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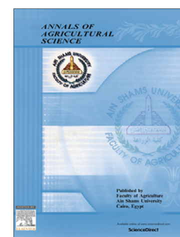

2,4-Dichlorophenoxy acetic acid, abscisic acid, and hydrogen peroxide induced resistance-related components against potato early blight (*Alternaria solani*, Sorauer)

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RT-RNA

Abstract Induction of acquired resistance activates defense-related genes. Current study aimed to (a) initiate potato acquired resistance to the Early Blight disease caused by *Alternaria solani* through treating potato plants with low and repetitive doses of 2,4-dichlorophenoxy acetic acid (2,4-D), abscisic acid (AB), and hydrogen peroxide (H_2O_2) and (b) test the success of the use of the chemical inducers along with the application of fungicides. Potato cultivars Nicola and Spunta were treated once per wk for 6 wk and challenged 1 wk later by in the control of *A. solani*. Results showed that peroxidase (POD), phenylalanine ammonia lyase (PAL), and polyphenoloxidase (PPO) enzyme activities and gene regulation were significantly increased after 1 d of infection and lasted for more than 15 d. The 2,4-D and H_2O_2 inducers significantly increased both enzyme activities and gene expression of PAL, PPO, and POD for more than 15 d post inoculation. PAL was the most increased at the enzyme activity and gene expression levels. Incorporation of such in-expensive treatments might reduce management costs and reduce the environmental pollution.

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Introduction

Potato (*Solanum tuberosum* L.) is one of the top five consumed crops worldwide because it is common, affordable, and nutritious (Lovat et al., 2015). It was the fifth most produced crop in Egypt with 4.8 MMT in 2013 (FAO, 2015). A plethora of diseases affects potato and causes significant loss of yield

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and quality (Wale et al., 2008). Among them is Early Blight (*Alternaria solani* (Sorauer)). It causes defoliation and reduces tuber yield and quality by 20–30% (Wale et al., 2008). Management strategies for *A. solani* depend mainly on the application of fungicides. These fungicides work as either protectants (chlorothalonil and dithiocarbamates) or curative/systemic (azoxystrobin, difenoconazole, and tebuconazole) and are effective at the initial developmental stage of infection (Tomlin, 2003; Wale et al., 2008). New experimental methods involve the application of abiotic or biotic inducers to help promote plants' immune response against pathogenic attack.

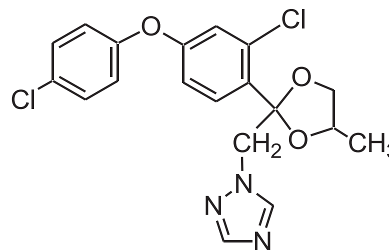
Diverse abiotic and biotic inducers, including virulent or avirulent pathogens, non-pathogenic microorganisms, cell wall fragments of microbes, plant extracts, and/or synthetic chemicals have been widely examined to induce resistance, both locally and systemically (Walters et al., 2005a,b; Choudhary et al., 2007; Wale et al., 2008). The inducers result in programmed cell death; often, an oxidative burst signals cell death (Heath, 1998), so the pathogen is surrounded by dead cells that prevent its movement to adjacent cells. At the same time, the plant is stimulated to synthesize antimicrobial compounds such as polyphenolics that hinder pathogen growth (Hammerschmidt, 1999). Accordingly, cell priming leads to the elicitation of defense-related genes in a stronger manner than under normal condition of infection (Conrath et al., 2001; Walters et al., 2005a).

The auxin-like compounds and abscisic acid have repeatedly been implicated in inducing defense responses in plants and promoting resistance to pathogens. For example, foliar sprays of the synthetic auxins 2,4-dichlorophenoxy acetic acid (2,4-D) and 3,5-dichlorophenoxy acetic acid (3,5-D) applied at or just before tuber initiation reduced common scab disease (*Streptomyces scabies*) in potato by 50% and 90%, respectively (McIntosh et al., 1981; McIntosh et al., 1985). It was later found that 2,4-D activated defense- and stress-related genes in *Arabidopsis thaliana* (Fode et al., 2008). Following below-ground herbivory in maize (*Zea mays*), abscisic acid (AB) was involved in induced systemic resistance (Erb et al., 2009) affecting development processes, including regulation of gene expression (Vleesschauwer et al., 2010).

