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Abstract Root rot, caused by *Pythium aphanidermatum*, is a major disease of sugar beet in warm regions with high levels of soil moisture. Use of resistant cultivars is an effective strategy to control this destructive disease. However, the mechanisms involved in resistance of sugar beet to the pathogen is still unknown. In this research, 10 sugar beet lines were evaluated in greenhouse for resistance to *P. aphanidermatum* at 6 and 12 weeks growth stages. Our results revealed higher level of resistance in the mature plants. Also, there is no interaction between growth stages and sugar beet lines to *P. aphanidermatum* suggested the possibility of evaluating resistance of sugar beet genotypes to this oomycetous pathogen at earlier growth stages, which leads to save time and expenses. Activity of defense-related enzymes, including peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO), and ascorbate peroxidase (APX) in the resistant (S2–24-P.107) and susceptible (SB-191) lines inoculated with the pathogen at two different growth stages viz. 6 and 12 week, was evaluated. Obtained results revealed that the activity of POX, CAT, and PPO enzymes was enhanced earlier and to the higher extent in the resistant compared to the susceptible line at both growth stages. The activity of APX was not significantly affected by plant inoculation. Phenolic content of the resistant line was higher than that of the susceptible line at both growth stages investigated. Therefore, our results indicated the prominence of POX, CAT, PPO, and phenolics in sugar beet resistance against *P. aphanidermatum.*

Keywords *Beta vulgaris* · *Pythium aphanidermatum* · Defense responses · Resistance

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Introduction

Root rot disease of sugar beet (*Beta vulgaris*), caused by the soilborne Oomycete *Pythium aphanidermatum* (Edson) Fitzp, is one of the most important diseases of this crop in temperate area with high level of soil moisture (Hine and Ruppel 1969). This disease has been reported from Arizona (Hine and Ruppel 1969), California, Colorado and Texas states in the USA (Jacobsen 2006), Canada (McKeen 1949) and Iran (Fatemi 1971). In Iran, *P. aphanidermatum* has the highest frequency among various species of the *Pythium* genus causing sugar beet root rot and causes the highest damage in central and southern warm regions of this country (Fatemi 1971; Mahmoudi and Soltani 2005; Hendrix and Campbell 1973). The pathogen causes wilting and yellowing of the infected sugar beets and dying of

the older leaves. Disease symptoms on the taproot are wet, deep rots with brown to black color, which extend from the lower to the upper parts of sugar beet root (Cooke and Scott 1993). In addition to direct damage in the fields, this pathogen also causes root rot in storage (Bugbee 1986).

Use of resistant varieties is a promising, sustainable approach for effective control of the disease (Hendrix and Campbell 1973; Bannet et al. 2007; Kens 2008). However, information on the host defense responses and resistance mechanisms in sugar beet-*P. aphanidermatum* interaction is still scarce. There has not been much research in the world on development and introduction of sugar beet lines resistant to *P. aphanidermatum.* However, sources of resistance to this pathogen have been reported in other crops. The first investigations on resistance to *Pythium* spp. were carried out on sugarcane (Rands and Dopp 1938). Sources of resistance to *Pythium* spp. have been reported in bean (Lucas and Griffiths 2004), sunflower (Asgharipour 2012), alfalfa (Altier and Theis 1995), wheat (Higginbotham et al. 2004) and other crops. Studies conducted on sugar beet have been rather confined to germplasm evaluation for resistance to damping-off caused by *P. ultimum* (Luterbacher et al. 2005; Abrinnia et al. 2007). Fattahi et al. (2011) studied the resistance of four sugar beet genotypes to the important causal agents of sugar beet root rot disease. They found that the lines SB-19-P.16, SB-19-P.44 and SB-19-P.78 were partially resistant to *P. aphanidermatum*. This was the first report on presence of resistance sources in sugar beet to the root rot caused by this pathogen in Iran. Also, evaluation of 65 sugar beet lines for resistance to the root rot caused by *P. aphanidermatum* at the mature plant growth stage (12 weeks old) showed that the line S_2–24-P.107 had the highest level of resistance to the pathogen (Mahmoudi et al. 2014).

Plants react to pathogen attacks by activating different defense mechanisms, which prevent the entry and spread of pathogens (Malolepsza and Rozalaska 2005). One of these mechanisms is the production of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and superoxide radical (O_2^-) are among the most important ROS (Bhattacharjee 2012). Various types of ROS play several direct and indirect roles in plant defense mechanisms. They might be directly toxic for the attacking microorganisms (Haslan 1983), or could be indirectly involved in plant defense responses against various biotic and abiotic stresses (Dias et al. 2011; Taheri and Tarighi 2011). H_2O_2 is the most stable form of ROS, which is produced by different organelles in plant cells. Accumulation of H_2O_2 inhibits the growth of biotrophic pathogens, but can help necrotrophics in infecting host plant (Govrin and Levine 2000; Khaledi et al. 2016; Noorbakhsh and Taheri 2016). In addition, it might be important in defense responses against various types of pathogens as a second messenger, which activates several defense-related genes and interfaces with other signaling molecules and defense components such as phytohormones, nitric oxide, callose, and lignin (Dias et al. 2011; Noorbakhsh and Taheri 2016). H_2O_2 accumulation, which leads to protein cross-linking and integration of phenolic compounds in cell wall results in elevating plant resistance to necrotrophic and biotrophic phytopathogens (Asselbergh et al. 2007; Kang 2008).

