



Antioxidant Enzymatic Activity of Extracts from Hairy Roots of Root-Lesion-Nematode-Susceptible and -Resistant Cultivars of *Medicago sativa* [†]

Gonçalo Pereira ¹, Cláudia Vicente ²  and Jorge M. S. Faria ^{1,3,*} 

¹ INIAV, Instituto Nacional de Investigação Agrária e Veterinária, Quinta do Marquês, 2780-159 Oeiras, Portugal; goncalo.pereira@iniav.pt

² MED, Mediterranean Institute for Agriculture, Environment and Development & CHANGE—Global Change and Sustainability Institute, Institute for Advanced Studies and Research, Évora University, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal; cvicente@uevora.pt

³ GREEN-IT Bioresources for Sustainability, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB NOVA), Av. da República, 2780-157 Oeiras, Portugal

* Correspondence: fariajms@gmail.com

[†] Presented at the 4th International Electronic Conference on Agronomy, 2–5 December 2024; Available online: <https://sciforum.net/event/IECAG2024>.

Abstract

Diseases caused by phytoparasitic nematodes are still a heavy constraint on modern farming, causing losses in crop yields as well as increased production costs due to pest management. Root-lesion nematodes (RLNs) are soil-dwelling migratory endoparasites that infect the roots of several crop species. RLNs feed and reproduce in the cortical cells of affected plant roots typically characterized by development of necrotic spots. Injuries to plant tissues result in weakened plants that become more prone to attack from opportunistic pathogens. In alfalfa (*Medicago sativa* L.), resistance to *Pratylenchus penetrans* has been linked to increased transcription of key enzymes in the biosynthesis of phenylpropanoids, important molecules for countering oxidative stress. However, the mechanisms of resistance are still unknown. The present work analyzed indicators of oxidative stress in extracts from transgenic roots of susceptible (cv. Baker) and resistant (cv. MNGRN-16) alfalfa. On extracts of susceptible alfalfa transgenic roots, levels of lipid peroxidation were more than three times higher after seven and fourteen days of growth, while activity of guaiacol peroxidase (GPX) was approximately four times higher after fourteen and twenty-one days of growth, in comparison to the resistant cultivar. This suggests that resistance response may be dependent on plant redox state. Future work will focus on metabolomic characterization of these varieties in contact with RLNs.

Keywords: alfalfa; peroxidase; *Pratylenchus penetrans*; resistance; susceptibility



Academic Editor: José David Flores-Félix

Published: 24 September 2025

Citation: Pereira, G.; Vicente, C.; Faria, J.M.S. Antioxidant Enzymatic Activity of Extracts from Hairy Roots of Root-Lesion-Nematode-Susceptible and -Resistant Cultivars of *Medicago sativa*. *Biol. Life Sci. Forum* **2025**, *41*, 13. <https://doi.org/10.3390/blsf2025041013>

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Root-lesion nematodes (RLNs) (*Pratylenchus* (Nematoda: *Pratylenchidae*)) are among the most agriculturally significant phytoparasitic nematodes worldwide. RLNs are migratory endoparasites. This means they remain vermiform throughout their life cycle, and can move in and out of plant roots to the surrounding rhizosphere. Their life cycle involves six stages: egg, four juvenile stages, and adult form. Unlike sedentary phytoparasitic nematodes, all stages are mobile, including juveniles and adults, and can penetrate and infect plant roots. Inside roots, RLNs feed on cortical cells and vascular tissue, while in

soil they feed on epidermal cells and root hairs [1]. The progression of RLN infection in the host's root system is typically characterized by the formation of necrotic lesions on the root tissue, leading to impaired water and nutrient uptake, stunted growth, wilting, and reduced yield [2]. RLN reproduction and movement within the root tissue results in enlargement of these necrotic lesions, making the plant more susceptible to infection by other opportunistic pathogens [3,4].