With respect to *A. solani* control, several compounds have been used to increase plant resistance. For example, application of salicylic acid (1.5 mM) and β-amino-butyric acid (BABA; 15 mM) increased the activity of pathogenesis-related proteins; chitinase (32%) and 1,3-β-glucanase (56%) in susceptible tomato cultivars and increased resistance to *A. solani* compared to control (Raut and Borkar, 2014). Also, treating cucumber with *Pseudomonas azotoformans* GC-B19, *Paenibacillus elgii* MM-B22, and DL-β-amino-n-butyric acid effectively induced rapid elicitation of hypersensitive reaction-like cell death with the generation of H_2O_2 and increased accumulation of defense-related enzymes (β-1, 3-glucanase, chitinase, and peroxidase) against *Colletotrichum orbiculare* (Sang et al., 2014). The current study tried to (a) elevate the resistance of potato plants against Early Blight disease through multiple applications of low doses of 2,4-D, AB, and H_2O_2 and (b) test the success of these treatments along with the application of recommended fungicides in management of *A. solani*.

Materials and methods

Fungicide difenoconazole (SCORE®)



cis,trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether.

The fungicide difenoconazle (SCORE®, Syngenta, Basel, Switzerland) was purchased from a local distributor. This is a broad-spectrum systemic triazole fungicide with both preventative and curative properties against *A. solani*. It suppresses the fungal infection by inhibiting the biosynthesis of ergosterol in fungal cell membranes (Tomlin, 2003).

Chemicals

Potato dextrose agar (PDA), 2,4-dichlorophenoxy acetic acid (2,4-D, 98% purity, MP Biomedicals Inc., ICN10155380), abscisic acid (AB, >98% purity, MP Biomedicals Inc., AC133485000), hydrogen peroxide (H_2O_2, 30% certified ACS, H325-500), catechol (MP Biomedicals Inc., ICN215225), 2-mercaptoethanol (Fisher BioReagents, BP176100), polyvinylpyrrolidone (PVP; Acros Organics, AC227541000), pyrogallol (Sigma, P0381, ⩾98% HPLC grade), and L-phenylalanine (Sigma–Aldrich, P2126) were purchased from local supplier.

Plant material and growth condition

Seed tubers of potato cultivars that are resistant (Nicola) and susceptible (Spunta) to infection with *A. solani* were used in the current study (ECPD, 2015). Tubers were obtained from the International Potato Center (CIP), Chili, through the importer at Kafr El-Zayat, Gharbiya Governorate, Egypt.

Potato planting and application of chemical elicitors

Seed tubers of cvs Nicola and Spunta were surface sterilized in 1% sodium hypochlorite (Chlorax® 15%) for 5 min and washed 3 times with autoclave-sterilized water. Tubers were planted in plastic pots (30 cm diameter, one tuber per pot) filled with autoclave-sterilized peat moss. Plants were grown under greenhouse (GH) conditions (25 ± 2 °C and 65 ± 5% RH), watered once every 3 to 4 d. Fertilization was done every 6–8 d with commercial fertilizer that has N (19%), P (19%), K (19%), S (5.94%), Fe (1000 ppm), Zn (500 ppm), and Mn (500 ppm) (Farmers VASCON, Moubarak Industries City, Quesna, Menoufia, Egypt) at 1 kg/1000 L of water. Chemical inducers: 2,4-D (18 μM), AB (10 μM), H_2O_2 (5 mM), and

water (control) were sprayed onto 2-leaf-old plants to run off once every week for 6 wk in 4 separate units of the GH. The experiments were repeated twice over time.

A. solani isolate and inoculum preparation

The isolate of Early Blight pathogen, *A. solani*, was obtained from Department of Plant Pathology, Faculty of Agriculture, Damanhour University. It was maintained on potato dextrose agar (PDA) medium at 20 ± 1 °C with 16/8 (L/D) photoperiod. Sporulation was induced by maintaining cultures at 24 ± 1 °C for 6 d under near ultraviolet light (310–400 nm) with 16/8 L/D photoperiod. Conidia were collected by scraping fungal growth from the Petri dishes with sterilized distilled water. Spore suspension was filtered through cheesecloth. Spore concentration was adjusted to 10^6 spore ml^{-1} for inoculation.