In order to avoid oxidative damage caused by high levels of ROS, plants have a complex antioxidative defense systems. These systems are comprised of enzymatic antioxidants (including peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), etc.) and non-enzymatic components (such as ascorbic acid, glutathione, proline, carotenoids, phenolic acids, flavonoids, etc.) (Chopra and Selote 2007; Kasote et al. 2015). The accumulation of ROS is regulated by a balance of scavenging and producing enzymes (Garcia-Brugger et al. 2006).

Antioxidative defense enzymes, such as POX, CAT, and APX, scavenge ROS which might be correlated with formation of several compounds associated with plant resistance to pathogens including lignin, suberin, and callose (Nikraftar et al. 2013; Noorbakhsh and Taheri 2016).

The enzyme polyphenol oxidase (PPO) has both pro- and anti-oxidant functions (Boeckx et al. 2015). PPO catalyzes the oxidation of phenolics to o-quinones (Araji et al. 2014). PPO-mediated production of o-quinones has also been involved in the indirect generation of ROS (as secondary reaction products) to trigger plant defense pathways. Also, PPO activity can be associated with non-enzymatic ROS scavenging systems, including flavonoids and phenolic acid substrates (Boeckx et al. 2015).

Most of the evaluations for resistance to sugar beet root rot are conducted from 12 weeks growth stage

onward, which is a time-consuming process. If resistance to root rot could be evaluated at early stages of root growth on 6 weeks old plants, when a complete root structure has been developed (Biancardi et al. 2005), it would be possible to evaluate more genotypes in a shorter period of time. To our knowledge, reports on various aspects of *B. vulgaris* - *P. aphamidermatum* interaction are lacking. Especially, comparative biochemical analysis between resistant and susceptible sugar beet lines inoculated with this pathogen have never been carried out. Therefore, the objectives of this research were to: (i) evaluate the reaction of 10 sugar beet lines at 6 and 12 weeks growth stages, (ii) investigate the changes in the activity of defense-related antioxidative enzymes such as peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and polyphenol oxidase (PPO) during *B. vulgaris* - *P. aphanidermatum* interaction, and (iii) quantify phenolics accumulation in a resistant and a susceptible sugar beet line infected with this soil borne phytopathogen.

Materials and methods

Plant materials

Eight sugar beet lines derived from a Rhizoctonia root rot resistant population, SB-19 (Panella 1999), including S1–89004, S1–92533, S1–92535, S1–92588, S1–92633, S1–92637, S1–92638, and O.T.201–15 along with the line S2–24-P.107 (as a resistant control for sugar beet root rot caused by *P. aphanidermatum*) (Mahmoudi et al. 2014) and the line SB-191 (as a susceptible control) were obtained from the Sugar Beet Seed Institute (SBSI), Karaj, Iran. The seeds were disinfected with sodium hypochlorite 0.5% for 2 min and washed with sterile distilled water for 3 min. Plastic pots of 9 cm diameter for 6-week-old plants and of 20 cm diameter for 12-week-old plants were used. The pots contained sterilized combination of field soil: sand (1:2 *v*/v). Five disinfected seeds were sown per pot. One week after emergence, the seedlings were thinned to one seedling per pot. During the growth period, the greenhouse temperature and relative humidity were set at 25 ± 2 °C and 30%, respectively. Two weeks after sowing, the seedlings were weekly fertilized with 30 ml Hoagland solution (Hoagland and Snyder 1933) and irrigated when needed.

Preparation of inocula, inoculation and disease evaluation

In order to prepare the inoculum, the isolate 8P of *P. aphanidermatum* was used, which had been isolated from the infected sugar beets and introduced as a very aggressive and virulent isolate in a previous research (Kakueinezhad et al. 2013). For this purpose, the containers containing 0.5 g grass (*Echinochloa colonum*) leaf sections of 1–2 cm length and 100 ml distilled water (Grass leaf blade water cultures) were used (Al-Sheikh 2010) and these containers were sterilized by autoclave (at 120 °C for 20 min) three times in three successive days. Ten agar plugs obtained from the 5-day-old cultures of *P. aphanidermatum* on potato dextrose agar (PDA) were put into the container and kept in dark at 31 °C for 5 days and then, the produced oospores were collected by blending and filtering the mycelia through a 4-layer cheese cloth. The oospores were counted by haemocytometer and the suspension was diluted by sterile distilled water for preparing the desired concentration of inoculation (Kakueinezhad et al. 2013).

In order to inoculate the 6-week-old plants, 20 ml oospore suspension with density of 6×10^3 oospores per ml and for inoculating the 12-week-old plants, 20 ml suspension with density of 10^4 oospores per ml were used (Deng et al. 2005). Accurately before inoculation, the surface of root epidermis was scratched. From two weeks before inoculation, the greenhouse temperature was gradually increased from 25 ± 2 °C to 35 ± 2 °C and relative humidity from 30% to 70% at time of inoculation. Then, the pots were placed in plastic saucers. For inoculation, the pot soil was dug to 3 cm from the soil surface and superficial lesion of 2 mm dimension was created in the sugar beet root. Then, the oospore suspension was immediately poured in the base of each plant and the pots were irrigated to create saturated conditions. After 48 h, the saucer's water was depleted and irrigation was conducted daily. For uninoculated control plants, the blended and filtered grass leaf blade water cultures containing PDA without *P. aphanidermatum* were used.

Evaluation of disease severity

Two weeks after inoculation, the root rot severity was scored on the basis of 1–9 scale (Büttner et al. 2004). In

this scale, the score 1 is given to the completely healthy roots and the score 9 to the completely rotted roots. The disease index was calculated as mentioned below (Taheri and Tarighi 2010):

$$\textit{Disease index}\ (\mathrm{DI}) = \frac{\sum(\textit{Disease score} \times \textit{number of plants with that score})}{\textit{Total number of plants tested} \times \textit{The highest score}} \times 100$$

In order to complete the Koch's Postulates and confirm the presence of *P. aphanidermatum* as infectious agent, the margin of the infected lesion (so that they contain both diseased and healthy tissue) was cultured.

