Physiology 31: 105–124.

Eisenback J.D., Triantaphyllou H.H. 1991. Root-knot nematodes: *Meloidogyne* species and races. p. 191–274. In: "Manual of Agricultural Nematology" (W.R. Nickle, ed.). CRC Press, USA. DOI: https://doi.org/10.1201/9781003066576

Elling A.A. 2013. Major emerging problems with minor *Meloidogyne* species. Phytopathology 103: 1092–1102. DOI: https://doi.org/10.1094/PHYTO-01-13-0019-RVW

Filipenko E., Kochetov A., Kanayama Y., Malinovsky V., Shumny V. 2013. PR-proteins with ribonuclease activity and plant resistance against pathogenic fungi. Russian Journal of Genetics: Applied Research 3: 474–480. DOI: https://doi.org/10.1134/S2079059713060026

Gheysen G., Fenoll C. 2002. Gene expression in nematode feeding sites. Annual Review of Phytopathology 40: 191–219. DOI: https://doi.org/10.1146/annurev.phyto.40.121201.093719

Hiraga S., Sasaki K., Ito H., Ohashi Y., Matsui H. 2001. A large family of class III plant peroxidases. Plant and Cell Physiology 42: 462–468. DOI: https://doi.org/10.1093/pcp/pce061

Holbein J., Grundler F.M., Siddique S. 2016. Plant basal resistance to nematodes: an update. Journal of Experimental Botany 67: 2049–2061. DOI: https://doi.org/10.1093/jxb/erw005

Hussey R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57: 1025–1028.

Jain D., Khurana J.P. 2018. Role of pathogenesis-related (PR) proteins in plant defense mechanism. p. 265–281. In: "Molecular Aspects of Plant-Pathogen Interaction." (A. Singh, I.K. Singh, eds.). Springer, Singapore. DOI: https://doi.org/10.1007/978-981-10-7371-7

Kyndt T., Nahar K., Haegeman A., De Vleesschauwer D., Höfte M., Gheysen G. 2012. Comparing systemic defence-related gene expression changes upon migratory and sedentary nematode attack in rice. Plant Biology 14: 73–82. DOI: https://doi.org/10.1111/j.1438-8677.2011.00524.x

Mahantheshwara B., Nayak D., Patra M.K. 2019. Protein estimation through biochemical analysis in resistant and susceptible cultivars of cowpea against infection by root-knot nematode, *Meloidogyne incognita*. Journal of Entomology and Zoology Studies 7 (4): 1191–1193.

MlÝčkovß K., Luhovß L., Lebeda A., Mieslerovß B., Peč P. 2004. Reactive oxygen species generation and peroxidase activity during *Oidium neolycopersici* infection on *Lycopersicon* species. Plant Physiology and Biochemistry 42: 753–761. DOI: https://doi.org/10.1016/j.plaphy.2004.07.007

Mohanty K., Ganguly A., Dasgupta D. 1986. Development of peroxidase (EC 1.11. 1.7) activities in susceptible and resistant cultivars of cowpea inoculated with the root-knot nematode, *Meloidogyne incognita*. Indian Journal of Nematology 16: 252–256.

Mohsenzadeh S., Esmaeili M., Moosavi F., Shahrtash M., Saffari B., Mohabatkar H. 2011. Plant glutathione S-transferase classification, structure and evolution. African Journal of Biotechnology 10: 8160–8165. DOI: https://doi.org/10.5897/AJB11.1024

Przybylska A., Kornobis F., Obrępalska-Stęplowska A. 2018. Analysis of defense gene expression changes in susceptible and tolerant cultivars of maize (*Zea mays*) upon *Meloidogyne arenaria* infection. Physiological and Molecular Plant Pathology 103: 78–83. DOI: https://doi.org/10.1016/j.pmpp.2018.05.005

Przybylska A., Obrępalska-Stęplowska A. 2020. Plant defense responses in monocotyledonous and dicotyledonous host plants during root-knot nematode infection. Plant and Soil 451: 239–260. DOI: https://doi.org/10.1007/s11104-020-04533-0

Siddiqui Z., Husain S. 1992. Response of twenty chickpea cultivars to *Meloidogyne incognita* race 3. Nematologia Mediterranea 20: 33–36.

Singh N.K., Paz E., Kutsher Y., Reuveni M., Lers A. 2020. Tomato T2 ribonuclease LE is involved in the response to pathogens. Molecular Plant Pathology 21: 895–906. DOI: https://doi.org/10.1111/mpp.12928

Veronico P., Paciolla C., Pomar F., De Leonardis S., García-Ulloa A., Melillo M.T. 2018. Changes in lignin biosynthesis and monomer composition in response to benzothiadiazole and root-knot nematode *Meloidogyne incognita* infection in tomato. Journal of Plant Physiology 230: 40–50. DOI: https://doi.org/10.1016/j.jplph.2018.07.013