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Biochemical defense responses of tolerant and susceptible lettuce accessions following infection by *Sclerotinia sclerotiorum*

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ABSTRACT

Sclerotinia sclerotiorum is one of the most destructive lettuce diseases, restricting lettuce cultivation globally. Here, we measured the activity of three antioxidant enzymes in two contrastingly responding lettuce accessions (susceptible ML1 and tolerant Jah accessions) challenged by *S. sclerotiorum*. These enzymes included superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Moreover, we applied the 2,2-diphenylpicrylhydrazyl (DPPH) method and the malondialdehyde (MDA) assay. The activities of SOD and GPx were much pronounced in the tolerant accession than in the susceptible one. Our results indicate that MDA concentration increased significantly in the leaves of tolerant accession compared with that of the susceptible one at 1 dpi whereas MDA level was pronounced in the later time points in the susceptible accession compared with tolerant accession. Taken together, we observed that the examined biochemical markers differentially fluctuated in the applied accessions, shedding light to understand better resistance mechanisms involved in restricting this notorious fungal pathogen.

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Biochemical markers; Lipid peroxidation; Lettuce and Sclerotinia sclerotiorum

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating fungal pathogen capable of colonizing more than 400 plant species. Some of them are either economically and agronomically important crops such as *Lactuca sativa* (garden lettuce) (Boland and Hall 1994; Bolton et al. 2006). This fungus causes lettuce drop on the leafy green lettuce, which is an annual plant, cultivating widely throughout the world and is consumed mostly

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as a leaf vegetable. This plant contains valuable sources of health-beneficial bioactive compounds, including fiber, iron, folate, and vitamin C (Kim et al. 2016). This disease is a central factor restricting lettuce cultivation globally and causes significant yield losses of around 60% in individual fields (Hao and Subbarao 2005).

S. sclerotiorum is an unusual and typical necrotrophic fungal pathogen, employing a variety of mechanisms to infect host species. These include secretion of hydrolytic enzymes, production of distinct metabolites acting as toxins, and generation of oxalic acid (Cessna et al. 2000; Kabbage et al. 2015). The lettuce drop cycle is established by the germination of the sclerotia either carpogenically or myceliogenically, resulting in the production of apothecia or hyphae, respectively. Subsequently, the generated apothecia release abundant ascospores that infect the above-ground parts of the lettuce plant by landing and germinating on them under favorable environments (Bolton et al. 2006). Under cold and moist environmental conditions, *S. sclerotiorum* rapidly colonizes the attacked host tissues, resulting in disease symptoms developments, including water-soaked lesions that subsequently develop into necrotic tissues covered with fluffy and white mycelium (Willetts and Wong 1980; Bolton et al. 2006).

Since *S. sclerotiorum* produces long-lived structures called sclerotia that reside in soils for up to 10 years, Lettuce drop control is a difficult task to achieve (Harper et al. 2002). Additionally, this fungus generates ample airborne ascospores, major primary inoculum, to establish the infection cycle (Bolton et al. 2006). Currently, the most effective agents providing a modest level of management against lettuce drop are fungicide applications, including dicloran (Botran), iprodione (Rovral), and vinclozolin (Ronilan) (Matheron and Matejka 1989; Matheron and Porchas 2004). However, the quick degradation of iprodione and vinclozolin in soils under repetitively application are documented reasons demonstrating that these compounds are not successful agents to manage lettuce drop under intensive lettuce cultivation (Martin et al. 1991). Additionally, the emergence of fungicide-tolerant isolates and cross-tolerant to various types of fungicides have been reported for these compounds (Davet and Martin 1993; Hubbard et al. 1997). Consequently, an integrated pest management (IPM) approach has been suggested as a certain and reasonable practice to manage lettuce drop in such a way to combine several methods such as chemical and cultural approaches. Breeding for resistance can consider as an environment-friendly and safe component in the IPM measures to control this notorious disease. Several studies reported evidence explaining the obstacle that existed in conducting a successful breeding program to discover and breed for

