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Candidate marker genes and enzymes for selection of potato with resistance to early blight, caused by *Alternaria alternata*

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Summary. Early blight, caused by *Alternaria alternata* (Fr.) Keissler is a serious disease of potato and other cultivated *Solanum* species. The molecular components defining defense responses to *A. alternata* in potato are limited. Host transcript accumulation after *A. alternata* inoculation of six potato genotypes (10/33/R1, 3/33/R2 and 21/33/R2, resistant to the pathogen, and 8707/106, 8703/804 and 8707/112, susceptible) was examined to develop understanding of mechanisms of their responses to *A. alternata* genotypes. The marker genes *PR-2*, *ChtA*, *PR-5*, *PR1-b*, *PIN2*, *ERF3*, *PAL* and *LOX*, activity of catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPOs) and phenylalanine ammonia-lyase (PAL), as well as biomass growth parameters, were analysed. Expression of *PR-2*, *ChtA*, *PR-5*, *PR1-b* and *PAL* genes was greatly increased in the inoculated resistant genotypes compared to the susceptible genotypes and un-inoculated controls. Transcription levels of *PIN2*, *ERF3* and *LOX* genes were decreased in resistant inoculated plants. Simultaneously, activities of POX, SOD and PPOs were greatly increased in the inoculated resistant host genotypes, compared to the susceptible and non-inoculated controls. CAT activity in genotype 21/33/R2 and PAL activity in resistant genotypes 21/33/R2 and 10/33/R1 increased in the susceptible and non-inoculated. Host growth parameters of inoculated plants decreased compared to un-inoculated controls. Knowledge of changes in gene expression levels and enzyme production in defense processes in infected potato plants can inform future studies to identify the defense mechanisms, and assist generation of potato cultivars resistant to early blight.

Keywords. Antioxidant enzymes, biomass, defense mechanism, *Solanum tuberosum*.

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INTRODUCTION

Potato (*Solanum tuberosum* L., *Solanaceae*) an important human source of carbohydrates, protein and vitamin C (Hussain, 2016; Rajiv and Kawar, 2016; Kumari *et al*., 2018). Potato crops can also be grown in different envi-

ronmental conditions, and these provide important staple food in many regions (Hussain, 2016).

Among the diseases impacting potato crops, early blight is considered the most devastating. This disease is caused by several species of *Alternaria*, including *A. solani* and *A. alternata* (Nasr-Esfahani, 2018; Zhai *et al*., 2018). *Alternaria alternata* (Fr.) Keissler (*Dothideomycetes*) is capable of infecting *Solanaceae* crops, including potato, at all stages of plant development, causing considerable yield losses (Pourarian *et al*., 2018; Moghaddam *et al*., 2019). Severe impacts of *Alternaria* species on potato production occur in Iran, with the potato pathotype of *A. alternata* being the dominant pathogen species, causing significant yield and nutritive value losses (Nasr-Esfahani, 2018; Moghaddam *et al*., 2019). This pathogen causes brown spots with concentric rings on potato leaves, and ultimately leaf senescence (Esfahani, 2018a; Pourarian *et al*., 2018; Nasr-Esfahani, 2019). Early blight has the potential to reduce the tuber production by more than 20% in susceptible cultivars under favourable environmental conditions (Raimo *et al*., 2018; Ding *et al*., 2019).

Alternaria alternata causes necrotic lesions on potato leaves and tubers, although these lesions are smaller than those caused by *A. solani* (Ding, 2018; Ding *et al*., 2019). Field symptoms of early blight are often confused with physiological damage caused by ozone or nutrient deficiencies (Evenhuis *et al*., 2020). Although applications of fungicides have been recommended for control of early blight, indiscriminate use of pesticides may increase hazards to humans and the environment (Meier *et al*., 2015; Nasr-Esfahani, 2018). Therefore, effective, economical, and harmless, disease management is to develop and/or select disease resistant potatoes capable of producing satisfactory tuber yields, even within heavily pathogen-infested environments (Nasr-Esfahani *et al*., 2018; Moghaddam *et al*., 2020). Three potato clones (BR3, BR5, and BR85) incorporating combined resistances to early blight from *S. palustre* and late blight from *S. bulbocastanum* into a *S. tuberosum* background yield well in temperate climate conditions. In addition to possessing heritable resistance to early and late blights, these clones have other desirable agronomic traits, are fertile, and readily cross to established potato cultivars (Meier *et al*., 2015). Similar results were obtained through screening of several potato genotypes for early blight disease resistance (Odilbekov *et al*. 2014; Xue *et al*., 2019; Ding, 2021).

Pathogenesis-related (PR) protein synthesis genes have provided resistance to pathogens in various crops (McNeece *et al*., 2019). The studies of Wang et al. (2008), Derksen *et al*. (2013), Moghaddam *et al*. (2019) and Hoegen *et al*. (2002) emphasize on this hypothesis, supporting the role of *PR* genes in enhancing resistance to biotic stresses and providing a strategy for development of disease-resistant transgenic food crops (Ali *et al*., 2018). Zhai *et al*. (2018) used RNAi technology to silence the tomato *PR5* gene for resistance to *A. alternata*, and Toufiq *et al*. (2018) isolated the *chitinase* (*ChtA*) gene from *Hordeum vulgare* L., which could inhibit important pathogenic fungi. In a similar study, Khan *et al*. (2017) generated transgenic potato, which overexpressed the *H. vulgare endo-chitinase* gene, indicating high resistance of transgenic potato plants to *A. solani*. Thus, knowledge of changes in expression levels of *PR* genes in resistant and susceptible potato genotypes to *A. alternata* could indicate how *PR* genes play roles in potato resistance to this pathogen (Moghaddam *et al*., 2019; Bagheri *et al*., 2020). However, knowledge is scarce of the molecular defense responses with systemic (leaf) defenses before and after *A. alternata* inoculation of potato. So the present study aimed to provides information on leaf expression levels of the *PR-2, ChtA, PR-5, PR1-b, PIN2, ERF3, PAL and LOX* analysis genes in six contrasting potato genotypes after inoculation with *A. alternata.* In addition to pathogenesis-related (PR) protein synthesis genes, plant hormone mediated signalling pathways may also play important roles in plant disease resistance.

Proteinase inhibitors (PIN) in plants are small proteins involved in defense mechanisms against pathogenic microorganisms, that may imperil the plant integrity (antimicrobial properties) (Rehman *et al.,* 2017). Therefore, studying the changes in enzymes involved in defense processes in response to biotic and abiotic stresses can identify biochemical pathways for creation of resistant crop varieties (Bektas and Eulgem, 2015; Zhang and Liu, 2019; Isah, 2019). Activity of stress-related enzymes (PPO, POX, SOD, PAL and CAT) has been reported in pepper by Bagheri *et al*. (2021), tomato by Moghaddam et al. (2020), and apple by Huang et al. (2016), in response to *A. alternata,* with increases in activity of the related enzymes. These findings were supported by Yang *et al*. (2017) for tomato fruit, with activating and increased expression of corresponding genes for resistance to *A. alternata*. Although defense mechanisms vary across different cultivars, the antifungal effects of chitinases and other hydrolytic enzymes have been determined against *A. solani* and other biotic stresses (Moghaddam *et al.,* 2019). Further research also showed that the genes responsible for the production of pathogenesis-related proteins increased resistance against various pathogens in different crops (McNeece *et al.,* 2019). Furthermore, the role of *Glomus mosseae* and *Trichoderma harzianum* in the protection of cucum-

ber (*Cucumis sativus*) against *A. alternata* indicated increased activity of catalase and peroxidase enzymes was associated with increased resistance to this pathogen (Matrood *et al*., 2020).