RLNs are distributed globally, being able to thrive in diverse climatic conditions, from temperate to tropical regions. Over 100 species have been identified, with *P. penetrans*, *P. thornei*, and *P. coffeae* being among those most notoriously damaging to crops [5]. Their broad host range and adaptability to various soil types make them particularly challenging to manage in either annual or perennial crop systems. Economic losses due to RLN infection can be substantial, often reaching 30–50% in susceptible crops under favorable conditions [6,7]. In alfalfa (*Medicago sativa* L.) resistance to RLN infection has been linked to upregulated transcription of genes for key enzymes in the biosynthesis of phenolic compounds [8,9], e.g., isoflavonoids [10], that play critical roles in plant defense by mitigating oxidative stress and limiting the accumulation of reactive oxygen species (ROS) [11–13]. Despite this, the redox mechanisms of resistance against RLN infection remain largely unknown.

Our study aimed to provide preliminary insights into the mechanism of resistance utilized by resistant alfalfa through an evaluation of antioxidant responses from in vitro hairy roots of two alfalfa cultivars, one susceptible to RLN infection, the other resistant. To this end, we analyzed the content of thiobarbituric acid-reactive substances (TBARS), a proxy for lipid peroxidation, and the activity of guaiacol peroxidase antioxidant enzyme, in extracts of these two cultivars. Oxidative stress markers and the enzymes used to prevent excess oxidative stress damage were utilized to develop a more comprehensive understanding of the mechanisms of resistance utilized by resistant alfalfa to mitigate RLN infection.

2. Materials and Methods

2.1. Chemicals

For determination of thiobarbituric acid-reactive substances (TBARS), trichloroacetic acid (99%), thiobarbituric acid (98%), and butylated hydroxytoluene (99%) were used. For determination of guaiacol peroxidase enzyme activity, di-potassium hydrogen phosphate (98%), potassium dihydrogen phosphate (99%), hydrogen peroxide (30%), and guaiacol (99%) were employed. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Growth of Plant Material and Bacteria

Seeds from two alfalfa cultivars, cv. Baker (susceptible) and cv. MNGRN-16 (resistant), were kindly supplied by Dr. Paulo Vieira from USDA-ARS. The seeds were washed under running tap water for 5 min, then treated with a common detergent solution (1 drop per 40 mL of water) to remove finer debris. Surface sterilization was performed by immersing the seeds in 80% (*v/v*) ethanol for 5 min with vigorous agitation. The ethanol was then discarded under a laminar flow hood, and the seeds were rinsed three times with sterilized demineralized water. The sterilized seeds were incubated at 25 °C in darkness within sterile Petri dishes containing hydrated filter paper to facilitate germination. After approximately two weeks, hypocotyls were excised for subsequent infection with *Rhizobium rhizogenes*. For hairy root induction, a strain of *R. rhizogenes* harboring the *gus* reporter gene co-integrated in the Ri plasmid and driven by a double 35S promoter (A4pRiA4::70GUS) was employed. Bacterial cultures were initiated by spreading the strain on Luria–Bertani

(LB) solid medium and incubating overnight at 26 °C. A single colony was then inoculated into 10 mL of liquid LB broth (LB medium without agar) within a sterile 50 mL flask and incubated overnight in the dark at 26 °C under agitation (180 r.p.m.). Bacterial growth was monitored spectrophotometrically until optical density at 600 nm (OD₆₀₀) reached 0.6, indicating the exponential growth phase. Bacteria at this stage were used for inoculation of plant hypocotyls.