resistance toward lettuce drop. These include the attribution of resistance to plant architecture (Hayes et al. 2010), missing standard protocol resulting in repeatable results (Grube and Ryder 2004), and the impact of environmental conditions in disease development (Subbarao 1998). Despite enormous efforts to identify genetic resources conferring qualitative resistance complying with the gene for gene relationship against this fungus, no immune lettuce accessions have been discovered so far (Hayes et al. 2010). Conversely, partial or polygenic resistance leading to the reduction of disease incidence reported in some accessions, and wild relative genotypes, but this was often attributed to plant development traits such as quick bolting and low leaf area rather than a physiological mechanism functioning during plant development (Whipps et al. 2002; Lebeda et al. 2014). Nevertheless, the independence of plant morphology and resistance was determined in cv. Eruption with a high level of resistance toward *Sclerotinia* spp., and subsequently, two quantitative trait loci (QTLs), termed as *qLDR1.1* and *qLDR5.1*, providing partial resistance in this cultivar toward lettuce drop was determined by developing a recombinant inbred line population coupled with genotyping by sequencing, and QTL analysis (Mamo et al. 2019).

Like other plants, lettuce can defend itself against fungal attacks through a variety of tactics to inhibit the growth and reproduction of the invading pathogens. These responses are pre-existing (consecutive) while others are triggered following infection. Investigating the differential response of tolerant and susceptible cultivars concerning plant biochemistry and physiology will provide valuable information to understand the resistance and susceptibility of the host plant upon infection by biotic agents. Several biochemical responses are induced in the host plant due to the activation of host enzymes.

In this study, we measured the activity of three enzymes, playing central roles in providing disease resistance. These include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). CAT is an antioxidant enzyme playing essential roles in degrading H_2O_2 into water and oxygen (Mhamdi et al. 2010), whereas GPx is a major ROS-scavenging enzymes catalyzing the reduction of H_2O_2 to suppress the toxic levels of H_2O_2 generated through oxidative stress event (Hasanuzzaman et al. 2017). SOD is a critical antioxidant enzyme contributing to scavenge free radicals accumulated in a plant under biotic and abiotic stress conditions (Alscher et al. 2002). Additionally, we applied the 2,2-diphenylpicrylhydrazyl (DPPH) method to estimate the properties of tolerant and susceptible lettuce accessions for scavenging free radicals (Liu et al. 2007). Furthermore, the malondialdehyde (MDA) assay was employed to detect the lipid peroxidation that occurred in two

contrastingly responding lettuce accessions following infection with *S. sclerotiorum* (Hodges et al. 1999). Eventually, we calculated total phenolic and protein contents in the two tested accessions to monitor their profiles upon infection by the *S. sclerotiorum*.

In the current study, we aimed to compare the biochemical responses of susceptible (ML1) and tolerant (Jah) lettuce accessions following infection by *S. sclerotiorum*. We employed a variety of assays to provide novel insights into how lettuce protects itself against this invading fungal pathogen.

