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ORIGINAL ARTICLE

Role of Extracellular Hydrolytic and Ligninolytic Enzymes as Virulence Factors of the *Dematophora necatrix* in the Activation of Basal Resistance of Walnut

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K E Y W O R D S

Basal resistance;
Defence responses;
Phenolics;
Soil-borne;
Virulence

A B S T R A C T

Dematophora necatrix is one of the most destructive soil-borne fungal pathogens causing white root rot disease on walnuts in Iran and worldwide. No walnut rootstock genotypes have shown complete resistance to this disease. This study aims to identify and monitor the prevalence of *D. necatrix* in traditional and commercial walnut orchards as well as to explore extracellular the hydrolytic and ligninolytic enzymes produced by the fungal isolates plus variations in virulence and aggressiveness *in vivo*. We also examined the role of biochemical mechanisms, including the activities of oxidative enzymes and defensive compounds involved in the basal resistance of walnuts to the necrotrophic *D. necatrix*. In this study, we investigated the presence of the causal agent of white rot disease in root and soil samples collected from commercial and traditional gardens in 17 regions of Iran. Then, we analysed the relationship between the activity of extracellular hydrolytic and ligninolytic enzymes produced by *D. necatrix* isolates together with variations in pathogenicity and aggressiveness *in vivo*. Next, the activity of guaiacol peroxidase (GPX), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), and level of defensive compounds were studied at various time points after inoculation by different *D. necatrix* isolates. All fungal isolates were identified as *D. necatrix* based on their morphological and molecular characteristics. Analysis of the activity of extracellular hydrolytic and ligninolytic enzymes produced by the fungal isolates revealed a higher activity of these enzymes for isolates presenting higher levels of virulence and aggressiveness in walnut seedlings. The current study suggests the critical role of GPX, PPO, and PAL in walnut resistance against *D. necatrix*, which was associated with lignification and phenolic production. These results provide novel information about the role of metabolism of phenolic compounds in basal resistance of walnut plant after inoculation by *D. necatrix*, where the level of defence response depends on the level of pathogenicity of isolates.

Introduction

The Persian walnut (*Juglans regia* L.) is one of the main nut crops with high nutritional and therapeutic values (Aryapak and Ziarati, 2014; Pakrah *et al*., 2021; Jahanbani *et al*., 2018 and 2021; Akca and Sahin, 2022). Following pistachio, walnut is the second most important nut crop in terms of production in Iran and vast researches has been done on this nut tree in this country (Farsi *et al*., 2018; Vahdati *et al.*,

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2019; Hassani *et al*., 2020). In 2022, the production of walnuts (in-shell) worldwide was approximately 3,874 thousand tons, with Iran ranking third after producing 355 tons in a cultivated area of around 52 thousand hectares (FAOSTAT, 2023). *Dematophora necatrix* R. Hartig is a prevalent soil-borne plant pathogenic fungus causing white root rot disease, which is destructive to numerous plant species, including walnut (Pasini *et al*., 2016). This pathogen spreads through infested soil and root-to-root by hyphae and rhizomorphs (Ruano-Rosa *et al*., 2007; Arjona-López *et al*., 2020). Understanding the defence mechanisms activated in the pathosystem of walnut and D. necatrix is necessary to adopt effective disease control strategies (Williamson-Benavides and Dhingra, 2021).

Plants activate different mechanisms to adapt to biotic stresses by changing signal transmission pathways (Hosseini-Zahani and Taheri, 2023). In response to oxidative stress, plants produce enzymatic antioxidants such as guaiacol peroxidase (GPX), as well as non-enzymatic antioxidants such as flavonoids and phenolics to scavenge for different types of reactive oxygen species (ROS) and protect the host (Kuvalekar *et al*., 2011). The enzyme polyphenol oxidase (PPO) can act as an antioxidant by oxidizing phenolic compounds to quinones (Araji *et al*., 2014). It can also act as a pro-oxidant by promoting ROS production (Boeckx *et al*., 2015).

Phenolic compounds and flavonoids are secondary metabolites with multiple functions, such as antioxidant, antifungal, and antimicrobial properties, which are involved in many vital processes of plant growth and development by inducing resistance against different stresses (Khodadadi *et al.*, 2016; Behdad *et al*., 2020). Most of the antioxidant capacity in walnuts results from phenolic compounds (Arranz *et al*., 2008; Jariteh *et al.*, 2011; Sarikhani *et al*., 2021; Amin Ghezel *et al*., 2022). The level and type of plant secondary metabolites change due to pathogen infection with variations in the activities of defense enzymes (Kuvalekar *et al*., 2011). The GPX acts as a potent scavenger of H_2O_2 and participates in oxidizing phenolic compounds, including lignin-derived phenolics (Nowogórska and Patykowski, 2015). Phenylalanine ammonia-lyase (PAL) plays a crucial role as an essential enzyme in the biosynthesis of lignin and phenolic compounds through the phenylpropanoid metabolism pathway (Rasool *et al*., 2021). Increased PAL and PPO activity enhances the biosynthesis of secondary metabolites in plant tissues, thereby assisting plants in inhibiting pathogen infection (Solekha *et al*., 2020; Kuvalekar *et al*., 2011).

Fungal pathogens, belonging to the genus *Dematophora*, do not have specialized structures to penetrate plant cells (Kubicek *et al*., 2014) and directly penetrate the epidermal cell walls with short infection hyphae (Pliego *et al*., 2009). *D. necatrix* produces various extracellular hydrolytic enzymes, such as cellulase, xylanase, and pectinase, during the infection process of avocado roots (Kim *et al*., 2017). The root rot pathogen of *D. necatrix* wields a versatile arsenal of enzymes via which it directly attacks the lignin barrier (Di Lella *et al*., 2022). White root rot fungi produce the most abundant ligninolytic enzymes, including laccase, manganese, and lignin peroxidase (Urbanek *et al*., 2018). The cell wall degrading enzymes are unable to degrade lignin on their own and help the lignin degradation by providing H_2O_2 for the ligninolytic enzymes and reduce the generated intermediates (Chan *et al*., 2020). Increased activity of extracellular enzymes secreted by plant pathogens is directly linked to increased virulence and invasion of the host plant (Gawade *et al*., 2017).

To our knowledge, there is no information on the role of defence mechanisms in the basal resistance of walnut to *D. necatrix*. Further, we have yet to investigate the comparative role of extracellular hydrolytic and ligninolytic enzymes produced by *D. necatrix* isolates and their association with pathogenicity plus aggressiveness. Thus, the objectives of this research were to: (i) determine the

health status of commercial and traditional orchards to white root rot disease, (ii) evaluate the correlation between various extracellular hydrolytic and ligninolytic enzymes as virulence factors of the *D. necatrix* and their role in the appearance plus progression of disease symptoms in walnut plants, and (iii) investigate changes in defence-related enzyme activities (GPX, PPO, and PAL) as well as levels of defensive compounds (total phenolic, flavonoid and lignin contents) as a part of defence biochemical mechanisms involved in this pathosystem.