In addition to a increased activity of antioxidant enzymes at different host growth stages, overexpression of pathogenesis-related (PR) proteins is a common and widely distributed defense mechanism in plants that minimize disease in non-infected plant organs (Zhang *et al*., 2012a, b; Ali *et al*., 2018). PR proteins are produced in plants after pathogen attack, and are induced as part of systemic acquired resistance (Moghaddam *et al*., 2019; Bagheri *et al*., 2020; Tehrani *et al*., 2020). Several studies have shown overexpression of related genes induced by pathogens encoding host PR proteins, Increase expression of PR1, PR2 and PR3 genes was observed in inoculated tomato genotypes resistant to *A. alternata* (Moghaddam *et al*., 2019; 2020). Furthermore, activation of PR-1 and PR-5 genes was affected by *Phytophthora infestans* in potato cultivars and proximity to the inoculation sites (Wang *et al*., 2008), and high accumulation of mRNA and protein of PR1-b occurred in response infection by this pathogen in potato leaves (Hoegen *et al*., 2002). Intact salicylic acid signalling is required for potato defense against the necrotroph *A. solani* with increases in expression of PAL1, PAL2, PR-1 and PR-2 genes in moderately resistant potatoes. This indicates the role of the salicylic acid pathway in plant defense response (Derksen *et al*., 2013; Brouwer *et al*., 2020). Hormone signalling pathways were induced in four potato genotypes by a concentrated culture filtrates of *P. infestans*. The genotypes were ranked according to their levels of resistance to *P. infestans*, and discriminant analysis of gene expression profiles separated the most resistant genotype from the three others, particularly because of a strong induction of the salicylic acid (SA) pathway. In this genotype, transcripts (*EDS1*, *WRKY1*, *PR-1* and *PR-2*) involved in the SA pathway were induced by concentrated culture filtrate. SA pathway involvement was confirmed by a peak of SA accumulation 12 h after elicitation and by the induction of *jasmonate Zim domain protein 1* transcripts, which inhibit defense responses mediated by jasmonic acid (JA) (Saubeau *et al*., 2016).

Studies of the molecular components defining defense responses to *A. alternata* in potato are limited. The present study aimed to provide knowledge to identify the genes involved in the resistance genes effective against this pathogen. Marker genes for phytohormones and defense-related enzymes, not all of which exclusively indicate defense responses, were examined after *A. alternata* inoculations to identify the genes involved in the resistance

MATERIALS AND METHODS

Plant material

Forty nine potato genotypes were obtained from the Potato and Onion Research Department, Seed and Plant Improvement Institute, Karaj, Alborz Province, Iran. These genotypes were from crosses between Lotta ♀*♂ Kaiser; Agria ♀*♂ Savalan; Agria ♀*♂ Kaiser; Kaiser ♀*♂ Savalan. Of these, the main parents were; Agria – Quarta ♀*♂ Semlo; Kaiser – Monalisa ♀*♂ Rop B 1178, and Savalan – 91/6122 ♀*♂ 88/05, and these were provided by the Plant Improvement Institute (PORD/SPII), Karaj, Alborz Province, Iran. Primary screening of the resistance levels of the potato genotypes to the potato pathotype of *A. alternata*, based on development of brown spot symptoms on leaves and tubers, were performed in the field under natural infection conditions. After this field screening, six genotypes, including three (10/33/R1, 3/33/R2 and 21/33/R2) resistant to the pathogen and three susceptible (8707/106, 8703/804 and 8707/112) were selected (Table 1). To confirm the resistance levels of these genotypes to *A. alternata*, re-screening experiments were carried out in a greenhouse in a completely randomized design experiment, with five replications for the inoculated and un-inoculated (control) host genotypes. These greenhouse experiments were carried out at the Isfahan Agriculture and Natural Resources Research Center, Isfahan, Iran (Esfahani, 2018a).

Fungus cultures, inoculum preparation and pathogenicity assessments

To prepare inoculum of the potato pathotype of *A. alternata*, active cultures previously isolated from infected potatoes sed. d. Comparisons with isolates available in the plant protection department of Isfahan Agriculture and Natural Resources Research Center, Isfahan, Iran were used to confirm identity of the prepared isolates. The isolates were sub-cultured on potato dextrose agar and maintained at 25°C for 10 d (Esfahani, 2018a; Ding *et al*., 2021).

Pathogenicity experiments were carried out by planting potato seed tubers in plastic pots (30 cm diam.) containing soil and perlite (1:1), in greenhouse conditions (18 to 25°C, 14 h of light). A potato seed tuber (approx. 50 g) treated with thiabendazole was planted in each pot. The experiments were arranged in completely randomized designs with five replicates (each of one potato tuber), for all the genotypes, and for experimental controls (non-inoculated tubers). The pots were irrigated each day (Nasr-Esfahani *et al*., 2017; Nasr

Esfahani, 2018b). Resulting 1-month-old potato plants were inoculated with sprayed conidium suspensions (10^3 conidia mL^{-1}), and each pot was then with aplastic bag for 48 h (Esfahani, 2018a). Seven days after inoculation, symptomatic leaves were harvested, and youngest newly emerged leaves were collected into aluminum foils and stored at −20°C for enzyme evaluations, and at −80°C for RNA isolation (Pourarian *et al*., 2018; Naderi *et al*., 2020; Yang *et al*., 2020).

Plant biomass growth parameters

Biomass growth parameters, including root fresh weight (RFW), root dry weight (RDW), stem diameter (SD), stem length (SL), stem fresh weight (SFW), stem dry weight (SDW), root diameter (RD), root length (RL), root volume (RV) and leaf length (LL), were measured for all plants. (Bagheri *et al*., 2020; Hashemi *et al*., 2020; Li *et al*., 2020). These parameters were measured 2 weeks after inoculation, by gently up-rooting each plant. Root volumes were measured using changes in the water volumes (mm^3). Each main root from the point of first secondary root initiation, and root collar diameter (mm) were measured using a digital caliper (accuracy = 0.01 mm). In addition, stem, root and the sixth leaf lengths were measured for each plant. Plant part dry weights were measured after drying at 80°C until constant weight (Hashemi *et al*., 2020).

RNA extraction and cDNA synthesis

The IRaizol kit (RNA Biotech Co.) was used for RNA extractions. For each sample, approx. 100 mg of fresh tip leaf tissue was ground to a fine powder in liquid nitrogen, and was the homogenized with 1 mL of extraction buffer containing 4 M guanidium thiocyanate, 25 mM sodium citrate/pH 7.0, 0.5% (w/v) *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol, and was then held at room temperature for 5 min. Chloroform (300 µL) was then added and mixed, the mixture was centrifuged at 13,000 g for 5 min. After transferring the supernatant, 1 mL of absolute ethanol was added and nucleic acid was precipitated (Moghaddam *et al*., 2019). After determining the quality and quantity of the extraction product using electrophoresis and nanodrop (NanoDrop 2000, Thermo Scientific), cDNA was synthesized with an RB M-MLV Reverse Transcriptase Kit (RNA Biotech Co.). Initially, extracted RNA was treated with DNase I (RNA Biotech, Co.), and cDNA synthesis was performed using the RB MMLV Reverse Transcriptase Kit (RNA Biotech, Co,) according to the manufacturer's instructions. First, 0.5 µg of treated RNA was mixed with 2 µM Oligo (dT) Primer and 1 mM dNTPs; this mix was then heated at 65°C for 10 min and then immediately placed on ice for 8–10 min. Then, RT buffer (5×; 4 µL) and 1 µL (200 units) of reverse transcriptase were added to each tube, and the tubes were incubated at 50˚C for 50 min followed by 15 min incubation at 72˚C to stop cDNA synthesis (McNeece *et al*., 2019; (Moghaddam *et al*., 2020).