Biochemical analysis

Leaf samples were taken from the both inoculated and uninoculated plants at two plant growth stages (6 and 12 weeks old plants) for biochemical analyses. The samples were then immediately frozen in liquid nitrogen and stored at −80 °C until used.

The samples taken for analysis of defense-related enzymes were collected at 24, 48, 72, 96, and 120 h after inoculation (hai). Total phenols were analyzed at the end of experiment (120 hai).

Determination of enzyme activities

The enzyme extract was obtained by macerating 1 g of leaf tissue from each sample in liquid nitrogen. To the fine powder obtained, 2% polyvinylpyrrolidone (PVP) was added and homogenized in 6 ml of 50 mM sodium acetate buffer at pH 5.2. Then, homogenized material was centrifuged at 12000×g for 15 min at 4 °C, and the supernatant was used as an enzyme source (Leite et al. 2014).

Total soluble protein concentration was determined using a standard curve of Bovine Serum Albumin (BSA) according to the Bradford (1976) method.

Peroxidase activity (POX; EC 1.11.1.7) was measured by adding 100 μl of the enzyme extract to the reaction mixture containing 100 mM potassium phosphate (pH 6.8), 500 mM H_2O_2, and 900 mM guaiacol in a volume of 2.5 ml. Absorbance was measured using a spectrophotometer (model UV-2100; UNICO company, USA) at 470 nm and the enzyme activity was calculated using the molar extinction coefficient of $2.66 \times 10^4\ M^{-1}\ cm^{-1}$ expressed in μmol of tetraguaiacol formed per milligram of protein per minute (Maehly 1954).

Catalase activity (CAT; EC 1.11.1.6) was determined according to the method described by Aebi (1984). The 200 μl reaction mixtures containing 100 mM potassium phosphate buffer (pH 7.0), 100 mM hydrogen peroxide, and 50 μl of the sample were used. Reduction of H_2O_2 was monitored by reading the absorbance at 240 nm for 3 min. The specific activity was estimated by using the molar extinction coefficient of 36 $M^{-1}\ cm^{-1}$ (Anderson et al. 1995). The CAT activity was expressed as μmol of H_2O_2 oxidized per milligram of protein per minute.

Polyphenol oxidase activity (PPO; EC 1.10.3.2) was determined by adding 75 μl of enzyme extract adjusted to 2.5 ml of a solution containing 100 mM potassium phosphate buffer (pH 6.5) and 25 mM catechol. The increase of absorbance was observed at 410 nm (Gauillard et al. 1993). The PPO activity was expressed as μmol of quinone produced per milligram of protein per minute.

Ascorbate peroxidase activity (APX; EC 1.11.1.1) was determined according to the method described by Nakano and Asada (1981), using ascorbic acid as a substrate. The 2.5 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, and 150 mM hydrogen peroxide was used in this assay. The reaction was started after adding 100 μl of the crude enzyme extract. Readings were taken in a spectrophotometer at 290 nm and ascorbate oxidation was monitored for 3 min. The specific activity was estimated using molar extinction coefficient of 2800 $M^{-1}\ cm^{-1}$ (Hana et al. 2008). The APX activity was expressed as μmol of H_2O_2 oxidized per milligram of protein per minute.

Total phenol quantification

For analysis of total phenolic content, plant leaf tissues were ground in liquid nitrogen using a mortar and pestle until obtaining a fine powder. The samples were freeze-dried and 30 mg aliquot was transferred to a 2-ml microtube, homogenized with 1.5 ml of 80% methanol and agitated for 15 h in a rotating agitator, protected from light at room temperature, for soluble phenol

extraction and depigmentation of the tissue. The suspension was then centrifuged at 12000×g for 5 min. Supernatant was transferred to a new tube for analysis (Leite et al. 2014).

Total phenolic contents were measured using Folin–Ciocalteu colorimetric method described by (Gao et al. 2000). Plant extracts (30 μl) were mixed with 0.2 ml of Folin–Ciocalteu reagent and 2 ml of H_2O, and incubated at room temperature for 3 min. After adding 1 ml of 20% sodium carbonate to the mixture, total phenols were determined after 1 h of incubation at room temperature. Absorbance of the resulting blue color was measured at 765 nm using a spectrophotometer. Quantification was done with respect to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE), micrograms per 1 g of fresh weight (FW) (Wojdyło et al. 2007). All determinations were performed in triplicate.

Experimental design and statistical data analysis

For evaluation of root rot resistance, the experiment consisting of 10 sugar beet lines was arranged in a completely randomized design with five replications and repeated three times. Severity of disease symptoms was analyzed using the analysis of variance (ANOVA) and Duncan grouping test at $P \leq 0.05$ using the SAS program (version 9.1; SAS Institute, Inc., Cary, NC).

For the POX, CAT, APX, and PPO activities, the experiment was repeated three times with three replications. The design was considered to be a 2 × 2 × 2 × 5 factorial experiment consisting of two growth stages (6 and 12 weeks), two sugar beet lines (S2–24-P.107 and SB-191), two inoculation treatments (uninoculated and inoculated plants), and five sampling times (24, 48, 72, 96, and 120 hai). For total phenolics, it was considered to be a 2 × 2 × 2 factorial experiment consisting of two growth stages (6 and 12 weeks), two sugar beet lines, and two inoculation treatments (uninoculated and inoculated plants).The data were analyzed using MSTAT-C software version 2.1.