Material and methods

Plant materials

We employed Mazandaran Line 1 (ML-1) and Jahrom (Jah) accessions in our infection assay as susceptible and tolerant accessions against infection by the *S. sclerotiorum*. Additionally, we included *L. serriola,* the wild progenitor of cultivated lettuce (Johnson et al. 2000), to compare the physiological changes fluctuated in the wild progenitor to the applied accessions that are Iranian lettuce accessions. The previously confirmed pathogenic isolate of *S. sclerotiorum* on the examined accessions was used in this study (Unpublished data). We considered *L. serriola* as a wild progenitor (WP) accession here. Seeds of Lettuce accessions were grown in the greenhouse with a minimum temperature of 15 °C in modular trays, and subsequently, re-cultivated into 9 cm plastic pots with watering from below. Grown plants with 5–7 fully expanded leaves were inoculated with *S. sclerotiorum* mycelial plugs. Once the seedlings were around 6-week-old, the inoculum, which was the PDA plugs, excised from actively growing edges of fungal colonies, were placed in wounds made on the leaves by a needle and covered with a parafilm. To have adequate the mycelial pathogen, we sub-cultured this pathogen on 5–7 Petri dishes, and we kept them at 25 °C for seven days to generate enough fungal mass required for the infection assay. The inoculated plants were subsequently enclosed with a plastic bag and kept under darkness for 48 h at 23–25 °C. Subsequently, the inoculated seedlings were transferred to the greenhouse, and disease development was monitored by four days after inoculation. We harvested the inoculated plants at various time points, including 1,2, 3- and 4-days post inoculations (dpi). Collected samples were immediately flash-frozen in the liquid nitrogen to avoid proteolytic activity, and they were kept in the −80 freezer to use in the subsequent biochemical assays. Three biologicals for each treatment were used.

Enzyme extraction and activity assays

The harvested samples were homogenized in an extraction buffer containing 15% acetic acid as well as 85% methanol, and the enzyme extract was kept at 4 °C for 24 hours. These homogenates were centrifuged at 12,000 rpm for 15 minutes at 0 °C. The sample supernatant was passed through a 0.45 µm disposable filter and assayed for enzyme activity (Bakhshi and Arakawa 2006).

Superoxide dismutase (SOD) (EC 1.15.1.1)

We determined the SOD activity by measuring its inhibition in the photoreduction of nitroblue tetrazolium (NBT) by this enzyme as reported earlier (Škrovánková et al. 2012; Alici and Arabaci 2016). To aim this, we prepared the reaction mixture containing 50 mM sodium phosphate buffer (pH7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 µM NBT, 10 µM riboflavin, and 100 µL of crude extract in a final volume of 3.0 mL. We kept this mixture under white light at room temperature for 15 min to conduce the SOD reaction, and subsequently, the absorbance was recorded at 560 nm via a spectrophotometer device. We defined one unit (U) of SOD activity as the amount of enzyme leading to the 50% inhibition of photochemical reduction of NBT.

Guaiacol peroxidase (GPX) (EC 1.11.1.9)

GPX activity was calculated spectrophotometrically at 25 °C through guaiacol and H_2O_2 as the hydrogen donor, and substrate as depicted previously (Hemeda and Klein 1990). We added 10 mL of 1% guaiacol (v/v),10 mL of 0.3% H_2O_2 and 80 mL of 50 mM phosphate buffer (pH 6.6) to prepare 100 mL of reaction mixture. The assay system contained a reaction mixture and 75 µL of enzyme extract in a final volume of 3 mL. We determined the kinetic evolution of absorbance at 470 nm for 1 min. Peroxidase activity was measured via the extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) and was expressed in units of µmol of guaiacol oxidized per min and g FW.

Catalase activity (CAT) (EC 1.11.1.6)

CAT activity was calculated by following the protocol described previously (Aebi and Lester 1984). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 20 mM H_2O_2, and a suitable aliquot of the enzyme in a final volume of 3 mL. A decrease in the absorbance was documented at 240 nm through a spectrophotometer machine. The

CAT activity was expressed as units of μmoles of H_2O_2 degraded per min and g FW.

Malondialdehyde (MDA) assay

The amount of malondialdehyde, a widely used biochemical marker to determine oxidative stress, was measured based on the previously reported protocol with minor modifications (Heath and Packer 1968). We used trichloroacetic acid (TCA, 5% (w/v), 5 mL) to homogenize 0.2 g of tested samples, and subsequently, the treated samples were centrifuged at 10000 rpm for 15 min. Afterward, 5 mL of trichloroacetic acid containing 0.5% of Thiobarbituric acid (TBA) was added to 1 mL of sample supernatant, and the resulting mixture was heated in a hot water bath at 95 °C for 30 min. Afterward, the treated mixture was cooled rapidly in an ice bath for 10 min and then, this was re-centrifuged again with the mentioned conditions. Finally, the absorbance was measured at 532 and 600 nM. The non-specific absorbance at 600 nm was subtracted from what measured at the absorbance of 532 nm. The concentration of MDA was measured via the extinction coefficient of 155 mM^{-1} cm^{-1}.