Primer design

The primer sequences of pathogenesis-related protein 2 (*PR-2*), acidic endochitinase (*ChtA*), pathogenesis-related protein 5 (*PR-5*), pathogenesis-related protein 1b (*PR-1b*), proteinase inhibitor II (*PIN2*), ethylene-responsive transcription factor 3 (*ERF3*), phenylalanine ammonia-lyase (*PAL*) and lipoxygenase (*LOX*) genes were selected from the study of Arseneault *et al*. (2014), and elongation factor 1-alpha (*ef1-α*) by that of Gangadhar *et al*. (2016). The sequence of primers were evaluated and approved by Oligo Primer Analysis Software (ver. 7, Molecular Biology Insights). These primers reproduce fragments in the range of 60 to 66 bp (Supplementary Table 1).

Real-time PCR conditions

Real-time PCR reactions were carried out an RB Sybr qRT-PCR 2X Master Mix (RNA Biotech, Co.,) and in a StepOne Real-Time PCR instrument (Thermo Fisher Scientific). The reaction mixtures were each prepared in 0.1 mL qPCR 8-Strip Tubes (Gunster Biotech) as follows: 250 ng of cDNA, 12.5 µL of RB Sybr qRT-PCR 2X Master Mix and 0.25 µM of each primer in a final volume of 25 µL. The reaction temperature program was set as follows: 4 min at 94°C, then 40 cycles each at 94°C for 20 sec, annealing temperature (specified for each primer pair) for 20 sec and 72°C for 40 sec (Sohrabipour *et al*., 2018)., two technical repetitions were used for each sample. After the qPCR reaction was complete, the threshold cycle (Ct) values for each cDNA were calculated using StepOne Software (ver. 2.3, Thermo Fisher Scientific), and the equation was used to determine the relative expression levels of the evaluated genes (Wan *et al*., 2020). The *ef1-α* housekeeping gene was used for data normalization (Lekota *et al*., 2019; Liu *et al*., 2020).

Evaluation of defense-related enzyme activities

Protein extract preparation

Sodium phosphate buffer (0.1 M, pH 6.8) was mixed with 200 mg of each potato leaf sample, and then homogenized. The mixture was then centrifuged at 13,000 g for 10 min, the supernatant (protein-extract) was separated, and the protein concentration was determined using the Bradford method with a known concentration of Bovine serum albumin (A8806, Sigma) (Bradford, 1976; Nasr-Esfahani *et al*., 2020; Bagheri *et al*., 2021).

Superoxide dismutase (SOD) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 50 mM, methionine 13 mM, EDTA 0.1 µM, riboflavin 2 µM) was mixed with 100 µL of protein extract. This was then placed in the light for 15 minutes. For experimental control samples, the reaction buffer without protein extract was placed in darkness. Mixture absorption was measured at 560 nm. SOD activity was expressed units per mg protein (Giannopolitis and Ries, 1977).

Catalase (CAT) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 50 mM, pH 7, hydrogen peroxide 15 mM) was mixed with 100 µL of protein extract, and a mixture absorption change curve was recorded at 240 nm for 3 min. The enzyme activity was measured based on unit changes at 1 min mg^{-1} protein (Dazy *et al*., 2009).

Phenylalanine ammonia-lyase (PAL) activity

For each sample, approx. 2 mL of reaction buffer (Tris-hydrochloric acid 0.5 mM, pH 8, Phenylalanine 6 µmol) was mixed with 100 µL of protein extract and then held at 40°C for 1 h. To inhibit the reaction of cinnamic acid production from phenylalanine, 50 µL of hydrochloric acid (5 N) was added to the mixture, and absorption was measured at 290 nm. Enzyme activity was based on nanomoles of cinnamic acid production min^{-1} mg^{-1} protein (Beaudoin-Eagan and Thorpe, 1985; Kroner *et al*., 2011).

Peroxidase (POX) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 25 mM, pH 7, guaiacol 5 mM) was mixed with 100 µL of protein extract, and the spectrophotometer was zeroed with this mixture at 470 nm. Then, 5 µL of 30% hydrogen peroxide was added to the mixture and absorption was immediately measured at 10 sec intervals for 1 min. Enzyme activity was based on absorption changes min^{-1} mg^{-1} protein (Radotić *et al*., 2000).

Polyphenol oxidase (PPO) activity

For each sample, approx. 2 mL of reaction buffer (phosphate buffer 200 mM, pH 6, pyrogallol 20 mM) was held at 40°C, and 100 µL of protein extract was then added, mixture absorption changes in the mixture were measured at 430 nm (Raymond *et al*., 1993).

Statistical analyses

All the experiments were carried out with completely randomized designs, each with three replications, and with two technical replications for gene expression analyses. The qPCR data were analyzed using StepOne software and two-way analyses of variance (ANOVA). Enzyme activity data were analyzed using one-way ANOVA, and the LSD method was used for comparisons of means. The statistical analyses were carried out using SPSS software (ver. 16.0) (Li *et al*., 2018).

RESULTS

Disease severity evaluations of potato genotypes inoculated with Alternaria alternata

Analysis of variance of data from potato genotype reactions to *A. alternata* indicated different effects ($P \leq 0.01$) of inoculations on the host genotypes (Table 1). The greatest mean proportions of infection were 80% for genotype 8707/112, 78% for 8707/106, and 77% for 8703/804. The least mean infection proportions were 12% for 10/33/R1, 18% for both the 3/33/R2, and 21/33/R2 genotypes.

Effects of inoculations on biomass growth parameters

Variance analyses of potato plant biomass growth parameter data (Figure 3, A to J) showed that host geno-

Table 1. The impacts of the potato genotypes[a] evaluated in this study, including genotype numbers, registered name, .origin, scientific name, company, disease severity (%) and reaction to disease to leaf spot disease, *Alternaria alternata.*

S/No	Genotype No	Registered name	Origin	Scientific name	Company	Disease severity*, ** (%)	Reaction
1	10.25	10/33/R1	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII[a]	11.66[b] ± 2.88	Resistance
2	3.25	3/33/R2	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	18.33[b] ± 5.77	Resistance
3	21.25	21/33/R2	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	18.33[b] ± 5.77	Resistance
4	4.23	8707/106	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	76.66[a] ± 5.77	Susceptible
5	10.23	8703/804	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	78.33[a] ± 5.77	Susceptible
6	7.23	8707/112	Karaj-Iran	*Solanum tuberosum* L.	PORD/SPII	80.00[a] ± 10.00	Susceptible

*, ** significant at 5 or 1% probability level.

[a] Potato and Onion Research Department, Seed and Plant Improvement Institute, Karaj, Alborz Province, Iran.