Results

Root rot severity evaluations

The symptoms of root rot on above-ground plant parts of sugar beet infected with *P. aphanidermatum* were observed at three days after inoculation at both growth stages tested (6 and 12 weeks growth stages) as leaf yellowing or browning from the lower part of petiole. The distinct symptoms of root rot appeared on the root as yellow to brown rot. Uninoculated controls were healthy and showed no symptoms on shoot and root. *P. aphanidermatum* was easily re-isolated from rotted roots.

Statistical analysis showed that there were significant differences between two growth stages (6 and 12 weeks) and also among lines for resistance to the root rot disease caused by *P. aphanidermatum* (Table 1). Interaction between growth stages and lines was not significant. This result indicated that resistance of sugar beet lines was not affected by the growth stages. Resistance rank to *P. aphanidermatum* was moderately stable in plant responses at two growth stages (Fig. 1). The resistant line (S2–24-P.107) displayed the lowest DI at both 6 weeks (32.6%) and 12 weeks growth stages (24.4%). Also, the DIs observed on the O-T.201–15 (27.4%) and S1–89004 (30.3%) lines were not significantly different compared to that of the resistant line (S2–24-P.107) at 12 weeks growth stage. The line SB-191 with a DI of 88.9% at 6-weeks and 81.5% at 12-weeks growth stages showed the highest susceptibility to *P. aphanidermatum* (Fig. 1).

In the resistant line (S2–24-P.107) at 6 and 12 weeks growth stages, few plants showed limited root rot on the root tip. Most of the plants were symptomless at two weeks after inoculation with *P. aphanidermatum* and the wound on roots was healed, especially in the plants inoculated at 12 weeks growth stage (Fig. 2a and c).

In susceptible line (SB-191) at 6 and 12 weeks growth stages, the root rot symptoms were started from the root tip as yellow to brown rot. Symptoms were developed on the upper part of plant very fast. Two weeks after inoculation, most of the inoculated plants were rotted completely (Fig. 2b and d).

Table 1 Analysis of variance for the effect of growth stages (GS) and sugar beet lines (L) on severity of root rot disease caused by *Pythium aphanidermatum*

Sources of variation	df	*P* values
GS	1	0.0001
L	9	0.0001
GS × L	9	ns

ns not significant

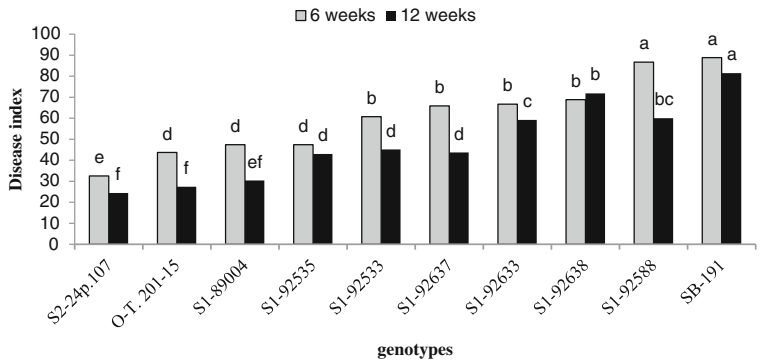

Fig. 1 Disease index of sugar beet lines inoculated with *Pythium aphanidermatum* at 6 and 12 weeks growth stages of the host plant. The number of infected plants under each grade (1–9 scale) was recorded and the disease index (DI) was calculated by the equation: DI = Sum of (Disease score ×number of plants with that score) / (number of plants assessed × the highest score) × 100. Columns with different letters differ significantly at 0.05 probability levels

Enzyme activities

Results of ANOVA and comparing the means of data obtained for growth stages (GS) (6 and 12 weeks), plant inoculation (PI)) (inoculated plants (I) and uninoculated (UI)), lines (L) (S2–24-P.107 (R) and SB-191(S)), sampling time (ST) (24, 48, 72, 96, and 120 h after inoculation (hai)), and their interactions for the activity of POX, CAT, PPO, and APX enzymes are presented in Table 2 and Table 3, respectively.

Activity of POX

The interaction of growth stages × lines × plant inoculation × sampling time were significant for the POX activity (P value ≤ 0.01), indicating the importance of these factors in explaining variation of the variable studied (Table 2). The peak of POX activity was found in the line S2–24-P.107 infected by *P. aphanidermatum* in 6 weeks growth stages at 48 hai (6 W/I/R/48 hai) and in 12 weeks growth stage at 72 hai (12 W/I/R/72 hai) with 4.78 and 6.78 μmol min^{-1} mg^{-1} protein, respectively. Peroxidase (POX) activity of treatments 6 W/I/R/48 hai, 12 W/I/R/48 hai, 12 W/I/R/72 hai, 12 W/I/R/96 hai, 12 W/I/R/120 hai and 12 W/I/S/72 hai were higher and classified in the same group (Table 3).There was no uninoculated treatment in this group. Therefore, sugar beet inoculation with the pathogen led to increase in the POX activity.