2.3. Establishment of Alfalfa Transgenic Roots

Alfalfa hairy roots were initiated by infecting hypocotyl segments of germinants of the two alfalfa cultivars with *R. rhizogenes*. Under aseptic conditions, the hypocotyl segments were wounded using a scalpel previously dipped in a diluted bacterial suspension. Briefly, the *R. rhizogenes* culture, at the exponential growth phase, was diluted 1:10 (v/v) in liquid Schenk and Hildebrandt (SH) medium supplemented with 30 g/L sucrose (pH 5.6). Following infection, the segments were briefly dried on sterile filter paper (1 min) and placed on solid SH medium (8 g/L agar). Co-cultivation with the bacteria was conducted for three days, after which the segments were transferred to SH solid medium supplemented with cefotaxime and carbenicillin (150 µg/mL each). The culture medium, including antibiotics, was refreshed weekly for over three months. After this time, the emerging hairy roots were excised and propagated on antibiotic-free SH solid medium. After approximately three months of routine subculture at four-week intervals, root segments were transferred to liquid SH medium and maintained on orbital shakers (60 r.p.m.). Cultures were subcultured every four weeks by aseptically transferring portions of root clumps (ca. 1 g fresh weight) to fresh SH culture medium [14]. Throughout the cultivation period, alfalfa hairy root cultures were maintained in darkness at 25 ± 1 °C. After ca. one year of routine subculture, the hairy roots were used for time-course characterization of root redox status. For the bioassay, 20 mL amounts of liquid SH medium were added to microboxes (8 cm base diameter, 6 cm height) equipped with green filters (XXL+) on the lids (SacO₂, Deinze, Belgium) to facilitate air exchange. A root clump (ca. 1 g fresh weight) was placed into each microbox, and cultures were maintained in darkness at 25 ± 1 °C under continuous agitation (80 r.p.m.). At 7, 14, and 21 days of root growth, hairy roots from three replicate microboxes were collected, washed with phosphate-buffered saline (pH 7.0), blotted dry on filter paper, weighed, flash-frozen, and stored at −80 °C until further analysis.

2.4. Thiobarbituric Acid-Reactive Substance (TBARS) Assay

A TBARS assay was employed to assess oxidative damage by quantifying lipid peroxidation [15]. The previously flash-frozen hairy root samples were ground in liquid nitrogen to a fine powder using a chilled mortar and pestle. Samples of ca. 50 mg weight were each then homogenized in 0.5 mL of 0.1% (w/v) trichloroacetic acid (TCA), the homogenate was centrifuged at 12,000 × g for 10 min at 4 °C, and the resulting supernatant was collected. For the reaction, 50 µL of the supernatant was mixed with 200 µL of assay solution consisting of 20% (w/v) TCA containing 0.65% (w/v) thiobarbituric acid (TBA) and 0.01% (w/v) butylated hydroxytoluene (BHT), to prevent further lipid oxidation. The control assay solution had the same composition but without TBA. Three replicates of hairy root extracts were used for bioassay and control tests. Each mixture was incubated in a thermoblock at 95 °C for 25 min, then rapidly cooled on ice. After centrifugation at 5000 × g for 10 min, the absorbance of the clear supernatant was measured at 440, 532, and 600 nm using a Thermo Scientific™ Multiskan™ Microplate Spectrophotometer (Waltham, MA, USA).

Malondialdehyde (MDA) equivalents, representing lipid peroxidation levels, were calculated using the following formulas sequentially:

$$A = (A_{532\text{bioassay}} - A_{600\text{bioassay}}) - (A_{532\text{control}} - A_{600\text{control}}) \quad (1)$$

$$B = 0.0571 \times (A_{440_{\text{bioassay}}} - A_{600_{\text{bioassay}}}) \quad (2)$$

$$\text{MDA equivalents (nmol/mL)} = 10^6 \times ((A - B)/157,000) \quad (3)$$

MDA equivalents were expressed as nmol MDA per gram of root fresh weight (nmol MDA g⁻¹ FW).

2.5. Guaiacol Peroxidase Activity

Guaiacol peroxidase (GPX) activity was determined spectrophotometrically [16]. The previously flash-frozen hairy root samples were ground in liquid nitrogen to a fine powder using a chilled mortar and pestle. Samples of ca. 50 mg weight were each then homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 7.0), the homogenate was centrifuged at 12,000× g for 20 min at 4 °C, and the resulting supernatant was collected. Protein content of the supernatant was determined with the Bradford reagent method, using a bovine serum albumin (BSA) calibration curve [17]. For GPX, 10 µL of a 1:10 dilution of the supernatant was mixed with 190 µL of the reaction mixture, containing 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM guaiacol, and 10 mM hydrogen peroxide, and immediately measured at 470 nm using a Thermo Scientific™ Multiskan™ Microplate Spectrophotometer for 5 min. The increase in polymerized guaiacol (tetraguaiacol) due to enzyme activity was determined using an extinction coefficient of 26.6 mM⁻¹ cm⁻¹. Enzyme activity was expressed as µmol tetraguaiacol s⁻¹ per µg protein.