Pedigree/Properties ♀*♂: These lines are the outcome of the cross between Lotta ♀*♂ Kaiser; Agria ♀*♂ Savalan; Agria ♀*♂ Kaiser; Kaiser ♀*♂ Savalan. The main parents are: Agria- Quarta ♀*♂ Semlo; Kaiser – Monalisa ♀*♂ Rop B 1178, and Savalan- 91/6122 ♀*♂ 88/05.

The experiments were performed in a completely randomized design (CRD) with 3 replications and 2 technical replications for gene expression analysis.

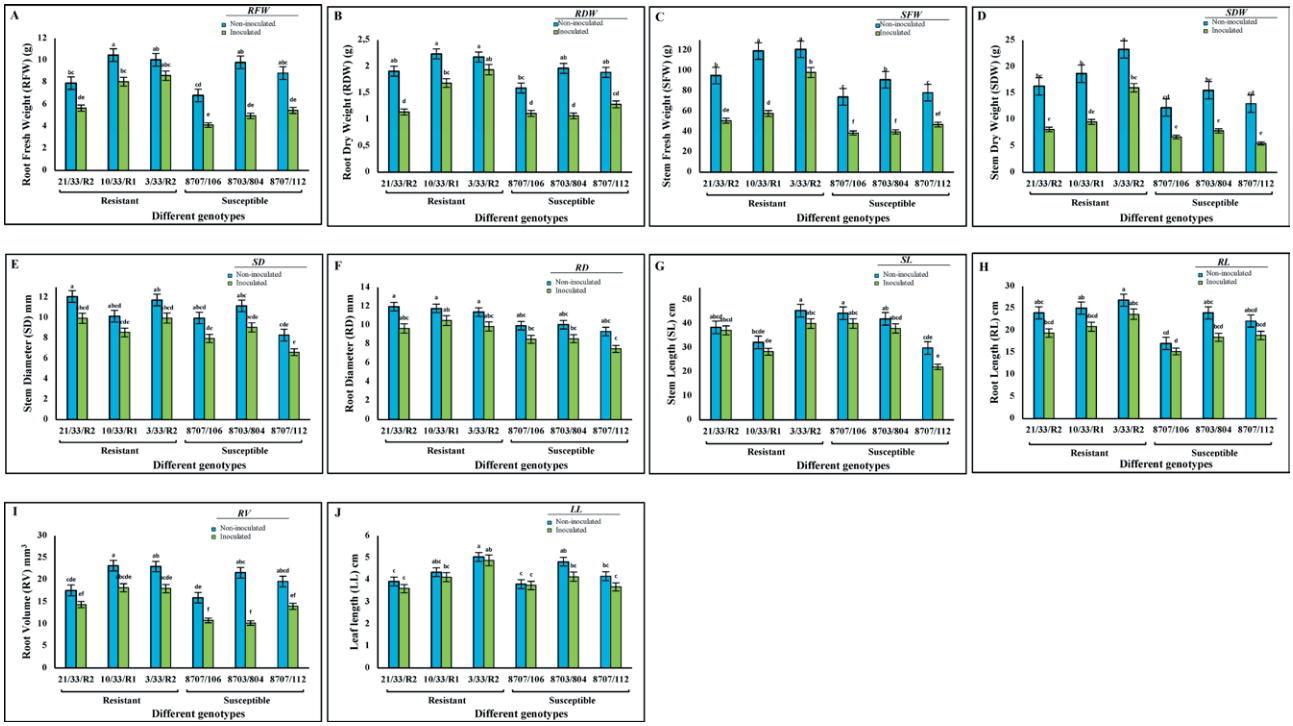

Figure 1. Variance analysis of potato plant biomass growth parameters, and comparison of the mean individual effects of inoculation treatment and host genotype in *Alternaria alternata* inoculated resistant and susceptible potato genotypes as compared to non-inoculated controls.

type and inoculation treatment affected ($P \leq 0.01$) plant growth (Supplementary Table 2). However, only the interaction of these two factors was statistically significant for SFW (Figure 1 C). Comparison of the individual means for the inoculation treatment effects showed reductions in parameters for inoculated samples compared to uninoculated controls. Individual effects of genotype were also statistically significant except SL (Figure 1 G), and LL, between the resistant and susceptible genotypes. Overall, the mean growth parameters were greater in non-inoculated resistant genotypes than in the inoculated plants. Greatest mean RFW was 10.5 g and mean dry

Table 2. Mean squares of analysis of variance for the relative expression level of defense genes, for susceptible and resistant potato genotypes to leaf spot disease, *Alternaria alternata.*

S.O.V	df	*PR-2*[a]	*ChtA*	*PR-5*	*PR-1b*	*PIN2*	ERF3	PAL	LOX
Inoculation treatment (I)	1	60.723**	22.040**	7.327**	22.070**	3.958**	0.014**	0.591**	10.731**
Genotype (G)	5	12.483**	4.237**	5.620**	6.257**	7.413**	0.833**	0.942**	2.173**
Interaction I × G	5	11.213**	3.681**	1.791**	6.734**	0.815**	1.158**	0.206**	0.834**
Error	11	0.280	0.139	0.061	0.087	0.067	0.034	0.036	0.076

Ns, *, ** not significant or significant at 5 or 1% probability level, [a]*PR-2* (pathogenesis-related protein 2), *ChtA* (acidic endochitinase), *PR-5* (pathogenesis-related protein 5), PR-*1b* (pathogenesis-related protein 1b), *PIN2* (proteinase inhibitor II), *ERF3* (ethylene-responsive transcription factor 3), *PAL* (phenylalanine ammonia-lyase) and *LOX* (lipoxygenase).

weight was 2.2 g for the non-inoculated resistant genotype 10/33/R1, and greatest mean SFW was 120.9 g and SDW was 23.3 g for 3/33/R2. Genotype 3/33/R2 had the greatest mean SD (11.8 cm), mean RD (11.4 cm), mean SL (45.5 cm), mean RL 26.9 cm), mean LL (23.0 cm), and mean RV (5.1 cm^3) (Figure 1). Correlation between plant growth parameters were statistically significant for all the evaluated factors, except for the relationship between SL with RFW, RDW, RL) RV (Figure 1).

Expression pattern analyses for defense genes

Analysis of variance of changes in expression levels of marker genes *PR-2*, *ChtA*, *PR-5*, *PR1-b*, *PIN2*, *ERF3*, *PAL* and *LOX* are presented in Table 2. These showed a significant ($P \leq 0.01$) effects of the inoculation treatment and host genotypes and their interactions on the transcription rates of the evaluated genes.

Expression of marker genes increased after inoculations with *A. alternata* in the resistant host genotypes (10/33/R1, 3/33/R2 and 21/33/R2), which had the lowest disease severities in the primary field resistance screening tests. The susceptible genotypes (8707/106, 8703/804 and 8707/112) and the non-inoculated controls genotypes had the lowest gene expression values (Figure 2, A, B, C, D and G). Up-regulation of *PR-2* in the host genotypes 10/33/R1, 3/33/R2 and 21/33/R2 was 4.95, 2.95, and 4.59 fold greater than that of controls. Up-regulation of *ChtA* was, respectively, 3.34, 3.29 and 4.46 fold greater in these three genotypes, and for *PR-5* was, respectively, 2.33, 1.87 and 1.73 fold greater than for the susceptible genotypes and the non-inoculated controls. Transcription of the *PR1-b* gene in these genotypes was, respectively, 2.46, 3.48, and 2.35-fold greater compared with non-inoculated controls. For the *PAL* gene, this increase was 1.49 and 1.30-fold, respectively, in genotypes 10/33/R1 and 3/33/R2. In genotype 21/33/R2, however, there was no change in *PAL* expression. Expression levels of these genes in the susceptible genotypes (8707/106, 8703/804 and 8707/112) decreased or were un-changed (Figure 2, A, B, C, D, and G). In genotype 8707/106, transcription changes of all the five genes assessed were not statistically significant. In genotype 8707/112, expression of *PR-2*, *ChtA* and *PR1-b* genes were un-changed, but increased by 1.58-fold got *PR-5* and 1.42-fold for *PAL* compared to non-inoculated controls. In genotype 8703/804, expression of *PR-2* and *PAL* genes remained un-unchanged, *ChtA* was up-regulated (1.69-fold), and *PR-5* and *PR1-b* genes were down-regulated (respectively, 1.58 and 1.88-fold) compared to susceptible genotypes and the non-inoculated controls genotypes.