Inoculation with A. solani

Eleven-week-old potato plants were inoculated by spraying with a spore suspension (10^6 spore ml^{-1}) of *A. solani* until the spore suspension was dripped from plant leaves using a high quality Kinlong Garden watering 28/410 plastic trigger sprayer (Jiangxi, China) according to Bokshi et al. (2003) and Nadia et al. (2007). Plants were covered with clear plastic bags (55 × 85 cm) for 48 h to increase humidity and facilitate fungal infection. The bags were removed and plants grown under greenhouse conditions at 25 ± 2 °C and 16/8 L/D photoperiod (Pelletier and Fry, 1989; Bokshi et al., 2003). Forty-eight hours after inoculation, plants within each treatment (2,4-D, AB, H_2O_2, and control) were divided into two groups. The first group was treated once with the fungicide difenoconazole at the recommended dose (50 ml/100 L water) and the second group was not treated. Each treatment was represented by five replicates and each replicate was represented by 3 pots.

Assessment of disease severity

Disease severity of inoculated leaves was assessed after 15 d of inoculation and categorized using a modified scale of Pandey and Pandey (2002). The percent of disease severity (PDS) was calculated using the following equation given by FAO (Anonymous, 1967) and Rodríguez et al. (2007).

$$\mathbf{PDS} = \left\{\sum(\mathbf{N} \times \mathbf{V})/(\mathbf{N} \times \mathbf{S})\right\} \times \mathbf{100}$$

where Σ = Summation, N = Number of leaves in each category, V = Numerical value of leaves observed, and S = Maximum numerical value/grade.

Defense-related enzymes

The activity of defense-related enzymes: peroxidase (POD), phenylalanine ammonia-lyase (PAL), and polyphenoloxidase (PPO) were estimated in the leaves after 0, 1, 2, 4, 8, and 15 d of inoculation. Also, the same leaf samples were used for the quantification of gene expressions of POD, PAL, and PPO using real-time PCR (RT-PCR) technique after 0, 2, and 8 d of inoculation. About 5 leaflets of the same height were randomly collected from 3 plants per replicate at each specific sampling time.

Estimation of peroxidase (POD) relative activity

Leaves of each replicate were homogenized and protein content of 1 g sample was extracted in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4 °C. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C (Universal 32R, Hettich Zentrifugen model D-78532, Germany). The supernatant was used for POD enzyme activity. The reaction mixture was 1.5 ml of pyrogallol (0.05 M), 0.5 ml of enzyme, and 0.5 ml of H_2O_2 (1%). The reaction mixture was incubated at 28 ± 2 °C. The absorbance of the mixture was recorded every 20 s for 3 min at 420 nm. The peroxidase activity was expressed as a change in the absorbance of the reaction mixture min^{-1} g^{-1} fresh matter (FM) (Hammerschmidt and Kuc, 1982).

Estimation of phenylalanine ammonia-lyase (PAL) activity

One gram of homogenized leaf sample of each replicate was extracted with 3 ml of ice-cold sodium borate buffer (0.1 M) pH 7.0 containing 2-mercaptoethanol (1.4 mM) and insoluble polyvinylpyrrolidone (PVP) (0.1 g). The extract was filtered through cheesecloth and the filtrate was centrifuged at 10,000 rpm for 15 min at 4 °C (Universal 32R, Hettich Zentrifugen model D-78532, Germany). The supernatant was used as enzyme source. About 0.4 ml of enzyme was incubated with 0.5 ml of borate buffer (0.1 M) pH 8.8 and 0.5 ml of L-phenylalanine (12 mM) for 30 min at 30 °C. The amount of *trans*-cinnamic acid generated was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1}. Enzyme activity was expressed as μmol *trans*-cinnamic acid min^{-1} g^{-1} FM (Dickerson et al., 1984).