Materials and Methods

Sample screening and collection

During the 2023-24 growing season, a total of 71 walnut plants with and without foliage yellowing and wilting symptoms throughout the entire plant or in just a portion of the canopy and fine white mycelia layer on collar and/or root were sampled from commercial and traditional orchards in different regions of Iran (Table 1). For all plants from which root samples were collected, a soil sample (about 1 kg) was also taken by mixing different sub-samples of soil from the rhizosphere at a depth of 5-30 cm. Then, the samples were individually placed in paper bags and transported in an ice box to the laboratory at the Seed and Plant Certification and Registration Institute (SPCRI) of Iran to isolate putative pathogens.

Fungal isolation and morphological identification

For isolation of fungal isolates from the collected root samples, we cut small sections of root tissue (approximately 1 × 1 cm), surface-sterilized in 2% sodium hypochlorite solution for 15 minutes, washed in sterile distilled water and left to air dry (Castro *et al*., 2013). These small sections were placed in Petri plates containing potato dextrose agar (PDA) amended with lactic acid (0.2%, v/v). Isolates from soil were obtained by the serial dilution plating method (Waksman, 1922). One gram of each soil sample was diluted in 9 mL of sterile distilled water. Then, 1 mL was taken from dilution and transferred to the second test tube. The dilutions continued until the 4th tube, and finally 0.5 mL from 4th tube was spread in Petri dishes containing PDA modified with lactic acid (0.2%, v/v) (Arjona-López *et al*., 2019). PDA plates were incubated at 25 ± 1 °C in dark for 10 days. *Dematophora* spp. colonies were identified initially under the light microscope (Olympus BX51) by the presence of the typical hyphal pear-shaped swellings near the septa (Pliego *et al*., 2012; Pérez-Jiménez, 2006).

DNA extraction, PCR amplification, and molecular identification

Total genomic DNA was extracted using the Genomic DNA isolation kit (Pishgam Biotech, Iran). Validation of morphological features of fungal isolates at the identified species level was performed using the species-specific primers of *D. necatrix* (forward: 5'-CAA AAC CCA TGT GAA CAT ACC A-3'and reverse: 5'-CCG AGG TCA ACC TTT GGT ATA G-3') according to Schena *et al*. (2002). The PCR reaction was performed in a volume of 25 µl, each reaction containing 1 µL of 2.5 µM every reverse and forward primers, 12.5 µL of PCR Master Mix (CinnaGen, Iran), and 3 µL of sample DNA and 7.5 µL PCR grade water. The PCR program involved a pre-incubation step for 5 min at 94 °C followed by 40 cycles of 45 s at 94 °C, 45 s at 60 °C, 60 s at 72 °C and final extension for 5 min at 72 °C. Amplified products were separated via gel electrophoresis in a 1.5% agarose and visualized with GelRed staining on a UV gel documentation system (Syngene Gene Flash Bio, USA). Negative control with molecular grade water instead of template DNA and positive control containing DNA of *D. necatrix* in PCR reaction mix were included with all PCR runs.

Plant materials

Persian walnut Damavand (Iranian and local

walnut cultivar) was used for virulence tests. 'Damavand' is a widespread walnut genotype planted in many walnut-growing regions of Iran because of its promising growth and high yield under Iran's climatic conditions. Walnut seedlings were planted individually in each of the 15 cm diameter (× 11 cm height) plastic pots containing sterilized mixture of peat moss, perlite, and field soil at 1: 1: 1 (v/v/v). The plants were grown under greenhouse conditions at 30 ± 4 °C; with a 16/8h light/dark photoperiod; 50 ± 5 % relative humidity; and irrigated when needed.

Virulence test on walnut plants

D. necatrix inoculum preparation

Fungal inoculum was prepared on autoclaved wheat grains as described by Freeman *et al*. (1986). In each pot, we carried out artificial inoculation using 30.0 g of colonized grains per approximately 1.0 kg of substrate soil. To ensure the spread of the inoculum, we placed it across four points distributed around the basal stem and root crown (~ 2.5 cm apart) and introduced it at two depths (~5 cm and ~ 10 cm, respectively). The sterilized wheat grains without the fungus served as the control.

Assessment of aggressiveness

To confirm Koch's postulates, fungal isolates were tested for virulence on walnut plants under controlled conditions. In the experimental design, four replicate walnut trees were planted for each isolate in a completely randomized design and the experiment was repeated twice. Aerial symptoms including chlorosis and defoliation were observed 21 days after inoculation, while control plants remained symptomless. Disease severity was evaluated by measuring the aerial symptoms of white root rot using a 0 (healthy) to 5 (plant died) scale as described Sun *et al*. (2008). Finally, the disease severity index (DSI) was calculated using the following formula (Khaledi *et al*., 2021):

$$DSI\% = [\sum(\text{Number of diseased plants at all levels}) \times (\text{The value of relevant level}) / (\text{Total number of investigated plants}) \times (\text{Highest disease level})] \times 100$$

Aggressiveness, as a quantitative component of virulence, was studied for each fungal isolate on walnut seedlings using the method described by Kleina *et al*. (2019) based on determining hours post-inoculation (HPI) for the onset of disease symptoms.

Extraction and enzymatic analyses

Five mycelial plugs (5 mm diameter) from each fungal culture actively growing on a PDA plate were transferred to 250 mL Erlenmeyer flasks containing 100 mL of sterile in primary culture media of hemicellulase (xylanase), pectinase, cellulose, and ligninolytic activities as described by other researchers (Khaledi and Hassani, 2018; Koroljova-Skorobogat'ko *et al*., 1998). Then, the flasks were incubated at 25 ± 2 °C on a rotating shaker at 120 rpm for 10 days *in vitro*. In order to obtain the crude enzyme solution, we filtered a volume of 5 mL of the medium in the flask at various times through a Whatman No.1 filter paper. Afterward, the obtained filtrate was centrifuged at 10,000 rpm at 4 °C for 20 min and then used the supernatant to determine enzyme activities. Xylanase activity was determined according to the method described by Bailey *et al*. (1992). Pectinase activity was determined according to the method described by Kaur and Gupta (2017), based on the amount of reduced D-galacturonic acid released in the culture supernatant. Cellulase activity was determined according to the method described by Wood and Bhat (1988). Ligninolytic enzymes, such as laccase, lignin peroxidase, and manganese peroxidase, were mainly secreted by white rot fungi, which can effectively degrade lignin. Therefore, we studied the activity of laccase, lignin, and manganese peroxidase enzymes. Laccase activity was determined according to the method described by other researchers (Praveen *et al*., 2011; Das *et al*., 1997; Kuwahara *et al*., 1984).