Changes in the transcription levels of *PIN2*, *ERF3* and *LOX* genes in resistant and susceptible genotypes showed decreasing trends (Figure 2, E, F and H). For *PIN2* the relative expression level was 1.79-fold less in genotype 3/33/R2, 1.27-fold less in 21/33/R2 and 3.43-fold less in 8707/106 than for the non-inoculated controls. In genotypes 10/33/R1 8707/112 and 8703/804 expression of *PIN2* was un-changed. Reductions in expression of *ERF3* in the three resistant and 8703/804 genotypes were, respectively, 1.82, 2.34, 1.91 and 1.74-fold. Down-regulations in *LOX* expression were 1.49-fold in genotype 3/33/R2, 3.35-fold in 21/33/R2, 7.07-fold in 8707/106, and 4.65-fold in genotype 8707/112, compared with susceptible genotypes and the non-inoculated controls.

Comparison of gene expression level changes between inoculated resistant and susceptible potato genotypes indicated expression of *PR-2*, *ChtA*, *PR-5*, *PR1-b* and *PAL* genes in the resistant genotypes increased more than in susceptible genotypes, and that gene expression was 8.31-fold greater for *PR-2*, 5.72-fold greater for *ChtA*, 2.47-fold greater for *PR-5*, 8.61-fold greater for *PR1-b*, and 2.36 fold greater for *PAL*. For the *PIN2* and *LOX* genes, expression decreased in the *A. alternata* inoculated plants, and this decrease in *PIN2* and *ERF3* genes in resistant genotypes was greater than in the susceptible plants, with 1.24-fold decrease for *PIN2* and 1.87 fold decrease for *ERF3*. In the *LOX* gene, reductions in

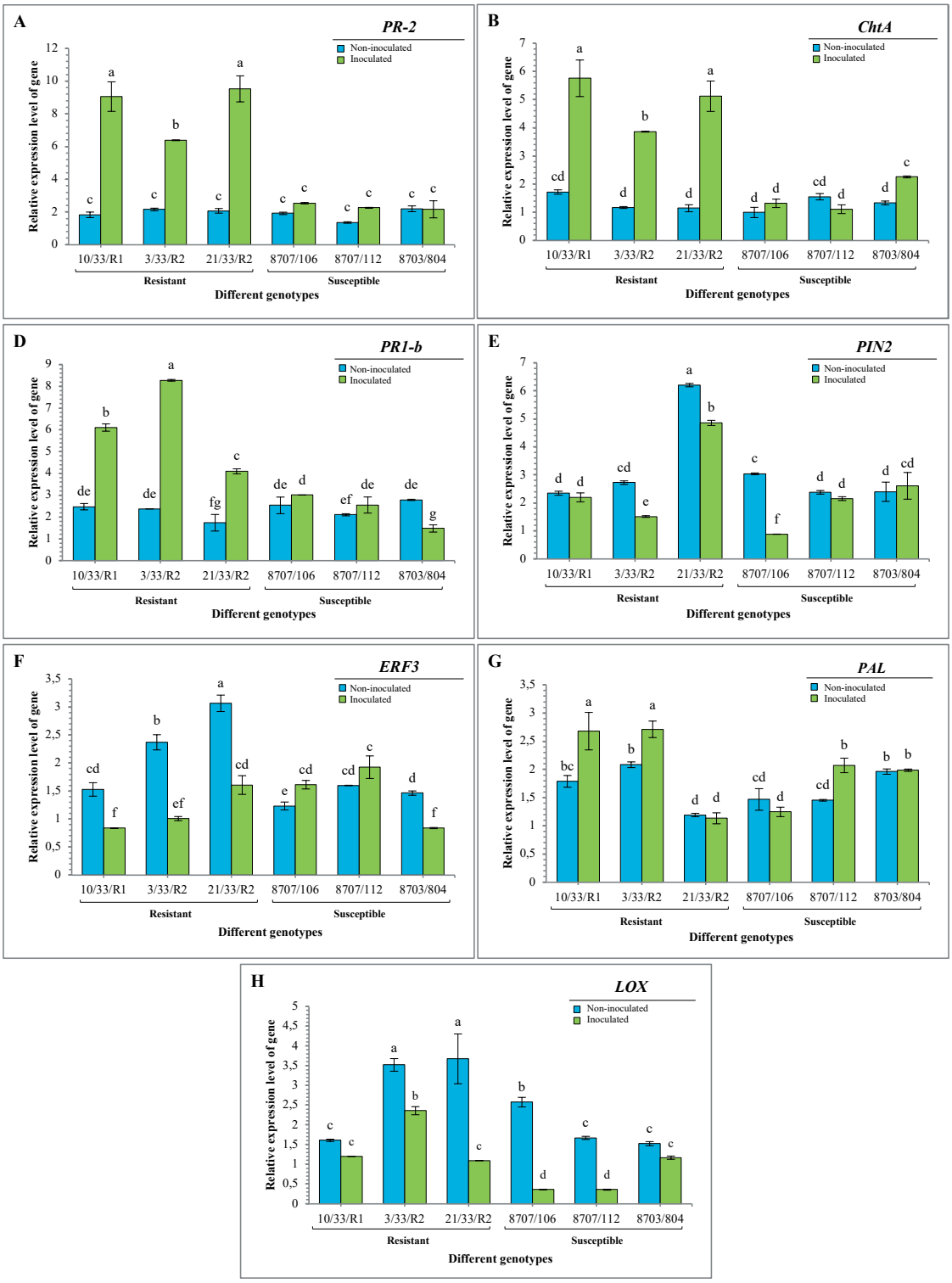

Figure 2. Relative expression levels of the genes for pathogenesis-related protein 2 (*PR-2*), acidic endochitinase (*ChtA*), pathogenesis-related protein 5 (*PR-5*), pathogenesis-related protein 1b (*PR-1b*), proteinase inhibitor II (*PIN2*), ethylene-responsive transcription factor 3 (*ERF3*), phenylalanine ammonia-lyase (*PAL*), and lipoxygenase (LOX), for *Alternaria alternata* susceptible (4.23, 7.23, 10.23) and resistant (10.25, 3.25, 21.25) potato genotypes. Normalization of the data obtained from the Real-time PCR reactions was carried out using the EF1α housekeeping gene. The experiment was carried out with three biological replicates for each sample and two technical replicates. Different letters indicate statistically significant differences ($P \leq 0.05$).

Table 3. Variance analysis of quantification of enzymes activities in inoculated resistant and susceptible potato genotypes as compared to controls, un-inoculated ones to leaf spot disease, *Alternaria alternata*.