Estimation of polyphenoloxidase (PPO) activity

Leaf samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) using a pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C (Universal 32R, Hettich Zentrifugen model D-78532, Germany) and supernatant was used as crude enzyme. Polyphenoloxidase activity was measured by mixing 1.5 ml of the phosphate buffer, 200 μl of the crude enzyme, and 200 μl of catechol (0.01 M). The reaction mixture was incubated at room temperature for 2 min and absorbance recorded at 495 nm. The changes in absorbance were recorded every 30 s interval for 2 min and the activity was expressed as change in absorbance min^{-1} g^{-1} FM (Mayer et al., 1965).

Quantification of POD, PAL, and PPO gene expression

Isolation of RNA

Total RNA was extracted from plant tissue using the GStract™ RNA Isolation kit II (Guanidium Thiocynate Method) (SA-40005; Maxim Biotech, Inc., USA) according to the manufacture's procedure.

Reverse transcription-polymerase chain reaction (RT-PCR) of mRNA

Reverse transcription (RT) or first strand reaction was performed to convert the mRNA to complementary DNA (cDNA) in the presence of dNTPS and reverse transcriptase.

The components were combined with the DNA primer in a reverse transcriptase buffer for 1 h at 42 °C. Reverse transcription reaction was performed using oligo (dT) primer (5′-TTT TTTTTTTTTTTT-3′). Each 25 μl reaction mixture contained 2.5 μl (5×) buffer with $MgCl_2$, 2.5 μl (2.5 mM) dNTPs, 1 μl (10 pmol) primer, 2.5 μl RNA (2 mg/ml) and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler (Rotor-Gene-6000-system (QIAGEN, USA)) programmed at 42 °C for 1 h at 72 °C for 10 min (enzyme killing) and the product was stored at 4 °C until use.

POD, PAL, and PPO gene expression using RT-qPCR

Samples were analyzed using the Fermentase kit (Sigma Egypt, Cairo) (Peng et al., 2004). Each reaction mixture had 12.5 μl of 2× Quantitech SYBR® Green RT Mix, 1 μl of 25 pm μl^{-1} forward primer (Table 1), 1 μl of 25 pm μl^{-1} reverse primer, 1 μl of the cDNA (50 ng), and 9.5 μl of RNase free water for a total of 25 μl. Samples were mixed by spinning before loading in the Rotor's wells. The real time PCR program was as the following: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Data acquisition was performed during the extension step (Rotor-Gene- 6000-system (QIAGEN, USA)).

Gene expression data analysis

Comparative quantification analysis was done using Rotor-Gene-6000 Series instrument software according to Rasmussen (2001). The ratio of the target gene was expressed in the sample versus control in comparison with the reference gene. The relative expression of genes was estimated and analyzed using bioinformatics and statistical software. Results were normalized to 18S rRNA housekeeping gene (reference gene). The data were statistically evaluated, interpreted and analyzed using Rotor-Gene-6000 software version 1.7.

Statistical analysis

Enzyme assays were carried out as repeated measures over time with 5 replicates per treatment. Data were statistically analyzed using the MIXED procedure of the statistical analysis software (SAS) version 9.4 Cary, NC, SAS Institute Inc. (SAS, 2014). Least significant means were compared using specific multiple comparison of Dunnett's post-hoc Test ($P < 0.05$).

Results and discussions

Disease severity

Results presented in Table 2 showed the disease severity (PDS) after 15 d of inoculation. A significant difference in the PDS was noticed between 'Nicola' and 'Spunta'. The resistant cultivar (Nicola) showed lower incidence of infections compared to the susceptible cultivar (Spunta). Inducers' treatments individually or in combination with difenoconazole (fungicide) showed significant protective effects against *A. solani*. The 2,4-D was the most effective in inducing response against the Early Blight, which was more pronounced in the resistant cultivar (Nicola). After 15 d of inoculation, PDS values were 42.6%, 19%, 9.2%, 14%, 6%, 6.2%, and 2.3% for Nicola and 61%, 32%, 16%, 20%, 8.5%, 9%, and 5% for Spunta after treatment with control, AB, AB + difenoconazole, H_2O_2, H_2O_2 + difenoconazole, 2,4-D, and 2,4-D + difenoconazole, respectively (Table 2).