Determination of defence enzyme activities

The plant aerial parts (500 mg) were ground collected from various treatments [seedling uninoculated, seedling inoculated by *D. necatrix* isolates MU57, RV08, ZA94, LX66, FP35, ND36, BE71, CT17, GD23, SC89 and HO42] into a fine powder with a mortar and pestle under liquid nitrogen, then homogenized in 2 ml of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 2% (w/v) polyvinylpyrrolidone (PVP), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was then centrifuged at 12,000 g and 4 $^{\circ}$C for 15 min to extract the supernatant for enzymatic analysis (Almeida *et al*., 2024). Sampling of root tissues was performed at various time points following inoculation by different *D. necatrix* isolates [1, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days post-inoculation (DPI)]. The method of Bradford (1976) was used to determine the total protein concentration, with bovine serum albumin (Sigma) serving as a standard.

Guaiacol peroxidase (GPX) activity was assessed according to Konieczny *et al*. (2014) and was expressed as μmol tetraguaiacol.min^{-1} mg^{-1} protein. Polyphenol oxidase (PPO) activity was measured according to Mayer *et al*. (1965) and was expressed as μmol.min^{-1} mg^{-1} protein. Phenylalanine ammonia-lyase (PAL) activity was determined based on the rate of cinnamic acid production as described by Ramamoorthy *et al*. (2002) and was expressed as nmol trans-cinnamic acid.min^{-1} mg^{-1} protein.

Determination of total phenolic, total flavonoid, and lignin contents

The total phenolic content was determined using the Folin-Ciocalteu reagent method as described by Wang *et al*. (2022). Total flavonoids and lignin content were determined according to the method of Zhang *et al*. and Suzuki *et al*. (2009). All experiments were performed in triplicate and repeated twice.

Statistical analysis

The obtained data were statistically analysed via SAS software (version 9.2; SAS Institute, Cary, NC, USA) and mean comparison was conducted using the Duncan's multiple range test at the level $P \leq 0.05$.

Results

Morphological and molecular identification of Dematophora isolates

A total of 142 samples including 71 soil samples and 71 root samples from approximately 17 walnut orchards in various regions of Iran (Table 1) were collected for screening the causal agent of white root rot disease (on average 6 to 14 samples per orchard plot). In this study, 11 fungal isolates were recovered from root and soil samples in the studied orchards. Eight isolates were isolated from root sections of walnut trees collected in four traditional orchards (Gorgan, Asadabad, Sahneh, and Chenaran). Three isolates were isolated from soil samples collected in three traditional orchards (Gorgan, Asadabad, and Sanandaj). Among the samples collected from the soil and roots, no *Dematophora* infection was observed and detected in all commercial orchards (Kuhin and Tuyserkan), and ten traditional orchards (Mobarakeh, Urmia, Damavand, Bojnord, Shahrud, Marvdasht, Jiroft, Shahzan, Joveyn, and Azarshahr) from different regions of Iran. The results of this research indicated that four, three, two, one, and one fungal isolates recovered of Gorgan, Asadabad, Chenaran, Sanandaj and Sahneh samples, respectively (Table 1). Among the samples collected from traditional orchards in the studied regions, the Gorgan sample showed the highest frequency of *Dematophora* isolates obtained from the collected soil and root samples (Table 1).

Table 1. Characteristics of soil and root samples based on sampling site and frequency of *Dematophora* isolates identified in traditional and commercial walnut orchards.

Orchard type	Orchard code	Sample site	NDI*	
			Soil sample	Root sample
Commercial orchards	KQ67	Qazvin - Kuhin	ND	ND
		Qazvin - Kuhin	ND	ND
		Qazvin - Kuhin	ND	ND
		Qazvin - Kuhin	ND	ND
		Qazvin - Kuhin	ND	ND
		Qazvin - Kuhin	ND	ND
		Qazvin - Kuhin	ND	ND
	TH91	Hamadan - Tuyserkan	ND	ND
		Hamadan - Tuyserkan	ND	ND
		Hamadan - Tuyserkan	ND	ND
		Hamadan - Tuyserkan	ND	ND
		Hamadan - Tuyserkan	ND	ND
		Hamadan - Tuyserkan	ND	ND
		Hamadan - Tuyserkan	ND	ND
Traditional orchards	SM21	Markazi - Shahzan	ND	ND
		Markazi - Shahzan	ND	ND
		Markazi - Shahzan	ND	ND
	SK23	Kurdistan - Sanandaj	1	ND
		Kurdistan - Sanandaj	ND	ND
		Kurdistan - Sanandaj	ND	ND
	AH18	Hamadan - Asadabad	ND	ND
		Hamadan - Asadabad	ND	2
		Hamadan - Asadabad	ND	ND
		Hamadan - Asadabad	1	ND
		Hamadan - Asadabad	ND	ND
		Hamadan - Asadabad	ND	ND
		Hamadan - Asadabad	ND	ND
	SK04	Kermanshah - Sahneh	ND	1
		Kermanshah - Sahneh	ND	ND
		Kermanshah - Sahneh	ND	ND
	MI96	Isfahan - Mobarakeh	ND	ND
		Isfahan - Mobarakeh	ND	ND
		Isfahan - Mobarakeh	ND	ND
	DT62	Tehran - Damavand	ND	ND
		Tehran - Damavand	ND	ND
		Tehran - Damavand	ND	ND
Traditional orchards	BK92	North Khorasan - Bojnord	ND	ND
		North Khorasan - Bojnord	ND	ND
		North Khorasan - Bojnord	ND	ND
	SS13	Semnan - Shahrud	ND	ND
		Semnan - Shahrud	ND	ND
		Semnan - Shahrud	ND	ND
	MS56	Fars - Marvdasht	ND	ND
		Fars - Marvdasht	ND	ND
		Fars - Marvdasht	ND	ND
	JK35	Kerman - Jiroft	ND	ND
		Kerman - Jiroft	ND	ND
		Kerman - Jiroft	ND	ND
	UA05	West Azerbaijan - Urmia	ND	ND
		West Azerbaijan - Urmia	ND	ND
		West Azerbaijan - Urmia	ND	ND
	JH73	Razavi Khorasan - Joveyn	ND	ND
		Razavi Khorasan - Joveyn	ND	ND
		Razavi Khorasan - Joveyn	ND	ND
	AG44	East Azerbaijan - Azarshahr	ND	ND
		East Azerbaijan - Azarshahr	ND	ND
		East Azerbaijan - Azarshahr	ND	ND
	CK87	Razavi Khorasan - Chenaran	ND	ND
		Razavi Khorasan - Chenaran	ND	ND
		Razavi Khorasan - Chenaran	ND	ND
		Razavi Khorasan - Chenaran	ND	1
		Razavi Khorasan - Chenaran	ND	ND
		Razavi Khorasan - Chenaran	ND	ND
		Razavi Khorasan - Chenaran	ND	1
	GG50	Golestan - Gorgan	ND	1
		Golestan - Gorgan	ND	ND
		Golestan - Gorgan	ND	ND
		Golestan - Gorgan	ND	ND
		Golestan - Gorgan	ND	1
		Golestan - Gorgan	1	1
		Golestan - Gorgan	ND	ND

The morphological characterization revealed that, all fungal isolates were classified only in the *Dematophora* genus. Fungal isolates form white mycelia with pear-shaped swellings near the septa in the hyphae, which is a diagnostic characteristic of *Dematophora necatrix* (Pliego *et al*., 2012; Pérez-Jiménez, 2006). The colony colour of the isolates varied from white to darker grey. The conidia were smooth, one-celled, hyaline, and elliptical shaped, measuring 3.6-4.8 × 1.9-2.2 µm. The conidiophores were branched, erect, brown, 2.2-2.6 µm wide, and more than 400 µm long. After presumptive identification, the fungal isolates were submitted to molecular identification by species-specific PCR. PCR amplification results confirmed that the morphological identification of *D. necatrix* isolates was performed correctly. All fungal isolates were identified by PCR amplification with *D. necatrix*-specific primers R2 and R8, which yielded about 493 bp.