S.O.V	df	Total protein	POX		SOD		PPOs		CAT		PAL	
			Activity	Specific activity	Activity	Specific activity	Activity	Specific activity	Activity	Specific activity	Activity	Specific activity
Inoculation treatment (I)	1	0.258**	6.352**	2.054**	0.605**	0.198**	4231.5**	1982.3**	98.3**	25.3**	1.021**	0.325**
Genotype (G)	5	0.316**	7.032**	3.004**	0.706**	0.252**	5054.4**	2007.6**	109.6**	31.1**	1.124**	0.354**
Interaction I × G	5	0.148**	5.264**	1.985**	0.352**	0.158**	3826.5**	2145.3**	85.3**	21.3**	1.251**	0.425**
Error	11	0.014	0.032	0.012	0.024	0.001	38.2	1.3	5.9	0.139	0.021	0.001

Enzymes activity of, superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPOs), catalase (CAT), and phenylalanine ammonia-lyase (PAL).
** = Significant at 1% probability level.

expression in susceptible and resistant genotypes were very similar (Figure 2).

Changes in defense enzyme activities

Variance analyses of enzyme activity quantification data for inoculated resistant and susceptible potato genotypes compared to non-inoculated controls showed that host genotype factor affected ($P \leq 0.01$) enzyme activities in *A. alternata*-inoculated plants (Table 3). The greatest increase in activity was detected for the POX and PPO enzymes in genotype 10/33/R1, with 7.4-fold increase in POX and 4.7-fold increase in PPO (Figure 3, A, B, C, D and E). Changes in the specific activity of POX, SOD and PPOs enzymes (Figure 3, A, B and C) in inoculated resistant genotypes (10/33/R1, 3/33/R2 and 21/33/R2) showed significant ($P \leq 0.05$) incremental trends (Table 4) in all the three genotypes compared to non-inoculated controls. Specific activity of PAL was up-regulated 3.3 and 2.0-fold, compared to the controls. In genotype 3/33/R2, specific activity of CAT was un-changed, while that of PAL was 1.5-fold less compared with control samples. The activities of all the enzymes (POX, SOD, PPOs, CAT and PAL) in the susceptible genotypes (8707/106, 8703/804 and 8707/112) did not change greatly, with the greatest increase of 1.3-fold in comparison to resistant genotyped, and 7.4-fold increase after inoculation compared to non-inoculated controls (Figure 3).

Relationships between of defense gene expression, enzyme activities and biomass growth parameters

The results in the Table 5 showed statistically significant positive correlations between *PR-2*, *ChtA*, *PR-5* and *PR1-b* genes with POX, SOD and PPOs enzyme activities; *ChtA*, *PR-5*, *PR1-b* and *PAL* genes with PAL enzyme; *PAL* gene with POX, PPOs, CAT and PAL enzymes; and *PIN2* gene with CAT enzyme activities. In addition, changes in expression levels of these genes also showed positive significant correlations with some of the potato plant parameters, such as *PIN2*, *ERF3* and *LOX* genes with RFW, RDW, SFW SDW and RL; *ERF3*-SD; *LOX*-RD; *PAL*-SL; *PIN2* and *LOX* with RV; and PIN2, *ERF3* and *PAL* with LL, respectively (Table 5). For the *PAL* gene, a significant positive correlation was recorded with PPOs and PAL enzymes and SL parameter. The *LOX* gene did not show any correlation with changes in defense enzymes, and a significant positive correlation was found with the parameters SFW, RDW, SDW, RD, RL or RV. No statistically significant correlations were detected between the activities of defense enzymes and plant growth parameters, except in CAT enzyme with SL (r = .617*) (Table 5).

DISCUSSION

There were considerable changes in biomass growth parameters, with decreasing trends in plants inoculated with *A. alternata*, compared to the non-inoculated plants. The reductions in these parameters in susceptible genotypes were greater than in the resistant plants. Similar effects of *A. alternata* have been demonstrated in cotton seedlings (Le and Gregson, 2019), tomato (Moghaddam *et al*., 2019), *Cucumis sativus* (Matrood *et al*., 2020), and American ginseng (Neils *et al*., 2021). Reductions in growth of potato due to the pathogen in the present study confirms the deleterious effects of *A. alternata* on host growth.

The present study has shown significant increases in relative expression of marker genes in all the inoculated resistant potato genotypes. McNeece *et al*. (2019) stated that pathogenesis-related (PR) protein synthesis