DPPH radical scavenging activity

We evaluated the free radical scavenging capacity based on the protocol described previously through the stable DPPH with slight modifications (Yu 2001; Zhou et al. 2004). 950 μL of 100 μM methanolic DPPH solution was mixed with 100 μL of examined samples dissolved in methanol at various concentrations. The antioxidant–radical reactions were kept for 30 min at ambient temperature under darkness and, subsequently, the decrease absorbance was measured at 517 nm against a blank of pure ethanol to calculate the radical scavenging activity of each tested sample. DPPH radical scavenging capacity was expressed as IC50 concentration where 50% inhibition of the DPPH radical is gained.

Total phenolic content (TPC)

We employed the Folin–Ciocalteu colorimetric (FCR) method to extract the total phenolic contents via gallic acid as a standard with a minor modification (Singleton et al. 1999; Yu et al. 2003). To aim this, 500 μL of each reaction mixture was mixed with 2.5 mL of Folin- Ciocalteu reagent (0.2 N) and then, 2 mL of Na_2CO_3 solution (75 g/L) was added. The treated reaction was kept at room temperature under darkness for 120 min and subsequently, the optical density was calculated at 760 nm

against a blank. The TPC was determined according to the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per gram of the dry mass of lettuce.

Total soluble protein content (TSP)

One hundred mg of harvested tissues were ground in 300 µL of 1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) as reported previously (Wang et al. 1996). The TSP content was quantified by the Bradford method against a known concentration of BSA as a standard (Bradford 1976).

Results

The results presented in this study show the physiological changes that occurred in the two contrastingly responding lettuce accession towards infection by the *S. sclerotiorum*. Our previous study resulted in the identification of two local lettuce accessions termed Mazandaran Line 1 (ML-1) and Jahrom (Jah), which were susceptible and tolerant accessions against infection by the respective fungus (Unpublished data). Here, we evaluated various biochemical markers fluctuated in the above-mentioned accessions following infection by the *S. sclerotiorum* at 1, 2, 3, and 4 days post-inoculation (dpi).

Changes in SOD, GPX, and CAT activities

To obtain novel insight on the potential roles of antioxidant enzymes playing important roles in the *S. sclerotiorum-L. sativa* interactions, we quantified the activities of three central antioxidant enzymes (SOD, GPX, and CAT) at various time courses following infection by this fungus. The highest SOD activity was found in the WP accession at 2 dpi whereas the lowest activity of this enzyme was noticed in the susceptible ML1 one at all studied time points. The activity of SOD was remarkably higher in the tolerant Jah accession compared with that of susceptible one at all tested time courses (Figure 1a). GPX was gradually activated in the tolerant Jah accession to reach a peak at 2 dpi and then down-regulated by 4 dpi compared with other tested accessions. The lowest GPX activity was observed in the susceptible and WP accessions compared with that of the tolerant accession (Figure 1b). The highest CAT activity was observed in the WP accession at 1 and 2 dpi while that of this enzyme was significantly higher in the susceptible one than the tolerant accession at 1 dpi. The activity of CAT remained at the same level in the tolerant

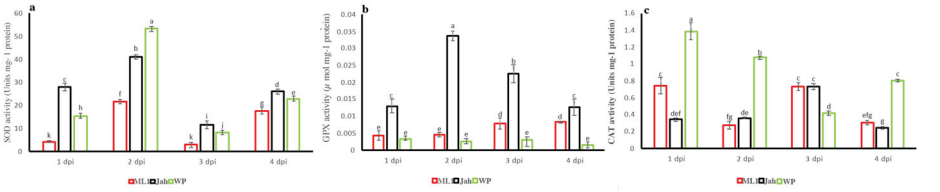

Figure 1. Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) antioxidative enzymes in the leaves of three lettuce accessions that are susceptible(ML1) and tolerant (Jah) lettuce accessions upon infection by the *Sclerotinia sclerotiorum*. We applied *Lactuca serriola* as a wild progenitor (WP) accession in this study. Data are mean ± SD of 3 biological replicates. Different letters on the top of bars indicate significant differences between accessions for each treatment at $p \leq 0.05$.