2.6. Data Treatment and Statistical Analysis

Statistical processing was performed with version 26 of SPSS Statistics software (IBM, New York, NY, USA). Statistical significance of the data was determined with one-way ANOVA, and individual means were compared using Tukey's post hoc test, with $p < 0.05$. The Shapiro–Wilk test was used to ensure data normality, and the Brown–Forsythe test was used to assess homoscedasticity. The results were presented as average and standard error values of three replicates.

3. Results and Discussion

3.1. Susceptible and Resistant Alfalfa Hairy Roots

Transgenic roots of alfalfa were obtained from the hypocotyls of seedlings at 2–3 weeks after germination. No substantial differences in transgenic root growth were observed between the cultivars susceptible (cv. Baker) (Figure 1a,c) and resistant (cv. MNGRN-16) to RLNs (Figure 1b,d) after 4 weeks of growth in SH semi-solid medium.

3.2. Thiobarbituric Acid-Reactive Substances (TBARS) and Activity of Guaiacol Peroxidase

Transgenic roots of both cultivars were sampled after 7, 14 and 21 days of growth in liquid SH medium to investigate the time-course evolution of oxidative stress markers. Oxidative damage, as evaluated through the TBARS method (which provides information on lipid peroxidation and membrane damage), was higher in hairy roots of the susceptible cv. Baker after 7 and 14 days of culture, suggesting an increased oxidative pressure. However, after 21 days there was no substantial difference in oxidative damage between the susceptible cv. Baker and the resistant cv. MNGRN-16 (Figure 2).

Likewise, GPX activity was higher in the susceptible cultivar (cv. Baker) when compared to the resistant cv. MNGRN-16 (Figure 3). However, substantial differences were observed only after 14 and 21 days of culture growth, not after 7 days. The higher GPX activity in cv. Baker suggests a more intense oxidative response.

The use of transgenic roots allowed a finer control over environmental and genetic variability. The two cultivars showed contrasting biochemical profiles, a result which

underscores fundamental differences in oxidative stress management. The susceptible cultivar seems to have, early on, a heightened oxidative stress state, showing 3-fold-higher lipid peroxidation levels. Contrarily, the resistant cultivar seems to have lower lipid peroxidation levels (except after 21 days, when these in vitro hairy roots were in their stationary growth stage), and lower GPX activity levels (after 1 week of growth), suggesting a more effective antioxidant response of the resistant MNGRN-16 alfalfa cultivar. This may be due to non-enzymatic antioxidant compounds such as the isoflavonoid phytoalexins that have previously been determined to be higher in this cultivar [8].