Virulence and aggressiveness assay

The results indicated that various *D. necatrix* isolates on walnut seedlings had various virulence capabilities (Fig. 1a). Based on the mean comparison of disease severity index, all *D. necatrix* isolates were found to be pathogenic or weakly pathogenic. The Koch's postulates were demonstrated by the re-isolation of the same fungus from the diseased roots. The results of virulence tests of different *D. necatrix* isolates on walnut seedlings indicated that the highest and lowest disease index values were recorded for the isolates of MU57 and HO42, respectively (Fig. 1a). The virulence test indicated that the disease index for fungal isolates ranged from 88.5 ± 1.5 to 16.5 ± 2.0 (Fig. 1a).

The data obtained from the aggressiveness test presented that the early development of disease symptoms by isolate MU57 (288 HPI) was comparable to that of the other tested isolates (Fig. 1b). Further, the results indicated that the lowest aggressiveness belongs to the HO42 isolates (420 HPI) (Fig. 1b). The results of the seedling infection tests showed different levels of aggressiveness of the isolates of *D. necatrix*. According to the results, symptoms of root disease appeared after 12 days post-inoculation (288 HPI) in inoculated plants, which died ultimately 86 days (2,068 HPI) after pathogen inoculation.

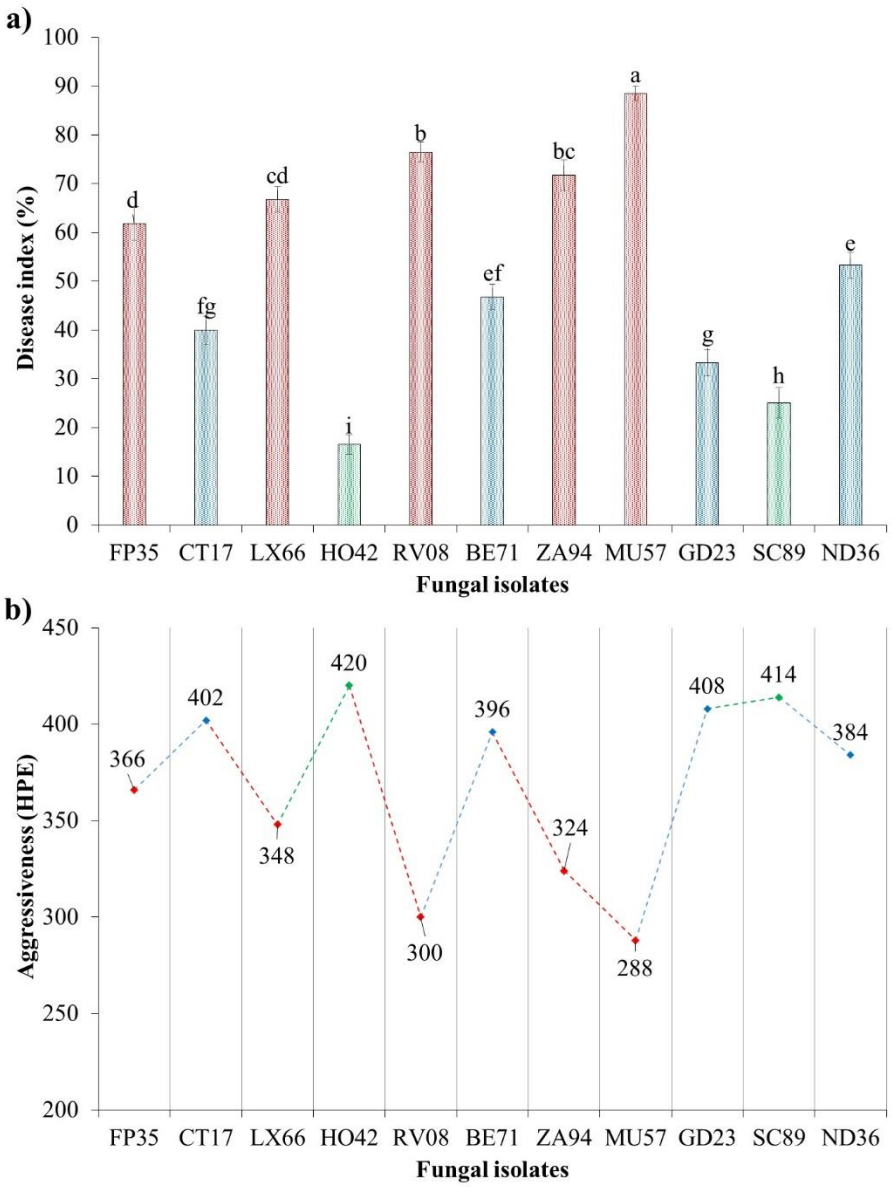

Fig. 1. Virulence (a) and aggressiveness (b) of *Dematophora necatrix* isolates inoculated on seedlings of walnut genotype Damavand.

Analysis of extracellular hydrolytic and ligninolytic enzyme activities

The study of extracellular enzyme activity revealed that different isolates of *D. necatrix* had different levels of enzyme activity *in vitro* (Fig. 2). The enzyme activity levels among the *D. necatrix*

isolates ranged from 355 to 211 µg mL^{-1} for cellulase, from 511 to 322 µg mL^{-1} for xylanase, from 782 to 676 µg mL^{-1} for pectinase, from 77 to 17 µg mL^{-1} for laccase, from 22 to 9 µg mL^{-1} for manganese peroxidase, and 146 to 95 µg mL^{-1} for lignin peroxidase (Fig. 2). The peak time of enzyme activity among *D. necatrix* isolates varied from 72 to 96 hours post-culturing on liquid medium (HPC) for cellulase, from 120 to 144 HPC for xylanase, from 192 to 216 HPC for pectinase, from 168 to 192 HPC for laccase, from 168 to 216 HPC for manganese peroxidase, and from 144 to 168 HPC for lignin peroxidase in liquid medium (Fig. 2). Isolate MU57 had the highest enzymatic activity in vitro compared to the other tested isolates. The isolate HO42 exhibited the lowest levels of extracellular hydrolytic and ligninolytic enzyme activities (Fig. 2).