Activity of CAT

There was a very significant difference (P value ≤ 0.01) in interaction among factors of growth stages × lines ×

Fig. 2 Root rot symptoms caused by *Pythium aphanidermatum* in the resistant line S2–24-P.107 (**a**) and susceptible line SB-191 (**b**) at 6 weeks growth stage and the resistant line S2–24-P.107 (**c**) and susceptible line SB-191 (**d**) at 12 weeks growth stage of sugar beet

Table 2 Analysis of variance of the effect of growth stage (GS), sugar beet lines (L), plant inoculation (PI), and sampling time (ST) on the activity of peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and polyphenol oxidase (PPO)

		P values			
Sources of variation	df	POX	CAT	PPO	APX
GS	1	0.0000	0.0024	0.0000	ns
PI	1	0.0000	0.0000	0.0001	0.0000
L	1	0.0013	0.0000	0.0682	0.0078
ST	4	0.0000	0.0204	0.0000	0.0000
GS × PI	1	ns	0.1503	0.2386	0.0001
GS × L	1	ns	0.0082	0.1230	0.1402
GS × ST	4	0.0000	0.0000	0.0000	0.0000
PI × L	1	0.0139	0.0000	0.0064	0.0998
PI × ST	4	ns	0.0155	ns	0.0219
L × ST	4	0.0431	0.0004	0.0001	0.0012
GS × PI × L	1	0.0791	0.0887	ns	ns
GS × PI × ST	4	0.0024	ns	0.0025	ns
PI × L × ST	4	ns	ns	0.1472	ns
GS × L × ST	4	0.1640	ns	0.0062	0.0028
GS × PI × L × ST	4	0.0019	0.0003	0.0280	0.1008

ns not significant

plant inoculation × sampling time in CAT activity (Table 2). Treatments 6 W/I/R/24 hai, 6 W/I/R/48 hai, 6 W/I/R/72 hai, 12 W/I/R/24 hai, 12 W/I/R/48 hai, 12 W/I/R/72 hai and 12 W/I/R/120 hai were in the same group with high CAT activity. All of these treatments belonged to resistant line (S2–24-P.107) infected by the pathogen. The peak of CAT activity was indicated in the line S2–24-P.107 infected by *P. aphanidermatum* at 48 hai in 6 weeks growth stage (6 W/I/R/48hai) and 72 hai in 12 weeks growth stage (12 W/I/R/72 hai) with 217.70 and 220.18 μmol min^{-1} mg^{-1} protein, respectively (Table 3).

Activity of PPO

Interactions of the factors growth stages × lines × plant inoculation × sampling time on PPO activity were significantly different (*P* value ≤0.05) (Table 2). Treatments 6 W/I/R/48 hai, 6 W/I/S/96 hai, and 6 W/I/S/120 hai were in the same group with high activity of PPO, 53.82, 24.74, and 27.08 μmol min^{-1} mg^{-1} protein, respectively. In the line SB-191, there were significant increases in the activity of PPO at 96 and 120 hai compared with other treatments. There was only significant increase in the activity of PPO in the line SB-24-P.107 at the 6 weeks growth stage, at 72 hai, compared with the other treatments (Table 3).

Activity of APX

For the APX activity, the interaction of growth stages × lines × plant inoculation × sampling time was not significant. Among triple interactions only the interaction of growth stages × lines × sampling time were significant for the APX activity (*P* value ≤ 0.01), indicating that the activity of APX was not affected by plant inoculation (Table 2). The peak of APX activity was observed in the line S2–24-P.107 infected by *P. aphanidermatum* in 6 weeks growth stage (3.99 μmol min^{-1} mg^{-1} protein) at 48 hai (6 W/I/R/48 hai) and in 12 weeks growth stage (4.74 μmol min^{-1} mg^{-1} protein) at 72 hai (12 W/I/R/72 hai) (Table 3).

Total phenolic content

Results of ANOVA showed that the interaction of growth stages × lines × plant inoculation was significant (Table 4), which indicates the impact of these factors on phenolics accumulation in the plant tissues. The highest total phenolic content (12.82 mg/g fresh weight) was observed in the resistant line S2–24-P.107 infected with the *P. aphanidermatum* in 12 weeks growth stage. Total phenolics of both infected lines such as the resistant line S2–24-P.107 in 6 weeks growth stage and the susceptible line SB-191 in 6 and 12 weeks growth stages were in the same group (Table 5).

Discussion

This is the first report that has demonstrated biochemical defense responses of sugar beet at two different growth stages against *P. aphanidermatum*. Results of this study indicated the presence of significant differences for resistance levels against root rot caused by *P. aphanidermatum* in various sugar beet lines. The highest resistance was found in the line S2–24-P.107 at both growth stages. Also, the lines S1–89004 and O.T-201-15 displayed partial resistance to *P. aphanidermatum* at both growth stages. Whereas, the line SB-191 showed the highest level of susceptibility to the pathogen at both growth stages tested. This is in accordance with the results

Table 3 Means comparison for interaction of growth stage (GS), plant inoculation, (PI) sugar beet lines (L), and sampling time (ST) on the activity of peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), and polyphenol oxidase (PPO)