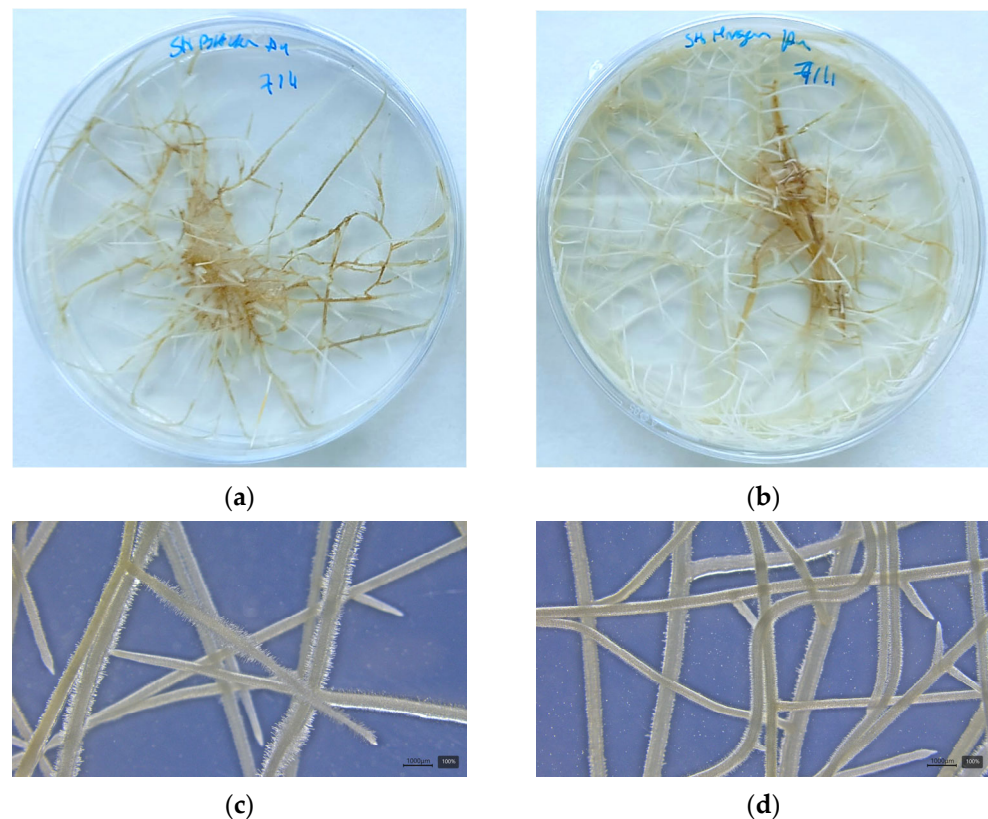


Figure 1. Examples of in vitro-grown alfalfa transgenic roots from susceptible (cv. Baker) (a) and resistant (cv. MNGRN-16) (b) cultivars after 4 weeks of growth in semi-solid culture medium. Microscopic details (bar = 1000 μ m) reveal no substantial morphological differences between susceptible (c) and resistant (d) alfalfa cultivar roots.

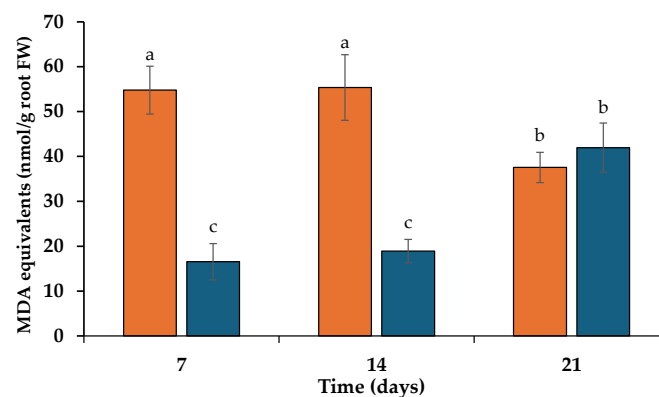


Figure 2. Malondialdehyde (MDA) equivalents, representing lipid peroxidation levels, determined through thiobarbituric acid-reactive substance (TBARS) assays on extracts of susceptible (cv. Baker) (orange columns) and resistant (cv. MNGRN-16) (blue columns) in vitro-grown alfalfa transgenic roots. Different letters indicate statistically significant differences ($p < 0.05$) on the basis of Tukey's test.