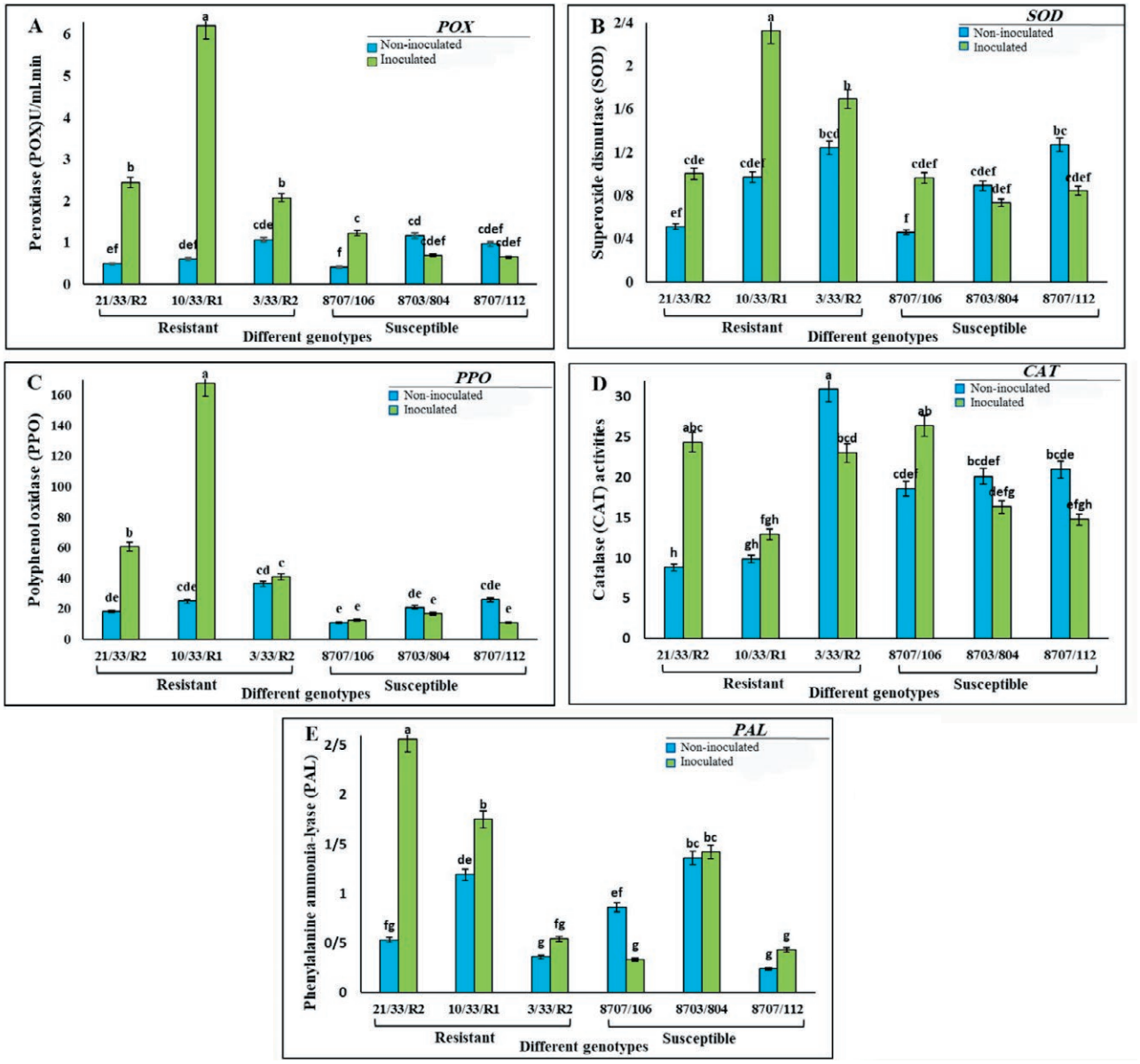

Figure 3. Activities of peroxidase (A), superoxide dismutase SOD (B), polyphenol oxidase PPO (C), catalase CAT (D), and phenylalanine ammonia lyase PAL (E) in *Alternaria alternata* inoculated potato genotypes that are resistant or susceptible to leaf spot.

genes provided resistance to pathogens in several crop plant types. Changes in *PR-2*, *ChtA*, *PR-5* and *PR1-b* in resistant potato genotypes indicate important roles of these genes in the direct defense mechanism of potatoes against *A. alternata*. Differences in resistance between the six potato genotypes can be partly explained by the potential for expression of defense-related proteins. Studies by Hoegen *et al.* (2002), Wang *et al.* (2008), Derksen, *et al.* (2013) and Moghaddam *et al.* (2019) have emphasized this hypothesis, supporting the role of *PR* genes in enhancing resistance to biotic stresses and providing an approach for development of disease-resistant transgenic products (Ali *et al.*, 2018). Zhai *et al.* (2018) used RNAi technology to silence the tomato *PR5* gene to *A. alternata,* and Toufiq *et al.* (2018) isolated *ChtA* gene from *Hordeum vulgare* L., which could inhibit important pathogenic fungi. Khan *et al.* (2017) generated transgenic potato which overexpressed the *H. vulgare endo-chitinase* gene, which gave high resistance of transgenic potato plants to *A. solani*. Thus, changes in expression levels of *PR* genes in resistant and susceptible potato genotypes to *A. alternata* detected in the present study,

Table 4. Quantification of enzymes activity in inoculated resistant and susceptible potato genotypes as compared to controls, non-inoculated ones to *Alternaria alternata*.

Resistance	Treatment	POX	SOD	PPOs	CAT	PAL
Resistant	Control	15.33b	0.225c	34.74b	7.32ab	0.717c
	Inoculated	3052a	0.885a	76.10a	8.26a	1.583a
Susceptible	Control	15.11b	0.239c	29.78b	4.79c	0.750b
	Inoculated	17.11b	0.409b	27.12b	5.57bc	0.667d

Enzymes activity of Superoxide dismutase (SOD), peroxidase (POX), polyphenol oxidase (PPOs), catalase (CAT), and phenylalanine ammonia-lyase (PAL).
Means in each column having same letter are not significantly different according to LSD test (P≤0.05).

Table 5. Correlation between expression of defense genes, enzymes activities and biomass growth parameters in inoculated resistant and susceptible potato genotypes as compared to controls, un-inoculated ones to leaf spot disease, *Alternaria alternata.*

Genes	POX[a]	SOD	PPOs	CAT	PAL	RFW	RDW	SFW	SDW	SD	RD	SL	RL	RV	LL
PR-2[b]	0.694*	0.943**	0.588*	0.320	0.471	-0.099	-0.154	-0.133	-0.161	-0.055	-0.033	-0.046	-0.021	-0.117	0.013
ChtA	0.693*	0.933**	0.596*	0.233	0.515*	-0.116	-0.184	-0.176	-0.224	-0.065	-0.066	-0.152	-0.009	-0.110	-0.067
PR-5	0.706*	0.904**	0.658*	0.173	0.661*	0.110	0.014	0.053	0.069	0.244	0.274	0.033	0.248	0.078	0.153
PR-1b	0.948**	0.828**	0.925**	-0.237	0.787**	-0.105	-0.194	-0.306	-0.301	-0.142	0.077	-0.212	-0.156	-0.103	-0.189
PIN2	-0.161	0.108	-0.180	0.604*	-0.365	0.528*	0.562*	0.686*	0.729**	0.474	0.350	0.401	0.635*	0.520*	0.712**
ERF3	-0.394	-0.344	-0.335	0.296	-0.326	0.521*	0.553*	0.667*	0.766**	0.516*	0.498	0.475	0.594*	0.487	0.689*
PAL	0.514*	0.461	0.590*	-0.562*	0.766**	-0.170	-0.303	-0.391	-0.440	-0.290	-0.032	-0.621*	-0.158	-0.141	-0.506*
LOX	0.030	0.036	0.154	0.014	0.040	0.750**	0.750**	0.755**	0.736**	0.466	0.727**	0.125	0.617*	0.813**	0.408

*, **: Correlation is significant at the 0.05 or 0.01 level.
[a] POD = Peroxidase, superoxide dismutase (SOD), polyphenol oxidase (PPOs), catalase (CAT), phenylalanine ammonia-lyase (PAL), root fresh weight (RFW), root dry weight (RDW), stem fresh weight (SFW), stem dry weight (SDW), stem diameter (SD), root diameter (RD), stem length (SL), root length (RL), root volume (RV) and leaf length (LL).
[b] *PR-2* (pathogenesis-related protein 2), *ChtA* (acidic endochitinase), *PR-5* (pathogenesis-related protein 5), PR-*1b* (pathogenesis-related protein 1b), *PIN2* (proteinase inhibitor II), *ERF3* (ethylene-responsive transcription factor 3), *PAL* (phenylalanine ammonia-lyase) and *LOX* (lipoxygenase).The bolded numbers show the significant correlations between the defense genes, enzymes activities and biomass growth parameters in inoculated resistant and susceptible potato genotypes as compared to controls, un-inoculated ones to leaf spot disease, *Alternaria alternata.*

and consistency of the results with the other reports, indicates that *PR* genes play important roles in creating resistance in potato genotypes against this pathogen (Moghaddam *et al*., 2019; Bagheri *et al*., 2020). However, knowledge of the molecular defense responses with systemic (leaf) defenses before and after *A. alternata* inoculation in potato is scarce. The present study is the first to provide information on leaf gene expression levels of *PR-2, ChtA, PR-5, PR1-b, PIN2, ERF3, PAL* and *LOX* in six contrasting genotypes of potato after inoculation with *A. alternata*.