In general, the highest level of activity was observed for pectinase, followed by xylanase, cellulase, laccase, lignin, and manganese peroxidase. We compared the activity of extracellular enzymes produced by *D. necatrix* isolates to examine a potential association between extracellular enzymes and virulence. Isolates HO42 and MU57, which showed the lowest and highest levels of extracellular hydrolytic and ligninolytic enzyme activities, respectively, presented the lowest and highest levels of pathogenicity in walnut seedlings (Fig. 1, Fig. 2).

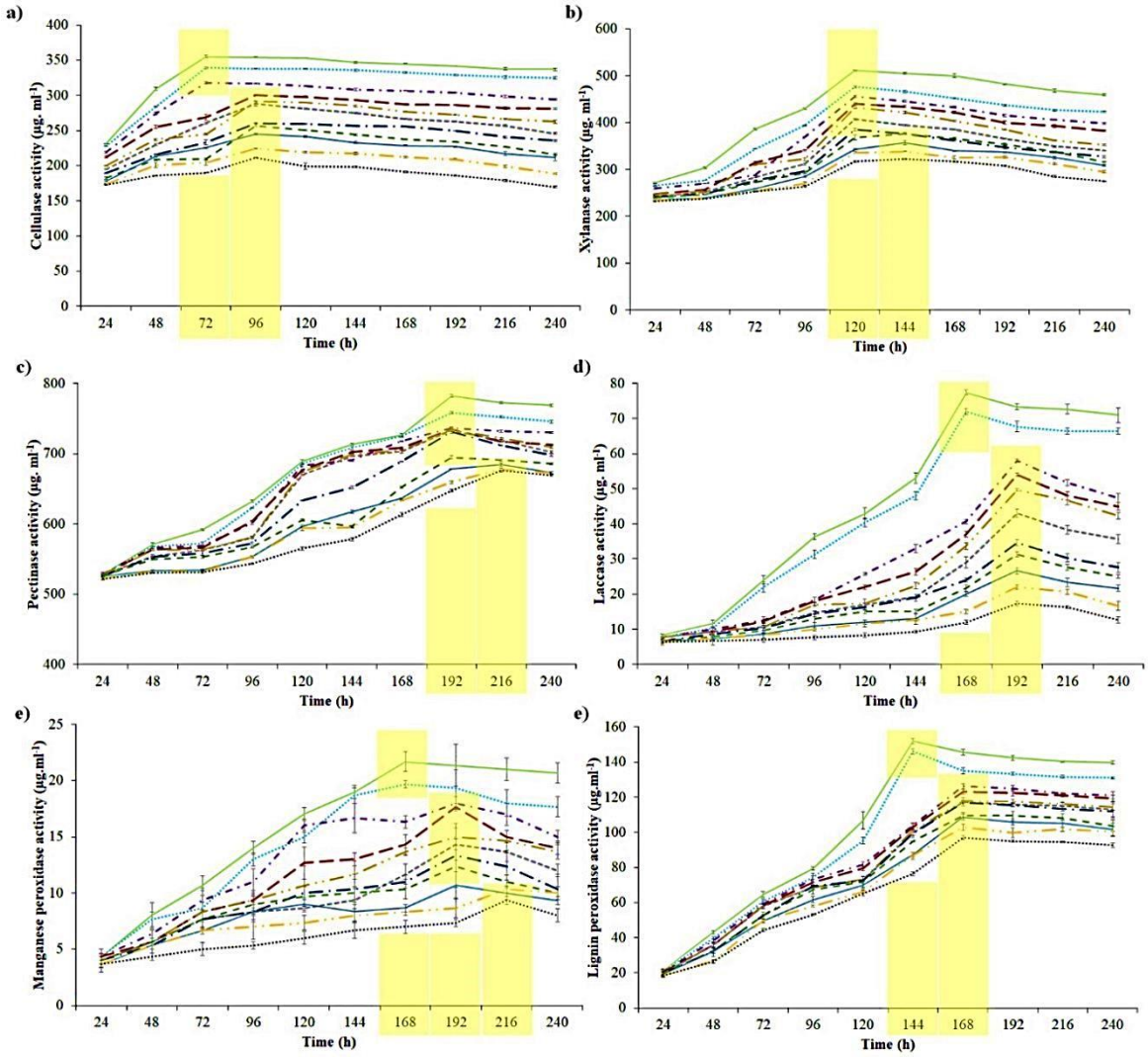

Fig. 2. Analysis of extracellular hydrolytic and ligninolytic enzyme activity produced by *Dematophora necatrix* isolates during a 240-hour incubation period. Cellulase activity (a), xylanase activity (b), pectinase activity (c), laccase activity (d), manganese peroxidase activity (e), lignin peroxidase activity (f). The bars indicate standard errors (SE), Values are means of three replicates. MU57; RV08; ZA94; LX66; FP35; ND36; BE71; CT17; GD23; SC89; HO42

Determination of antioxidant enzymes and phenylalanine ammonia-lyase activities

We examined the antioxidant activity of GPX and PPO to determine the time point of induction and their role in the basal resistance of walnuts against *D. necatrix* (Fig. 3). To study the biochemical defence mechanisms involved in basal resistance, we inspected PAL activity as key markers of the phenylpropanoid pathway. A comparative analysis of GPX, PPO, and PAL activities revealed that all plants inoculated with *D. necatrix* contained significantly higher activity than the uninoculated control at various time points. The highest activities of GPX, PPO, and PAL were detected for walnut plants inoculated with the HO42 isolate at most times studied (Fig. 3). The lowest activities of GPX, PPO, and PAL were observed for plants inoculated with MU57, presenting the highest virulence among the *D. necatrix* isolates used in this research (Fig. 3). GPX and PAL activity increased up to 6 DPI. They dropped up to 12 DPI, followed by an ascending trend in enzyme activity up to 15 DPI and finally, diminished up to 30 DPI (Fig. 3a, Fig. 3c). Plants with different treatments showed peak GPX and PAL activities at 15 DPI. However, the PPO activity continued to grow until nine DPI. It decreased until 15 DPI, followed by an upward trend of enzyme activity until 18 DPI, and finally, it fell until 30 DPI (Fig. 3b). However, in control plants without fungal inoculation, no significant changes were observed in GPX, PPO, and PAL activities and the plant showed a steady state during the period examined (Fig. 3).