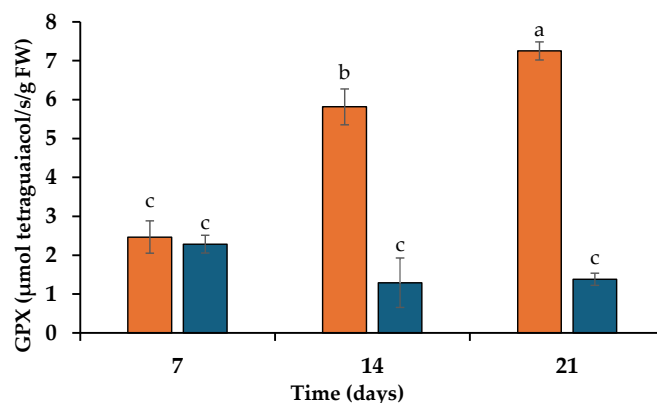


Figure 3. Activity of guaiacol peroxidase (measured as μmol of tetraguaiacol produced per s per μg of protein) on extracts of susceptible (cv. Baker) (orange columns) and resistant (cv. MNGRN-16) (blue columns) in vitro-grown alfalfa transgenic roots. Different letters indicate statistically significant differences ($p < 0.05$) on the basis of Tukey's test.

Future work will focus on metabolomic profiling to identify and quantify specific phytoalexins and other redox-active metabolites in these genotypes. Such data will help elucidate the integrated network of enzymatic and non-enzymatic defenses that confer resistance to RLNs in alfalfa.

Author Contributions: Conceptualization, J.M.S.F.; methodology, J.M.S.F.; software, J.M.S.F.; formal analysis, G.P. and J.M.S.F.; investigation, G.P. and J.M.S.F.; resources, C.V. and J.M.S.F.; data curation, J.M.S.F.; writing—original draft preparation, J.M.S.F.; writing—review and editing, G.P., C.V. and J.M.S.F.; funding acquisition, C.V. and J.M.S.F. All authors have read and agreed to the published version of the manuscript.

Funding: Partly funded by Fundação para a Ciência e a Tecnologia (FCT/MCTES) through projects ref. 2022.00359.CEECIND, <https://doi.org/10.54499/2022.00359.CEECIND/CP1737/CT0002> (NemAct, J.M.S.F.); ref. CEECIND/00040/2018, <https://doi.org/10.54499/CEECIND/00040/2018/CP1560/CT0001> (C.S.L.V.); and structural funding ref. LA/P/0121/2020, <https://doi.org/10.54499/LA/P/0121/2020> (CHANGE); ref. UIDB/05183/2020, <https://doi.org/10.54499/UIDB/05183/2020> (MED); and GreenIT (<https://doi.org/10.54499/UIDB/04551/2020> and <https://doi.org/10.54499/UIDP/04551/2020>).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data is available from the corresponding author (Jorge M. S. Faria) upon reasonable request.

Acknowledgments: The authors wish to thank Paulo Vieira from USDA-ARS for technical support.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Castillo, P.; Vovlas, N. *Pratylenchus (Nematoda: Pratylenchidae): Diagnosis, Biology, Pathogenicity and Management*; BRILL: Leiden, The Netherlands, 2007; Volume 6, ISBN 9789004155640.
- Jones, J.T.; Haegeman, A.; Danchin, E.G.J.; Gaur, H.S.; Helder, J.; Jones, M.G.K.; Kikuchi, T.; Manzanilla-López, R.; Palomares-Rius, J.E.; Wesemael, W.M.L.; et al. Top 10 Plant-Parasitic Nematodes in Molecular Plant Pathology. *Mol. Plant Pathol.* **2013**, *14*, 946–961. [[CrossRef](#)] [[PubMed](#)]
- Viketoft, M.; Flöhr, A.; Englund, J.-E.; Kardell, J.; Edin, E. Additive Effect of the Root-Lesion Nematode *Pratylenchus Penetrans* and the Fungus *Rhizoctonia Solani* on Potato Yield and Damage. *J. Plant Dis. Prot.* **2020**, *127*, 821–829. [[CrossRef](#)]
- MacGuidwin, A.E.; Rouse, D.I. Role of *Pratylenchus Penetrans* in the Potato Early Dying Disease of Russet Burbank Potato. *Phytopathology* **1990**, *80*, 1077. [[CrossRef](#)]