GS/PI/L/ST	POX (μmol min^{-1} mg^{-1} protein)	CAT (μmol min-1 mg-1 protein)	PPO (μmol min^{-1} mg^{-1} protein)	APX (μmol min^{-1} mg^{-1} protein)
6 W/I/R/24hai	1.38 $^{g-l}$	138.21 $^{a-d}$	15.35 $^{b-g}$	1.34 $^{c-g}$
6 W/I/R/48 hai	4.78 ^{ab}	217.70 ^{ab}	53.82 ^{a}	3.99 ^{ab}
6 W/I/R/72 hai	0.76 $^{i-p}$	127.90 $^{a-e}$	10.11 $^{c-i}$	1.59 $^{a-e}$
6 W/I/R/96 hai	0.73 $^{k-q}$	67.66 $^{c-j}$	11.18 $^{b-h}$	2.42 $^{a-d}$
6 W/I/R/120 hai	2.31 $^{c-g}$	62.37 $^{c-k}$	17.07 $^{b-f}$	1.26 $^{c-g}$
6 W/I/S/24 hai	1.48 $^{f-k}$	89.56 $^{c-i}$	6.85 $^{f-i}$	0.77 $^{d-j}$
6 W/I/S/48 hai	1.86 $^{d-h}$	62.59 $^{c-j}$	8.81 $^{e-i}$	1.16 $^{c-g}$
6 W/I/S/72 hai	0.30 ^{rs}	35.43 $^{h-n}$	5.28 $^{g-i}$	0.54 $^{f-n}$
6 W/I/S/96 hai	1.04 $^{g-n}$	67.55 $^{c-i}$	24.74 $^{a-c}$	2.27 $^{a-d}$
6 W/I/S/120 hai	1.89 $^{d-i}$	75.39 $^{c-h}$	27.08 ^{ab}	2.09 $^{b-f}$
6 W/UI/R/24 hai	0.51 $^{o-r}$	55.69 $^{e-l}$	7.03 $^{f-i}$	0.65 $^{e-l}$
6 W/UI/R/48 hai	1.39 $^{g-l}$	32.01 $^{h-o}$	11.32 $^{c-h}$	0.62 $^{e-l}$
6 W/UI/R/72 hai	0.42 $^{p-s}$	18.62 $^{m-p}$	22.50 $^{b-d}$	0.23 $^{n-q}$
6 W/UI/R/96 hai	0.30 ^{rs}	19.74 $^{n-p}$	7.15 $^{f-i}$	0.29 $^{i-q}$
6 W/UI/R/120 hai	0.69 $^{j-q}$	57.81 $^{d-k}$	5.72 $^{g-i}$	0.19 $^{l-q}$
6 W/UI/S/24 hai	0.22 ^{s}	21.47 $^{l-p}$	5.76 $^{g-i}$	0.20 $^{m-q}$
6 W/UI/S/48 hai	0.82 $^{h-p}$	34.90 $^{h-o}$	18.55 $^{b-f}$	0.14 ^{pq}
6 W/UI/S/72 hai	0.33 $^{q-s}$	37.73 $^{h-n}$	8.59 $^{d-i}$	0.17 $^{o-q}$
6 W/UI/S/96 hai	0.60 $^{m-r}$	44.11 $^{g-m}$	22.65 $^{b-e}$	0.29 $^{h-q}$
6 W/UI/S/120 hai	0.63 $^{l-r}$	48.04 $^{g-l}$	16.99 $^{b-f}$	0.56 $^{e-m}$
12 W/I/R/24 hai	3.05 $^{b-f}$	121.23 $^{a-f}$	1.83 $^{j-l}$	0.51 $^{g-p}$
12 W/I/R/48 hai	3.42 $^{a-e}$	116.90 $^{a-f}$	3.68 $^{i-k}$	0.46 $^{g-o}$
12 W/I/R/72 hai	6.78 ^{a}	220.18 ^{a}	15.44 $^{b-f}$	4.74 ^{a}
12 W/I/R/96 hai	3.37 $^{a-e}$	91.13 $^{b-g}$	6.22 $^{f-i}$	0.90 $^{c-h}$
12 W/I/R/120 hai	3.51 $^{a-d}$	142.17 $^{a-c}$	7.56 $^{e-i}$	1.26 $^{c-g}$
12 W/I/S/24 hai	1.00 $^{g-o}$	17.26 $^{n-p}$	0.84 ^{l}	0.12 ^{q}
12 W/I/S/48 hai	1.54 $^{e-j}$	45.26 $^{g-n}$	1.46 ^{kl}	0.98 $^{c-g}$
12 W/I/S/72 hai	4.13 $^{a-c}$	85.79 $^{c-i}$	8.54 $^{d-i}$	2.41 $^{a-c}$
12 W/I/S/96 hai	2.98 $^{b-f}$	52.58 $^{f-l}$	9.42 $^{c-i}$	1.02 $^{c-g}$
12 W/I/S/120 hai	3.07 $^{b-f}$	30.62 $^{j-p}$	4.97 ^{hi}	0.77 $^{e-m}$
12 W/UI/R/24 hai	0.48 $^{n-r}$	15.19 ^{op}	0.84 ^{l}	0.20 $^{m-q}$
12 W/UI/R/48 hai	1.18 $^{g-m}$	16.34 $^{n-p}$	1.52 ^{kl}	0.19 $^{n-q}$
12 W/UI/R/72 hai	1.58 $^{d-i}$	72.76 $^{c-h}$	4.24 $^{h-j}$	1.82 $^{a-e}$
12 W/UI/R/96 hai	1.65 $^{d-i}$	18.37 $^{n-p}$	4.97 $^{g-i}$	0.61 $^{g-p}$
12 W/UI/R/120 hai	2.11 $^{c-g}$	25.18 $^{k-p}$	7.48 $^{e-i}$	0.25 $^{i-q}$
12 W/UI/S/24 hai	1.20 $^{g-l}$	14.69 ^{op}	0.99 ^{l}	0.27 $^{j-q}$
12 W/UI/S/48 hai	1.24 $^{g-l}$	12.56 ^{p}	0.79 ^{l}	0.22 $^{k-q}$
12 W/UI/S/72 hai	1.51 $^{f-k}$	26.56 $^{j-p}$	5.26 $^{g-i}$	0.71 $^{e-k}$
12 W/UI/S/96 hai	1.32 $^{g-l}$	29.67 $^{i-p}$	5.68 $^{g-i}$	0.75 $^{c-i}$
12 W/UI/S/120 hai	1.96 $^{d-g}$	37.27 $^{g-n}$	4.66 $^{h-j}$	0.44 $^{g-p}$