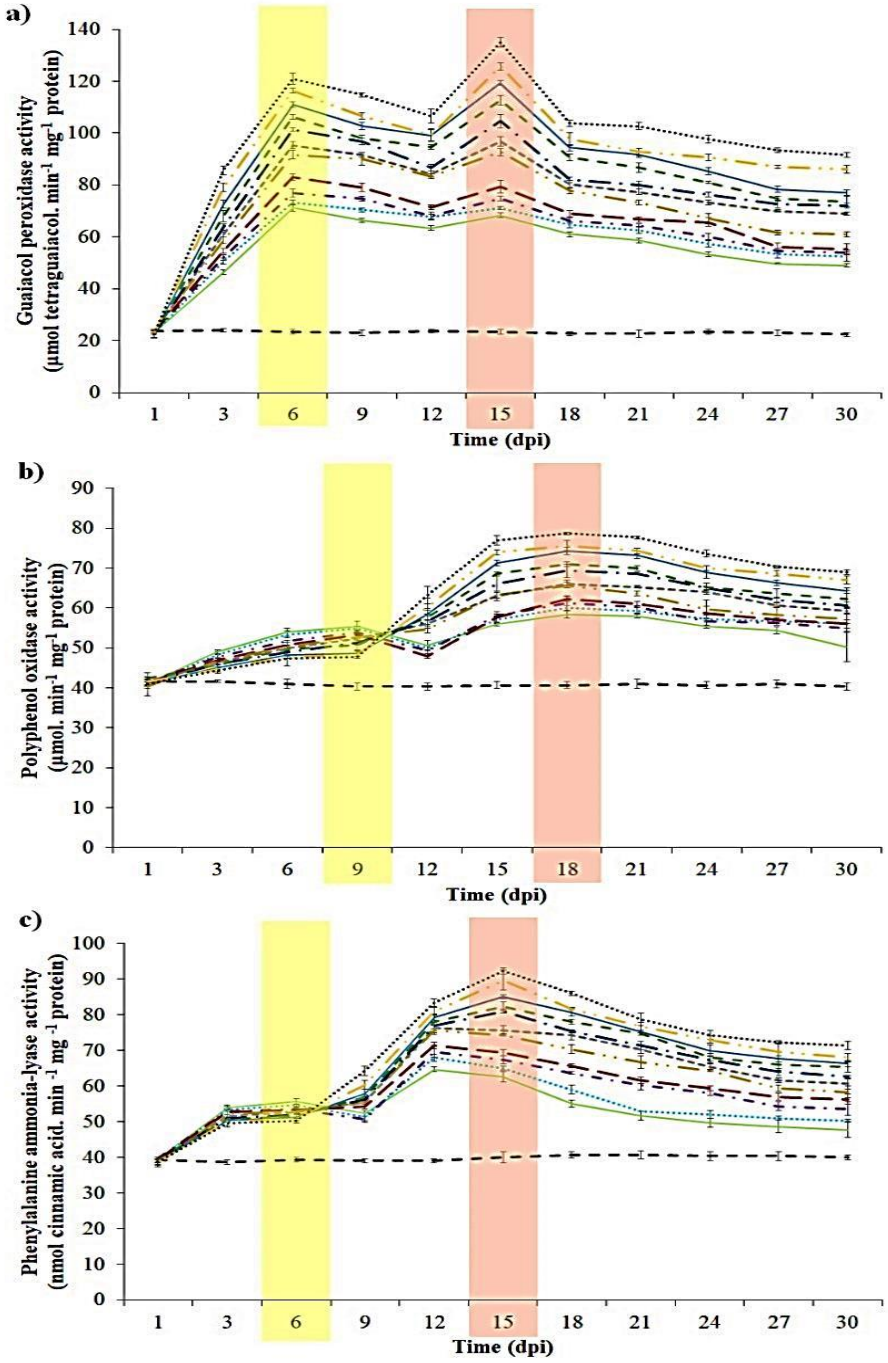

Fig. 3. Analysis of Guaiacol peroxidase (GPX; a), polyphenol oxidase (PPO; b) and phenylalanine ammonia lyase (PAL; c) activities in walnut genotype seedlings at different times after inoculation with *Dematophora necatrix* isolates. Data are means ± standard error. The experiment was repeated twice with similar results. Abbreviations in figure: control seedling uninoculated, seedling inoculated by *D. necatrix* isolates MU57; RV08; ZA94; LX66; FP35; ND36; BE71; CT17; GD23; SC89; HO42

Investigating the accumulation of defensive compounds

Our data indicated a significant difference in total phenolic, flavonoid, and lignin content between the different treatments used in this study (Fig. 4). The accumulation of flavonoid and phenolic compounds significantly increased after inoculating the walnut plants with *D. necatrix*, as compared to the non-

inoculated controls. Significant differences were observed among the walnuts treated with different isolates of *D. necatrix* regarding the relative increase and the timing of maximum accumulation of defensive compounds. The accumulation of defensive compounds increased in response to the walnut-*D. necatrix* interaction. The results demonstrated a significantly higher accumulation of total phenolic, flavonoid, and lignin in walnuts treated with isolate HO42 compared to other treatments. The results revealed that the plant treated with isolate HO42 exhibited increased phenolic and flavonoid contents until six DPI, followed by a decline until nine DPI. Subsequently, there was a rising trend from 9 to 15 DPI, ultimately decreasing to 30 DPI (Fig. 4a, Fig. 4b). The highest level of lignification belonged to the plant treated with isolate HO42 at 21 DPI (Fig. 4c). The accumulation levels of total phenolic, flavonoid, and lignin contents among plants treated with the different *D. necatrix* isolates varied from 563 to 495 mg GAE. g^{-1} DW for phenolic content, 283 to 379 mg QE. g^{-1} DW for flavonoid content, and 62 to 35 mg alkali lignin. g^{-1} DW for lignin content (Fig. 4). The least accumulation of phenolic compounds was observed for plants inoculated with the MU57 isolate, presenting the highest pathogenicity among the *D. necatrix* isolates used in this study. For the walnut treated with the MU57 isolate, the peak of phenolic and flavonoid content was observed at 15 DPI, followed by a descending trend for the rest of the time points tested (Fig. 4a, Fig. 4b). The peak of lignin content was observed at 18 DPI and gradually fell thereafter (Fig. 4c). Plants without fungal inoculation exhibited a consistent level of defensive compounds at the lowest level throughout the study (Fig. 4).