5. Fatemi, E.; Jung, C. Pathogenicity of the Root Lesion Nematode *Pratylenchus Neglectus* Depends on Pre-Culture Conditions. *Sci. Rep.* **2023**, *13*, 19642. [[CrossRef](#)] [[PubMed](#)]
6. Jones, M.G.K.; Fosu-Nyarko, J. Molecular Biology of Root Lesion Nematodes (*Pratylenchus* spp.) and Their Interaction with Host Plants. *Ann. Appl. Biol.* **2014**, *164*, 163–181. [[CrossRef](#)]
7. Nicol, J.M.; Turner, S.J.; Coyne, D.L.; Nijs, L.d.; Hockland, S.; Maafi, Z.T. Current Nematode Threats to World Agriculture. In *Genomics and Molecular Genetics of Plant-Nematode Interactions*; Jones, J.T., Gheysen, G., Fenoll, C., Eds.; Springer: Dordrecht, The Netherlands, 2011; pp. 21–43.
8. Vieira, P.; Mowery, J.; Eisenback, J.D.; Shao, J.; Nemchinov, L.G. Cellular and Transcriptional Responses of Resistant and Susceptible Cultivars of Alfalfa to the Root Lesion Nematode, *Pratylenchus Penetrans*. *Front. Plant Sci.* **2019**, *10*, 971. [[CrossRef](#)]
9. Hafez, S.L.; Sundararaj, P.; Miller, D. Reaction of Alfalfa Cultivars to the Root Lesion Nematode *Pratylenchus Penetrans*. *Nematol. Mediterr.* **2006**, *34*, 161–163.
10. Baldrige, G.D.; O'Neill, N.R.; Samac, D.A. Alfalfa (*Medicago sativa* L.) Resistance to the Root-Lesion Nematode, *Pratylenchus Penetrans*: Defense-Response Gene MRNA and Isoflavonoid Phytoalexin Levels in Roots. *Plant Mol. Biol.* **1998**, *38*, 999–1010. [[CrossRef](#)] [[PubMed](#)]
11. Wang, L.; Li, C.; Luo, K. Biosynthesis and Metabolic Engineering of Isoflavonoids in Model Plants and Crops: A Review. *Front. Plant Sci.* **2024**, *15*, 1384091. [[CrossRef](#)]
12. Polturak, G.; Misra, R.C.; El-Demerdash, A.; Owen, C.; Steed, A.; McDonald, H.P.; Wang, J.; Saalbach, G.; Martins, C.; Chartrain, L.; et al. Discovery of Isoflavone Phytoalexins in Wheat Reveals an Alternative Route to Isoflavonoid Biosynthesis. *Nat. Commun.* **2023**, *14*, 6977. [[CrossRef](#)]
13. Edens, R.M.; Anand, S.C.; Bolla, R.I. Enzymes of the Phenylpropanoid Pathway in Soybean Infected with *Meloidogyne Incognita* or *Heterodera Glycines*. *J. Nematol.* **1995**, *27*, 292–303. [[PubMed](#)]
14. Faria, J.M.S.; Barbosa, P.; Figueiredo, A.C.; Mota, M.; Vicente, C.S.L. In Vivo and In Vitro Infection of Potato Roots with Plant Parasitic Nematodes for the Assessment of Induced Structural Changes. *JoVE* **2025**, *216*, e67756. [[CrossRef](#)] [[PubMed](#)]
15. Hodges, D.M.; DeLong, J.M.; Forney, C.F.; Prange, R.K. Improving the Thiobarbituric Acid-Reactive-Substances Assay for Estimating Lipid Peroxidation in Plant Tissues Containing Anthocyanin and Other Interfering Compounds. *Planta* **1999**, *207*, 604–611. [[CrossRef](#)]
16. Aravind, P.; Prasad, M.N.V. Zinc Alleviates Cadmium-Induced Oxidative Stress in *Ceratophyllum demersum* L.: A Free Floating Freshwater Macrophyte. *Plant Physiol. Biochem.* **2003**, *41*, 391–397. [[CrossRef](#)]
17. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.