Means in the same column with different letters differ significantly at 0.05 probability levels. The design consisted of two growth stages (GS) (6 and 12 weeks), two inoculation treatments (PI) (inoculated plants (I) and uninoculated (UI)), two sugar beet lines (L) (S2–24 P.107 (R) and SB-191(S)), and five sampling times (ST) (24, 48, 72, 96, and 120 h after inoculation (hai))

Table 4 Variance analysis on the effect of growth stage (GS), plant inoculation (PI), and sugar beet lines (L) on total phenolic contents

Sources of variation	df	*P* values
GS	1	0.0353
PI	1	0.0000
L	1	0.0005
GS × PI	1	0.0007
GS × L	1	0.0084
PI × L	1	0.0003
GS × PI × L	1	0.0141

of Mahmoudi et al. (2014), who evaluated these lines at the adult plant stage (12 weeks old). Similarly, reports of other researchers showed different levels of resistance among various plant genotypes against *Pythium* root rot (Hendrix and Campbell 1973; Fattahi et al. 2011).

Our results showed that there were significant differences between 6 and 12 weeks growth stages for responses to the pathogen. Intensity of symptoms and disease index (DI) at 6 weeks growth stage was higher than those of 12 weeks growth stage and some of the partially resistant lines at 12 weeks growth stage displayed high level of susceptibility at 6 weeks growth stage. These findings indicated a higher level of resistance in mature sugar beet plants against the pathogen. Raftoyannis and Dick (2002) also documented that in sugar beet diseases caused by *Pythium* spp. and *Phytophthora* spp., disease severity is inversely correlated with plant age, which is in agreement with our data. However, we observed that the ranking of sugar beet lines did not change largely at two different growth stages tested and the lines resistant at 12 weeks were also resistant at 6 weeks growth stage. Absence of a significant interaction between growth stages and sugar beet lines for reaction to *P. aphanidermatum* indicated that it is possible to evaluate the lines at 6 weeks growth stage, which leads to saving the time and cost. Therefore, a large number of lines could be evaluated in a short period of time.

Table 5 Means comparison for interaction of growth stage (GS), plant inoculation (PI), and sugar beet lines (L) on the total phenolic contents assessed at 120 h after inoculation

Treatment	GS/PI/L	Total phenolic contents (mg/g fresh weight)
1	6 W/I/R	7.53 [b]
2	6 W/I/S	6.31 [b]
3	6 W/UI/R	2.98 [c]
4	6 W/UI/S	3.15 [c]
5	12 W/I/R	12.82 [a]
6	12 W/I/S	7.64 [b]
7	12 W/UI/R	3.43 [c]
8	12 W/UI/S/	3.41 [c]

Means in the same column with different letters differ significantly at 0.05 probability levels by *Duncan* test. The design consisted of two growth stages (GS) (6 and 12 weeks), two inoculation treatments (PI) (inoculated plants (I) and uninoculated (UI)) and two sugar beet lines (L) (S2–24-P.107 (R) and SB-191(S)), at 120 h after inoculation

Resistance and susceptibility to plant diseases is governed by the genetic of host and pathogen and depends on the exchange of complex signals and responses in certain environmental conditions (Yang et al. 1997). One major difference between resistant and susceptible plants is the on-time diagnosis of the attacking pathogen and the immediate and effective activation of the host defense mechanisms (Ebel and Cosio 1994). Therefore, by using the lines S_2–24-P.107 and SB-191, resistant and susceptible to *P. aphanidermatum* respectively, we investigated biochemical aspects of sugar beet resistance to root rot caused by this pathogen at two growth stages.

Increased activities of defense enzymes POX, CAT, PPO, APX, and production of phenolic compounds in different pathosystems have been introduced as the main plant defense mechanisms against various pathogens (Koc and Ustun 2012; Debona et al. 2012; Nikraftar et al. 2013; Balbi-Pena et al. 2012). These defense mechanisms neutralize the oxidative stresses induced by the pathogen. Oxidative stress triggers several defense-related mechanisms such as increased expression of antioxidant genes and elevated activity of antioxidative enzymes in plants, which promotes the host plant resistance to stress.

Invasion of the *Pythium* hyphae induces the production of ROS (Van Buyten and Höfte 2013) and results in H_2O_2 production as a stable form of ROS, which is created systemically in the plant (Salin 1988; Levine et al. 1994). Production of ROS in plant cells cooperate as a defense combination against pathogens in one hand and it could lead to cell damage that can facilitate tissue colonization by necrotrophic fungi on the other hand (Glazebrook 2005). As a result, presence of an effective and on-time antioxidative system that can balance ROS content and remove excess ROS for prevention of more

damage to plant cells, is very essential to achieve plant resistance against necrotrophic fungi.

The POX is known as one of the responsive enzymes, which could be induced rapidly in plant defense against various pathogens (Sulman et al. 2001). It plays several roles such as regulation of ROS by removing H_2O_2, lignification and suberization in response to biotic and abiotic stresses (Passardi et al. 2004). In the current research, the POX activity increased due to the pathogen inoculation from 24 hai in resistant line (S2–24-P.107) compared with the uninoculated control in both growth stages. Rate of increase in the POX activity of resistant line was much higher than that of the susceptible line. Significant increase of POX was observed at 48 hai in 6 weeks and at 48, 72 and 96 hai in 12 weeks growth stages. Decreased disease symptoms in resistant line (S2–24-P.107) in mature stage could be related with the increased and continued activity of POX enzyme in this stage. These results indicated the involvement of POX activity in resistance of sugar beet to *P. aphanidermatum*. Similarly, Zhang et al. (2013) reported the increased POX activity in pepper (*Capsicum annuum* L.) cultivars resistant to *Phytophthora capsici* and found that the POX activity was higher in the resistant cultivar and continued for a longer time, whereas in the susceptible cultivar, this increased activity was weaker and shorter. Similar to our results, inoculation of sunflower cultivars with the necotrophic fungus *Macrophomina phaseolina* resulted in increased peroxidase activity, especially in the resistant cultivars at seedling and adult plant stages (Aboshosha et al. 2008).