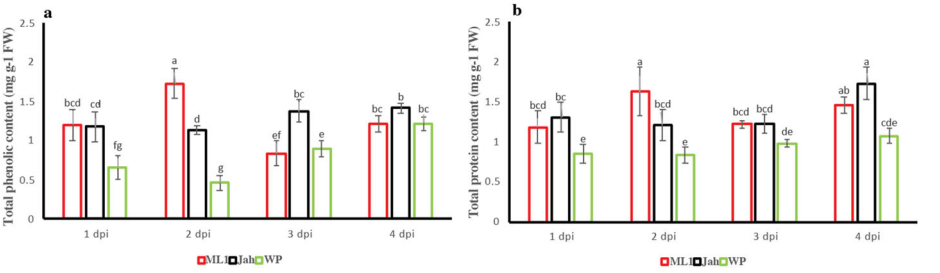

Figure 2. Concentration of the malondialdehyde (MDA) in the leaves of three lettuce accessions that are susceptible(ML1) and tolerant (Jah) lettuce accessions following infection by the *Sclerotinia sclerotiorum* and DPPH radical scavenging IC50 values of all tested tissues. We utilized *Lactuca serriola* as a wild progenitor (WP) accession in this study. Data are mean ± SD of 3 biological replicates. Different letters on the top of bars show significant differences between accessions for each treatment at $p \leq 0.05$.

Jah accession at 1 and 2 dpi but it reached the same level similar to that of the susceptible ML1 one at 3 dpi (Figure 1c).

Changes in malondialdehyde (MDA) content

To determine the level of cellular damages caused by *S. sclerotiorum* infection in the employed accessions, we quantified the MDA content, which is the final byproduct of lipid peroxidation during oxidative stress. MDA concentration increased significantly in the tolerant Jah accession at 1 dpi compared with that of the susceptible ML1 one, while it reached a peak in the susceptible accession at 2 dpi compared with that of the tolerant one. MDA content remained at the same level in the WP accession at 1 and 2 dpi whereas it increased rapidly and reached a significant peak at 3 dpi (Figure 2a).

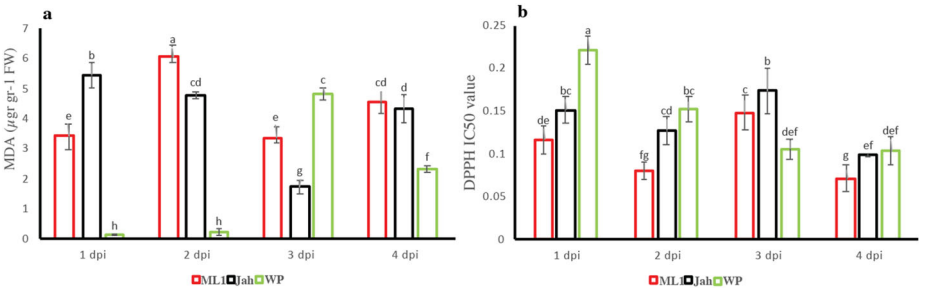

Figure 3. Comparisons of total phenolic compound (TPC) and total soluble protein (TSP) levels in the leaves of three lettuce accessions that are susceptible(ML1) and tolerant (Jah) lettuce accessions following infection by the *Sclerotinia sclerotiorum*. We used *Lactuca serriola* as a wild type (W) accession in this study. Data are mean ± SD of 3 biological replicates. Different letters on the top of bars display significant differences between accessions for each treatment at $p \leq 0.05$.