In addition to pathogenesis-related (PR) protein synthesis genes, plant hormone mediated signaling pathways also play an important roles in plant disease resistance. Up-regulation of PR protein synthesis genes, *PR-2*, *ChtA*, *PR-5*, *PR1-b* and *PAL* was a reaction to the presence of *A. alternata* infections. These genes likely encode key enzymes in the salicylic acid (SA) biosynthesis pathway (Tian *et al*., 2020). Potato plants exhibit increased resistance to *A. alternata* infections utilizing both salicylic acid (SA) and jasmonic acid (JA) signaling pathways (Derksen *et al*., 2013; Brouwer *et al*., 2020). Additionally, it is becoming increasingly evident that not only the JA and SA pathways are important in the host defense against necrotrophs, but that the plant hormones abscisic acid and indole acetic acid can also modulate host defense against necrotrophs, including *A. alternata* (Derksen *et al*., 2013; Brouwer *et al*., 2020). However, the mechanism was demonstrated by Tian *et al*. (2020), where the two defense-related hormones, salicylic acid and jasmonic acid signaling transduction pathways have antagonistic effects. In general, the jasmonic acid signaling pathway enhances resistance to hemi-biotrophic and necrotrophic pathogens, while resistance to biotrophic pathogens mainly depends

on salicylic acid signaling pathways. The present study confirms that up-regulation of PR protein synthesis genes and antioxidant enzymes occurs in pathogen-resistant potato genotypes (Hu *et al*., 2018).

Analysis of the expression level of the *PIN2* gene in resistant and susceptible potato genotypes in response to *A. alternata* indicated expression did not change or decreased in inoculated genotypes. Arseneault *et al*. (2015) reported no changes in *PIN2* expression in potato leaves to inoculated with *Pseudomonas fluorescens* LBUM223 or *Streptomyces scabies* (Arseneault *et al*., 2014). In the present study, changes in *PIN2* gene transcripts did not affect resistance in the potato genotypes to *A. alternata*. The effects of *PIN* genes are considered as not suitable for genetic engineering for the resistant plants against this pathogen. (Turra and Lorito, 2011).Previous and the present study therefore confirm that *PIN* gene expression is little-affected by the fungal pathogens.

Ethylene-response factors (ERFs) are transcription factors binding to specific motifs on DNA and regulate ethylene-dependent resistance responses (Debbarma *et al*., 2019). Kim *et al*. (2012) showed overexpression levels of the genes *ERF I* and *ERF II* in sweet potato leaves in response to *Pectobacterium chrysanthemi*. Ogata *et al*. (2012) showed similar responses in tobacco to tobacco mosaic virus , and potato also similarly responded to *P. infestans* (Chen *et al*., 2008; Gallou *et al*., 2011). RNA-Seq analysis in apples inoculated to the *A. alternata* apple pathotype showed induction of subfamilies of *ERF* and *DREB* genes (Huang *et al*., 2016). A model to explain the response of chrysanthemums to *A. alternata* based on RNA sequencing information showed that the products of genes for abscisic acid signalling, salicylic acid, EDS1, ethylene metabolism (ERF2) and extrusion compounds (MATE) could play important roles in defending against *A. alternata* (Li *et al*., 2020). Contrary to these studies, the present study found that expression of *ERF3* in infected resistant potato genotypes decreased compared to non-inoculated controls. This suggests that the *ERF3* protective function is indirect, and changes in *ERF3* patterns are likely to lead to expression of defense genes that may enhance resistance to *A. alternata*. This hypothesis could be confirmed by functional studies, such as where gene silencing was confirmed by application of *Streptomyces scabies* in infection of potato (Arseneault *et al*., 2014), and by *A. solani* (Tian *et al*., 2020), where reductive changes in *ERF3* gene were also reported.

Activity of lipoxygenases has also been identified in pathogenic defense response processes, confirmed by Kolomiets *et al*. (2000) for accumulation of *POTLX-3* mRNA in the leaves of potatoes infected by *P. infestans*, and by Hu *et al*. (2015) for susceptibility of a transgenic host to a *Cladosporium fulvum* was increased. Hou *et al*. (2018) also generated transgenic *Arabidopsis* which overexpressed the persimmon *9-LOX* gene, indicating responses of increased salicylic acid content and bacterial mortality, and decreased cell death occurred in *Arabidopsis* to *Pseudomonas syringae* pv. *tomato*. Increasing changes in *LOX* gene expression levels have also been reported in *Fusarium oxysporum*-inoculated *Iris* (Tehrani *et al*., 2020) and *P. melonis*-inoculated cucumbers (Hashemi *et al*., 2020). In the present study, expression of *LOX* in resistant and susceptible potato genotypes decreased after inoculation with *A. alternata*, which was not consistent with the results of Kolomiets *et al*. (2000) for *P. infestans*, Hu *et al*. (2015) for *Cladosporium fulvum* or Hou *et al*. (2018) for to *P. syringae* pv. *tomato*. This could be due to decreased expression of the *ERF3* gene, or to increased reactive oxygen species, or to weakening of the lipoxygenase pathway. Results of the present study indicated that the defense response induced by *A. alternata* inoculation was different in the six selected potato genotypes. Expression of marker genes *PR-2*, *ChtA*, *PR-5* and *PR1-b* was significantly increased in resistant infected plants, indicating that these genes are involved in the defense response. Decreasing expression of *PIN2*, *ERF3* and *LOX* genes may indicate the lack, or indirect, effect of these genes in the defense processes against to *A. alternata*.

The present study also revealed changes in the activity of candidate enzymes in all the resistant potato genotypes, where activity of CAT enzyme in 21/33/R2 genotype and PAL in 21/33/R2 and 10/33/R was significantly increased. In similar studies, the resistance-inducing substances, salicylic acid, abscisic acid and *Pseudomonas fluorescens* increased activity of POX, PPO and PAL enzymes in potato infected by *P. infestans* and tomato infected by *P. atrosepticum* (Kroner *et al*., 2011), tomato infected by *A. alternata* (Moghaddam *et al*., 2020), and in cucumber (Hashemi 2020; Nasr-Esfahani *et al*., 2020). Results of the present study are consistent with this research, which showed increasing activity of the enzymes created resistance in potato genotypes. Induction of oxidation reactions to *A. alternata* and production of free radicals leads to the formation of chain reactions that damage cells (Moghaddam *et al*., 2019; 2020). Increasing activity levels of POX, SOD, PPOs and PAL enzymes terminate chain reactions and create oxidative balance. As a result, increasing the activity of antioxidant enzymes will lead to the biochemical response of resistant in potato genotypes and other crops (Kroner *et al*., 2011; Nasr-Esfahani *et al*., 2020; Bagheri *et al*., 2021). There is a critical need for understanding of the genetic population and biochemical response of resistant and

susceptible potato to *A. alternata*, and for incorporating this knowledge into plant breeding strategies to develop *A. alternata*-resistant crops.

There are no previous reports of marker genes, protein profiles and changes of host growth parameters in response to of *A. alternata* infection of potato, using transcriptomics-proteomics-biomass approaches. The results presented here are a basis for future studies, to design efficient disease management strategies against early blight of potato.

CONCLUSIONS

From forty-nine potato genotypes screened for resistance to *A. alternata*, lowest infection percentages were recorded for the three genotypes 10/33/R1, 3/33/R2, and 21/33/R2 which are possible sources of resistance to this pathogen. Decreasing trends in biomass growth parameters were also recorded for plants inoculated with *A. alternata* compared to un-inoculated controls, and these decreases were greater in the susceptible than in the resistant genotypes. Molecular analyses of eight genes and five enzymes potentially involved in host resistance have demonstrated that inoculation of potato plants with *A. alternata* increased expression of marker genes and activity of enzymes in inoculated resistant potato genotypes compared to non-inoculated controls. Studies to evaluate genes and enzymes involved in defense processes in different potato genotypes can increase knowledge of the roles of these factors in plant defense processes. This knowledge can assist in identifying and selecting resistant genotypes. The use of resistance resources in breeding programmes will lead to production of new cultivars with high performance and resistance to biotic stresses.

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