Defense-related enzymes and induced resistance

Induction of systemic resistance might lead to direct activation of defense-related proteins and genes (Conrath et al., 2001). Among these defense-related proteins are POD (Fig. 1), PAL (Fig. 2), and PPO (Fig. 3) that catalyze the formation of lignin, wound responses, growth regulation, and synthesis of phytoalexins and phenolics (Ramamoorthy et al., 2002), which subsequently enhance plant's resistance to pathogens. Current results were in agreement with those of Theerthagiri et al. (2007) where increased POD and PPO were observed after the induction of tomato plants against *A. solani*. Latha et al. (2009) reported that defense enzymes: POD, PPO, PAL, chitinase, and β-1,3-glucanase were increased in tomato plants after the treatment with *Pseudomonas fluorescens* (Pf1 and Py15) and *Bacillus subtilis* compared to control or *A. solani*-inoculated plants. Moreover, Song et al. (2011) reported that tomato defense-related genes, β-1, 3-glucanase, PPO, POD, and superoxide dismutase (SOD) were significantly elevated by exogenous AB treatment. Moreover, subsequent challenging the AB-pretreated plants with *A. solani* resulted in greater expression of defense genes compared to water-treated or *A. solani* inoculated plants.

Table 1 Sequence of primers used in the real-time PCR.

Primers		Primer sequence 5 → 3′	Annealing (°C)
Phenylalanine ammonia-lyase	F	TTCAAGGCTACTCTGGC	60
	R	CAAGCCATTGTGGAGAT	
Peroxidase	F	GCTTTGTCAGGGGTTGTGAT	60
	R	TGCATCTCTAGCAACCAACG	
Polyphenol oxidase	F	CATGCTCTTGATGAGGCGTA	60
	R	CCATCTATGGAACGGGAAGA	
18S rRNA	F	GGGCATTCGTATTTCATAGTCAGAG	60
	R	CGGTTCTTGATTAATGAAAACATCC	

Table 2 Percent disease severity ± SD caused by Early Blight fungus to potato cultivars Nicola and Spunta after 15 d of inoculation.

Treatment	Disease severity (%)	
	Nicola	Spunta
A. solani	42.60 ± 4.21	61.00 ± 7.48
AB + *A. solani*	19.00 ± 2.56	32.30 ± 4.08
AB + *A. solani* + difenoconazole	9.20 ± 1.26	16.12 ± 3.15
H_2O_2 + *A. solani*	14.00 ± 2.64	20.00 ± 3.54
H_2O_2 + *A. solani* + difenoconazole	6.00 ± 0.84	8.50 ± 1.05
2,4-D + *A. solani*	6.22 ± 0.95	9.00 ± 1.08
2,4-D + *A. solani* + difenoconazole	2.30 ± 0.49	5.00 ± 0.85

Enzyme activity and gene expression of peroxidase (POD)

Results in Fig. 1A showed that the activity of POD (OD min^{-1} g^{-1} FM) was significantly increased up to 4-folds for 'Spunta' and 5-folds for Nicola compared to control after treating plants with the chemical inducers. Induction of plants' responses lasted for more than 15 d post inoculation. POD activity was significantly greater in induced plants compared to control and *A. solani*-inoculated plants. The greatest POD enzyme activity was noticed after 1 d of infection.

Similarly, data in Fig. 1B show that levels of gene expression of POD were significantly increased in induced plants compared to control. The single treatment with the fungicide difenoconazole after the chemical induction increased the POD gene expression in both resistant and susceptible cultivars. The 2,4-D treatment exhibited the most POD expression (Fig. 4). Also, the highest increase in the gene expression of POD was reached at 2 d of infection for all treatments. The POD gene expression of was greater in Nicola than in Spunta.

Results reported herein showed significant resistance to *A. solani* with the increased activity and gene expression of POD. These findings might be related to the increased amounts of hydroxyl cinnamic acids and/or oxidative burst generated after inducers treatment, which were supported in other plant species. For example, peroxidase catalyzed the formation of polymers of ferulic acid in the cell wall of *Phaseolus vulgaris* plants (Zimmerlin et al., 1994). PODs were involved in the oxidative burst and the generation of H_2O_2 in the membranes of infected cells of *Phaseolus vulgaris* that increase systemic response against invading pathogen (Brown et al., 1998; O'Brien et al., 2012; Tohid and Taheri, 2015).