Changes in DPPH free radical scavenging activity

We employed the DPPH free radical scavenging assay to determine the antioxidant activity fluctuated in the inoculated lettuce accessions following an attack by the *S. sclerotiorum*. IC50 is considered as the total antioxidant required to reduce the initial concentration of DPPH free radicals by 50%. The higher IC50 value means less antioxidant capacity and vice-versa (Bhoyar et al. 2011). The WP accession had the highest DPPH IC50 capacity at 1 dpi compared with that of other ones while the lowest DPPH activity was found in the susceptible ML1 accession at 2 and 4 dpi compared to other examined accessions (Figure 3a).

Changes in total phenolic and protein contents

To gain insight into the potential roles of total phenolic and protein contents in the *S. sclerotiorum*-lettuce pathosystem, we measured these factors in the studied accessions challenged by the *S. sclerotiorum*. The highest and lowest total phenolic content were observed in the susceptible and WP accessions at 2 dpi while the highest total phenolic content of tolerant Jah accession was observed at 3 and 4 dpi. The lowest changes in total protein content was observed in the WP accession at all selected time points whereas the highest total protein content was observed for susceptible and tolerant accessions at 2 and 4 dpi, respectively (Figure 3b).

Discussion

Lettuce drop is one of the most destructive diseases of lettuce causing, significant yield losses annually and restricting its cultivation all over the

world. Applications of tolerant genotypes are a cost-effective and environmentally-safe measure to reduce damage caused by this disease. In this study, we investigated the biochemical responses that occurred in the two contrastingly responding lettuce accessions following inoculation by *S. sclerotiorum* to understand mechanisms mediated tolerance or resistance toward lettuce drop. Here, we found that there are significant fluctuations in the activity of the selected biochemical markers upon attack by this fungus that could be employed to determine the tolerant and susceptible accessions.

Plant cells are equipped with antioxidative defense mechanisms to protect themselves against the high concentrations of reactive oxygen species (ROS) generated under biotic stresses such as a fungal attack, and various environmental stresses. ROS molecules are considered as by-products of plant metabolism, functioning as an essential signaling molecule in generating the programmed cell death (PCD), and maintaining typical plant growth. Nevertheless, ROS at high concentrations causes irreversible DNA damage and impacts various cellular elements resulting in hindering their normal roles (Mittler et al. 2004). There is a deliciated balance between the generation and the detoxification of ROS in plant cells and antioxidant enzymes such as SOD, GPX and CAT, playing pivotal roles in scavenging ROS in the stressed cells (Das and Roychoudhury 2014). Activities of SOD and GPX showed a similar trend in comparison of tolerant and susceptible accessions, revealing that both activities were induced significantly in the tolerant accessions compared with that of susceptible ones. These findings suggest that both enzymes are involved in conferring tolerance toward oxidative burst caused by the *S. sclerotiorum* attack. Our hypothesis could be supported by the fact that excessive oxidative burst is prevented in the tolerant accession as ROS could act as a messenger to induce PCD, and this phenomenon is favorable for this necrotrophic fungus, nourishing from dead tissues (Govrin and Levine 2000). These results were in agreement with previous studies where elevated SOD activity was attributed to the resistance response of barley to the necrotrophic phytopathogen *Pyrenophora teres* fsp. *teres* infection and increased GPX activity was recorded in the leaves of tolerant sunflower lines challenged with the *S. sclerotiorum* (Able 2003; Malenčić et al. 2004). Furthermore, our data demonstrated that *S. sclerotiorum* infection triggered significantly the CAT activity in the WP accession at 1 and 2 dpi compared with other accessions. This result suggests that H_2O_2 scavenging enzyme catalase likely plays an important role in providing resistance response in the WP accession. However, the marked CAT activity was observed in the susceptible accession at 1 dpi compared with that of the tolerant one while both activities at 3 dpi were

comparable. This trend was in disagreement with the previously reported studies where contrastingly responding sunflower accessions to infection by the *S. sclerotiorum* were investigated (Peluffo et al. 2010; Davar et al. 2013).