The CAT is a key enzyme in scavenging H_2O_2. Different results on its activity in various pathosystems have been reported. In the current study, 24 h after sugar beet root inoculations with *P. aphanidermatum* at both 6 and 12 weeks growth stages, significant increase of CAT activity was observed in the resistant line compared with other treatments and this increase was monitored up to 72 hai. Inoculation of the susceptible line with *P. aphanidermatum* resulted in reduction or a minor increase in the CAT activity. Increased and continued activity of the CAT indicated that this enzymatic antioxidant played a major and important role in sugar beet resistance to *P. aphanidermatum* in both growth stages. Similar to our data, Mandal et al. (2008) reported that the reduced CAT activity caused by a less-efficient system of ROS scavenging enzymes results in high damages induced by infection of tomato cultivars with *Fusarium oxysporum* f. sp. *lycopersici*. Also, Debona et al. (2012) showed that the induced level of the CAT enzyme was significantly higher in resistant wheat cultivars inoculated with the hemi-biotrophic fungus *Pyricularia oryzae* than in the susceptible cultivars. Similar to our observations, inoculation of pepper cultivars with *Phytophthora capsici* increased the activity of CAT and POX in the resistant compared to the susceptible cultivar (Koc and Ustun 2012).

The PPO, which converts phenolics to a fungitoxic compound called quinone and hence prevents the tissues from being colonized by the pathogen, had higher activity in the resistant compared to the susceptible sugar beet cultivar at two growth stages tested. The PPO activity increased significantly at 48 hai at 6 weeks growth stage, which revealed the role of PPO in disease resistance of immature sugar beet to *P. aphanidermatum*. These results are in agreement with those of Sahoo et al. (2009), who reported the increased phenolics concentration and PPO activity in the leaves of taro [*Colocasia esculenta* (L.) Schott] resistant genotypes inoculated with *Phytophthora colocasia* compared to the susceptible genotype. El-Argawy and Adss (2016) showed that activities of POX and PPO could be used as biochemical markers to differentiate between resistant and susceptible potato cultivars against *Ralstonia solanacaerum*.

The APX is an antioxidative enzyme, which uses ascorbate as a specific electron donor (Asada 1992) and converts H_2O_2 into H_2O that leads to removal of H_2O_2 from chloroplast, peroxisome and mitochondrion (Quan et al. 2008). Despite the fact that the APX activity wasn't significantly affected by plant inoculation, however the level of APX activity was higher in the resistant (S2–24-P.107) compared to the susceptible (SB-191) cultivar at both 6 and 12 weeks growth stages in response to inoculation by *P. aphanidermatum*. In accordance with the results of our research, Djébali et al. (2011) reported that the level of APX activity in a partially resistant cultivar of alfalfa inoculated with *Aphanomyces euteiches* was higher than that of susceptible cultivar. Overexpression of APX-like 1 gene (*CAPOA1*) in transgenic tobacco plants resulted in increased resistance to *Phytophthora nicotianae*. Also, overproduction of APX enhanced the activity of POX and hence supports the scavenging system of ROS, which results in tolerance to oxidative stress and resistance to the pathogen in tobacco cultivars inoculated with *P. nicotianae* (Sarowar et al. 2005).

The response of both resistant and susceptible sugar beet cultivars to the pathogen resulted in increased total phenolics but the level of increase was significantly higher in the resistant cultivar at 12 weeks growth stage. These data indicated the role of phenolics in defense of mature sugar beet plants against *P. aphanidermatum*. Similar to our results, the highest increase in total phenolic content was observed in a resistant tobacco cultivar inoculated with *P. aphanidermatum* compared to the susceptible cultivar (Khan et al. 2012). Similarly, direct relationship of bean resistance with phenolics in response to *R. solani* inoculation was reported by Abdel-Fattah et al. (2011), which supports our findings on the important function of phenolics in sugar beet defense against *P. aphanidermatum* as a necrotrophic pathogen.

In overall, the absence of significant correlation between plant growth stages and sugar beet cultivars in reaction to *P. aphanidermatum* was reported in this paper for the first time, which suggested the possibility of evaluating resistance of sugar beet genotypes to this oomycetous pathogen at earlier growth stages. This earlier assessment would be helpful for saving time and expenses in resistance assessment trials. Although, higher level of resistance to *P. aphanidermatum* root rot could be observed by maturity of sugar beet plants. The results of current research clearly revealed the role of increasing total phenolic content and enhanced activity of three defense enzymes, including POX, CAT, and PPO in resistance of sugar beet to *P. aphanidermatum* at both growth stages. This indicates the prominence of these systemic defense mechanisms in balance of ROS, reduction of root rot damages caused by *P. aphanidermatum* and expression of resistance in sugar beet cultivars. Improving our knowledge on defense responses in sugar beet – *P. aphanidermatum* pathosystem would be useful in breeding programs leading to the introduction of novel sugar beet cultivars with higher resistance levels to this destructive phytopathogen.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest to declare.

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