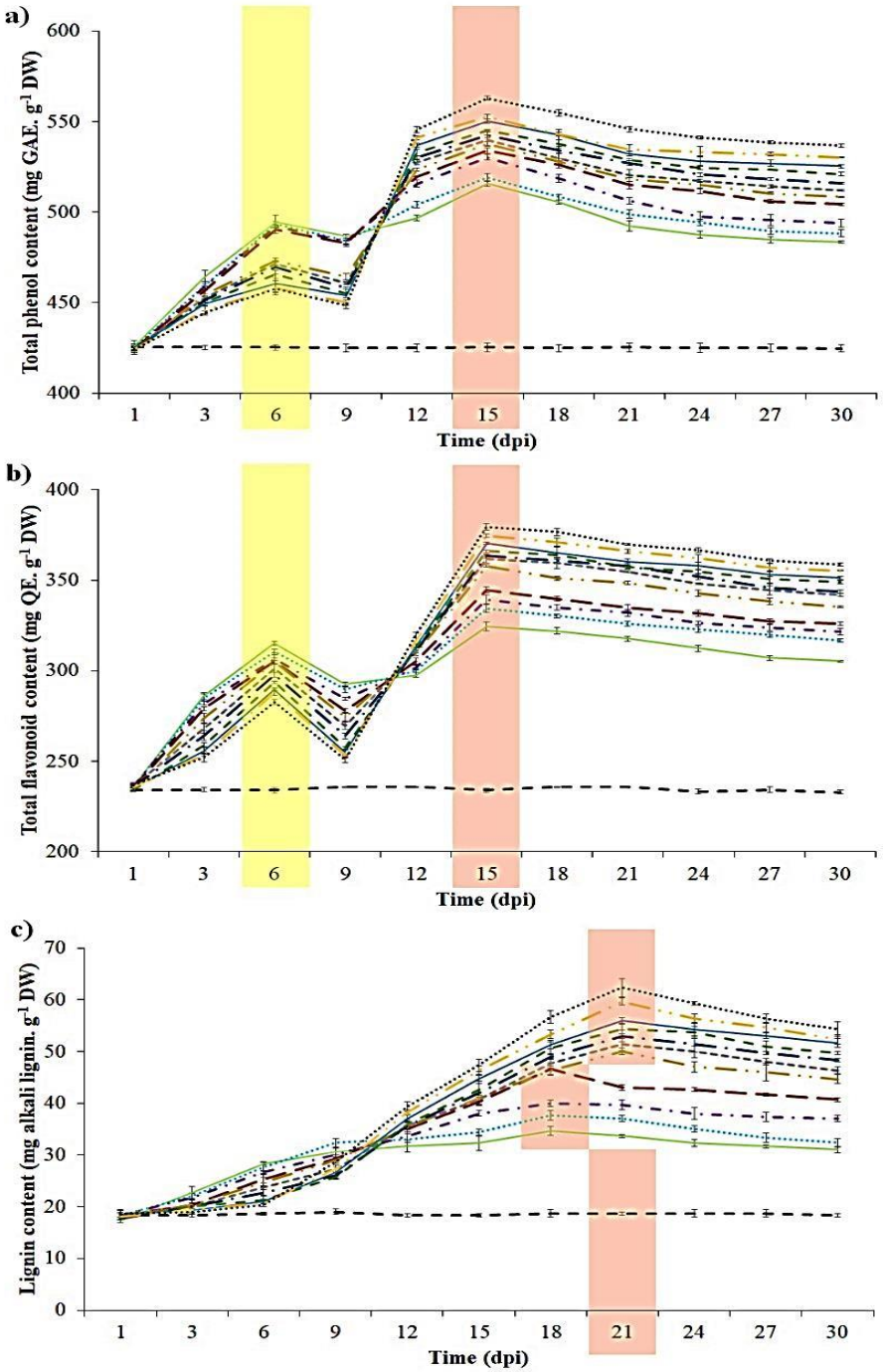

Fig. 4. Levels of total phenolic (a), flavonoid (b) and lignin (c) content in seedling of walnut genotype plants at different times after inoculation with *Dematophora necatrix* isolates. Data are means ± standard error. The experiment was repeated twice with similar results. Abbreviations in figure: control seedling uninoculated, seedling inoculated by *D. necatrix* isolates MU57; RV08; ZA94; LX66; FP35; ND36; BE71; CT17; GD23; SC89; HO42

Discussion

This study is the first report on the biochemical mechanisms involved in the basal resistance of walnuts to the necrotrophic fungus *D. necatrix*. The role of plant defence responses, including the accumulation of phenolic compounds and flavonoids, lignification and induction of GPX, PPO and PAL activities in the pathosystem of walnut and *D. necatrix*, has been demonstrated in this research. In

addition, this is the first detailed study on the relationship between virulence, aggressiveness, and the activity of extracellular enzymes produced by *D. necatrix* which have been evaluated and compared.

In this study, 11 fungal isolates from different traditional orchards in Iran were isolated and identified using molecular and morphological characteristics. Similar findings were also reported by Behdad (1975), who demonstrated that the fungal isolates were isolated from the diseased roots of walnut plants in the Isfahan orchards of Iran and were identified as *D. necatrix* (previously *Rosellinia necatrix* Berl. ex Prill.) based on morphological characteristics. Our observations revealed that pyriform hyphal swellings form at the junctions of septa in mature hyphae (older than seven days) and not in young mycelia, as reported by others (Pliego *et al*., 2012; Pérez-Jiménez, 2006). Similar results of the morphological and molecular identification of *D. necatrix* were obtained in nurseries and orchards of different fruit crops by Fusco *et al*. (2022), Arjona-López *et al*. (2019), Pliego *et al*. (2012), Pliego *et al*. (2009), Takemoto *et al*. (2011), and Ruano-Rosa *et al*. (2007). The genus *Rosellinia* has recently been segregated from *Dematophora* based on multi-locus phylogenetic analysis, morphological characteristics and considering the "One Fungus - One Species" concept (Suwannasai *et al*., 2023; Wittstein *et al*., 2020). Thus, according to the new research, the revival of *Dematophora* is established for the taxon *D. buxi* (Pourmoghaddam *et al*., 2023), *D. necatrix* (Fusco *et al*., 2022; Pourmoghaddam *et al*., 2022), *D. pepo* (Wittstein *et al*., 2020) and *D. bunodes* (Wittstein *et al*., 2020).

The *Dematophora* infection was only observed from samples collected in the traditional orchards. Our results showed that the highest frequency of fungal isolates was observed in the Gorgan region, followed by the Asadabad, Chenaran, Sanandaj, and Sahneh regions. This incidence of *Dematophora* may be attributed to cultivated sensitive genotypes, poor management of irrigation water, improper drainage, environmental conditions, lack of proper orchard management practices, presence of fungal infection in soil, and not using the proper fungicide causing the high incidences of fungal in the traditional orchard of Gorgan region. The rate of spread of *D. necatrix* varies across studied walnut orchards. Some orchards remain uninfected, while others experience rapid development of diseased and dying plants. Still other orchards have only one or two affected plants (Carlucci *et al*., 2013; Pérez-Jiménez, 2006), which is in line with our observation in this research.

Pathogen aggressiveness, an elementary quantitative trait, establishes the pathogen's potential for causing pathogenicity and yield losses on the host (Khaledi *et al*., 2021). Furthermore, aggressiveness is essential to understand host-pathogen interactions in the pathosystem of walnut and *D. necatrix*. Our research has presented different levels of virulence and aggressiveness among different isolates of *D. necatrix*. This finding aligns with the observations made by other researchers (Fusco *et al*., 2022; Kleina *et al*., 2019; Pasini *et al*., 2016; López *et al*., 2008).