The extent of cellular damage happend during oxidative burst event is commonly measured by determination of the MDA, which is the final products of lipid peroxidation in the stressed cells. Our results indicate that MDA concentration increased significantly in the leaves of tolerant accessions compared with that of the susceptible one at 1 dpi, coinciding with the suggested short biotrophic stage existed in the lifestyle of the *S. sclerotiorum* whereas MDA level was pronounced in the later time points in the susceptible accession compared with tolerant accession. This finding suggests that excessive cellular damage occurred in the tolerant accession at 24 dpi to confine the *S. sclerotiorum* growth but MDA concentration was elevated in the later sampling time points corresponding with extensive cellular damages occurred in the susceptible accession resulting in symptom expressions. Our data was consistent with the previously documented studies where an increased MDA concentration was reported following infection by the invading plant-pathogenic fungi in the compatible interaction (Mandal et al. 2008; Debona et al. 2012).

Our DPPH radical scavenging assay confirmed the highest IC50 value was found in the WP accession at 1 dpi compared with that of other accessions. The susceptible accession showed higher DPPH scavenging capacity than the tolerant accession. This finding was consistent with the previous report where the susceptible rice cultivars exhibited higher DPPH scavenging activity than the tolerant varieties when challenged with the rice blast fungus (*Pyricularia grisea*) (Toan et al. 2017).

There are several lines of evidence suggesting that phenolic compounds are implicated in plant disease resistance through the rapid synthesis of phenolics and their polymerization in the cell wall (Matern and Kneusel 1988). Our results indicate that the total phenolic compounds of tested accessions varied significantly depending on the kind of applied accessions and sampling time points. No significant differences were found between susceptible and tolerant accessions at 1 dpi while total phenolic content reached the highest peak at 2 dpi in the susceptible accession. The highest peak of this parameter in tolerant accession was noticed at 3 dpi. This result suggests that total phenolic compounds are involved in susceptibility and resistance responses of the lettuce plant at various time-courses of the infection process. There are some reports confirmed that total phenolic content plays a role in conferring resistance reactions against invading fungal pathogen (Saini et al. 1988; Arici et al. 2014; Medić-Pap et al. 2015).

It is increasingly evident that protein components are implicated in plant defense mechanisms toward invading pathogens (Sudisha et al. 2012). Attacked plants by (a)-biotic stress contain the high level of protein contents that are attributed to the activation of host defense mechanism and tactics employed by pathogens (Agrios 2005). In our study, quantification of total protein contents demonstrated that this marker was elevated significantly in the susceptible accession at 2 dpi compared with that of the other accessions. Moreover, total protein concentrations were decreased remarkedly in the WP accession at 1,2, and 3 dpi compared to other examined accessions. However, total protein contents in comparisons of tolerant and susceptible accessions remained unaffected by pathogen attack.

Taken together, we observed that the examined biochemical markers differentially fluctuated in the tolerant and susceptible accessions in the *L. sativa- S. sclerotiorum* interactions shedding light to understand better resistance mechanisms involved in restricting this notorious fungal pathogen. Interestingly, we found antioxidant enzymes (SOD and GPX) that are expressed differentially in the tolerant genotypes. Genes encoding these defense-related enzymes are likely promising candidates to be manipulated genetically in susceptible backgrounds through novel technology such as CRISPR-Cas9 (Ran et al. 2013). Nevertheless, it is worth profiling the expression pattern of the mentioned genes in the subsequent studies in comparison to the tolerant and susceptible genotypes.

Disclosure statement

The authors declare that they have no conflict of interest for the submitted manuscript, and this research does not involve Human Participants and/or Animal. In addition, all author agreed about this submitting.

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