The complex virulence factors of *D. necatrix* are not well understood, though researchers suggest that the production of extracellular hydrolytic enzymes and secondary metabolites contributes to the infection process (Shimizu *et al*., 2018; Pliego *et al*., 2012). Ghatge *et al*. (2020) reported that the fungus *D. necatrix* can degrade lignin components. We focused on a detailed investigation of xylanase, cellulase, pectinase, laccase, manganese peroxidase, and lignin peroxidase activities at different time points as these are the main hydrolytic enzymes produced by *D. necatrix* (Pointing *et al*., 2003). During the 240-hour study of the extracellular hydrolytic and ligninolytic enzymes, the rate of production and the time required for each enzyme to reach maximum activity differed between the different isolates of *D. necatrix*. The maximum activity of the extracellular enzymes studied was observed for the isolate MU57, presenting the highest levels of virulence and aggressiveness. Cellulase was the first enzyme to reach the peak,

while the maximum activity of the other enzymes was found to be delayed. Schwarze (2007) obtained similar results, observing that *Armillaria* spp. primarily decays the cellulose, xylanase, and pectin components of the plant cell wall while leaving lignin untouched during the early stages of decay. Other researchers obtained similar results, demonstrating that *D. necatrix* has a high level of cellulolytic enzyme activity compared to pectinase (Pérez-Jiménez, 2006). The extracellular oxidative enzymes showed maximum activity levels at different periods of incubation time *in vitro*, which was in line with those of Huang *et al*. (2017). Furthermore, Martínez *et al*. (2002) reported that lignin peroxidase activity reaches its maximum level after a short incubation time compared to manganese peroxidase and laccase. This finding aligns with our observation. Similar results were also reported by Sousa *et al*. (2019). They mentioned that lignin peroxidase was the main lignin-modifying enzyme with a far higher activity than manganese peroxidase and laccase. Song *et al*. (2022) demonstrated that the degradation of lignin and cellulose does not rely on a single enzyme but rather on the interactions of multiple enzymes, aligning with our findings.

The extracellular enzymes produced by *D. necatrix* can degrade lignocellulose constituents of plant cell walls (Ruiz-Dueñas *et al*., 2013). Therefore, it is difficult to determine the role of these secreted extracellular hydrolytic and ligninolytic enzymes in virulence because of functional redundancy. The comparison of extracellular enzyme activities and virulence of the isolates indicates that cellulase and lignin peroxidase had a more significant impact on virulence compared to other hydrolytic and ligninolytic enzymes studied in this research, which is parallel to other reports (Villavicencio *et al*., 2020; Pérez-Jiménez, 2006). Thus, we found a relationship between the virulence plus aggressiveness of walnut plants and the hydrolytic as well as ligninolytic enzymes produced by *D. necatrix* isolates *in vitro*. These results are similar to those obtained by Kim *et al*. (2017). Our *in vitro* and *in vivo* observations have shown that the levels of extracellular enzyme activity correlate with the variation of virulence and aggressiveness of *D. necatrix*. Therefore, these results suggest that extracellular hydrolytic and ligninolytic enzymes play a crucial role in disease progression. This is the first report demonstrating a strong correlation between the level of extracellular enzyme activity and the virulence of *D. necatrix* isolated on the host plant.

In order to address the destructive impact of biotic stresses, plants employ various defence mechanisms, such as antioxidant enzymes and defensive compounds (Hosseini-Zahani and Taheri 2023). The current study has revealed that the basal resistance of walnuts against *D. necatrix* is associated with changes in the levels of defense enzymes (GPX and PPO) and the accumulation of defensive compounds synthesized in the phenylpropanoid pathway. GPX is an essential type of peroxidase, which uses guaiacol as a specific electron donor (Sharma *et al*., 2012) and occurs in the presence of stressors (Fijalkowski and Kwarciak-Kozlowska, 2020). GPX may play a role in the production of phenolic, serving as an efficient resistance mechanism (Nikraftar *et al*., 2013). Walnut plants showed the first symptoms of the disease 12 days post-infection. The GPX activity increased significantly at six DPI and 15 DPI, which revealed the role of GPX in diminished oxidative stress and symptoms of white root rot in the walnut. When biotic stress affects the plant, PPO activity redirects phenolics to produce quinone, thereby inhibiting colonization of the tissues by the pathogen (Kakueinezhad *et al*., 2018). PPO activity increased significantly at 9 and 18 DPI, which highlights the role of PPO in disease resistance of walnut to *D. necatrix*. Araji *et al*. (2014) reported that PPO activity in walnut is associated with non-enzymatic ROS scavenging and acts as an indirect antioxidant regulator of cell death, aligning with our observations. Numerous studies have shown that PAL is essential for the resistance of plants to various abiotic and/or

biotic stresses (Zhang *et al*., 2022; Amri *et al*., 2021). The PAL activity significantly increased at six DPI and 15 DPI, highlighting the vital role of PAL in the plant's resistance to stress. This result is consistent with the observations of Yan *et al*. (2019). Tomás-Barberán and Espín (2001) reported that the higher amount of phenols and increase in PAL and PPO activity in plants are attributed to the pathogen infection, which is supported by our results. Our results indicate that the activities of defence enzymes increase in walnut trees after inoculation with *D. necatrix*. Thus, this increase in defence enzyme activity may be related to the biosynthesis of secondary metabolites in plant defence pathways, which is consistent with the research of Mandal *et al*. (2010).

The phenylpropanoid pathway produces secondary metabolites known as phenolic and flavonoid compounds (Lin *et al*., 2016). The defence compounds can act through several mechanisms, including scavenging radicals, reducing the effects of oxidative stress, and forming a physical barrier against infection. This protects plants from biotic agents and abiotic stresses (Treutter, 2006). Lignin, a phenolic complex polymer, is a primary defence strategy to hinder the advancement of pathogens in infected tissue (Choi *et al*., 2023). The data obtained revealed that the total content of phenolics, flavonoids, and lignin increased after infection with *D. necatrix* isolates compared to uninfected plants. These observations suggest that increased defensive compounds in walnut plants may be a defence strategy against *D. necatrix*. Similar results were obtained by Jiang *et al*. (2019), who reported that the phenolic compound content significantly increased in all walnut genotypes after inoculation by bacterial pathogens. Additionally, Zhang *et al*. (2022) reported a significant growth in flavonoid and phenolic compounds in walnut infected by bacterial pathogen compared to control plants, aligning with our findings. This study represents the initial comprehensive demonstration of how phenolic compounds, flavonoid content, and lignin contribution to the basal resistance of walnuts to *D. necatrix*. Based on the results obtained, we can conclude that the increased level of pathogenicity of *D. necatrix* influenced the reduction of GPX plus PPO activity and the distribution of phenolic compounds and flavonoids in this pathosystem, ultimately leading to the manifestation of the symptoms of white root rot.

Conclusions

These observations provided new insights into the crucial role of defence enzyme activity and the production of compounds related to the phenylpropanoid pathway as defence mechanisms involved in the basal resistance of walnuts to *D. necatrix*. Therefore, our knowledge on the defence mechanisms involved in the basal resistance as well as on the identification of virulence factors of *D. necatrix*, in particular the extracellular hydrolytic enzymes, and their relationship with virulence and aggressiveness, can be useful in breeding programs which would lead to the introduction of walnut genotypes with increased immunity to many pathogens.

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Conflict of interest

The authors declare that they have no conflict of interest.

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