Enzyme activity and gene expression of phenylalanine ammonia-Lyase (PAL)

Similar to POD, activity of PAL enzyme (nmol *trans*-cinnamic acid min^{-1} g^{-1} FM) was increased significantly after treating potato plants with the chemical inducers (Fig. 2A). Enzyme activity of PAL was greater in Nicola compared to Spunta. Folds of increase in PAL of Spunta were more than 2 for 2,4-D and 2,4-D with the fungicide treatments compared to *A. solani*-infected plants. Moreover, the enzymatic activity of PAL of Nicola potato was more than 5-folds compared to control plants. The chemical inducer treatments increased the activity of PAL significantly for longer than 15 d after inoculation greater compared to controls.

The relative expression of PAL genes in the two cultivars is presented in Fig. 2B. The levels of PAL gene expression of

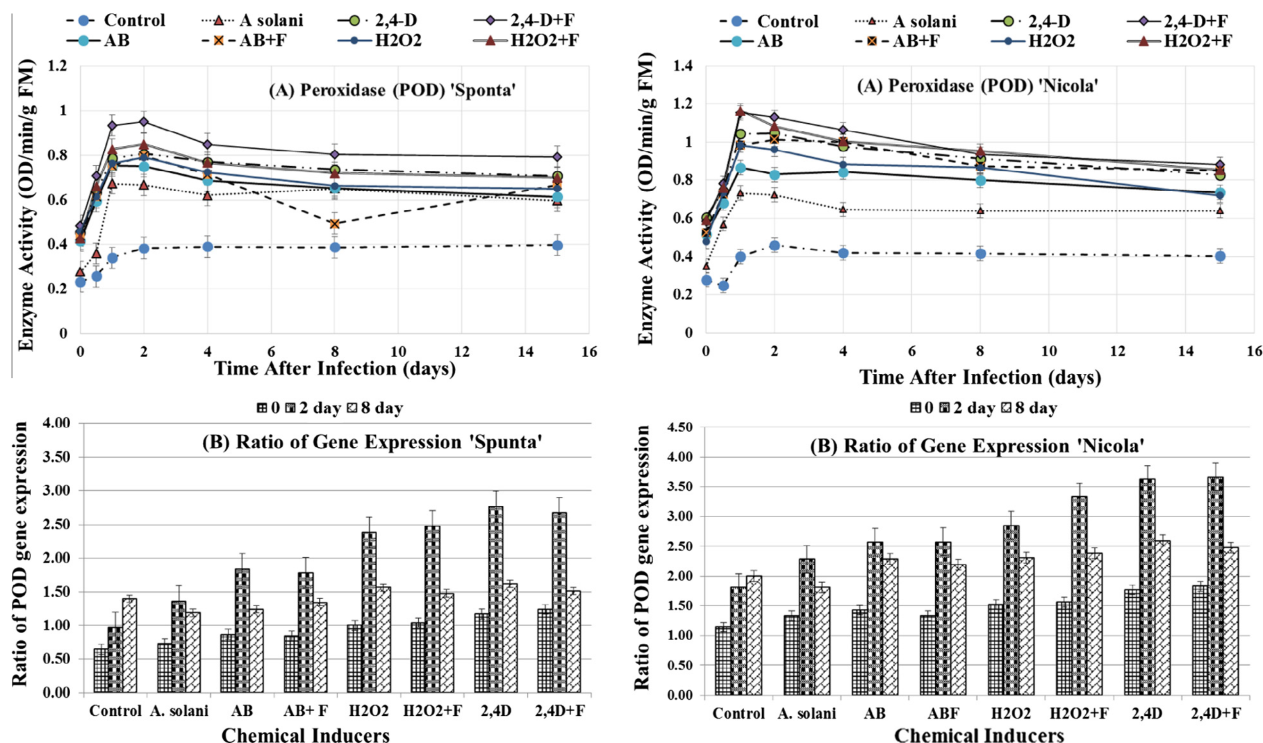


Fig. 1 Specific enzyme activity and ratio of gene expression of peroxidase (POD; OD min^{-1} g^{-1} FM) of potato cultivars Spunta and Nicola after the application of Abssisic acid (AB), 2,4-dichlorophenoxy acetic acid (2,4-D), and hydrogen peroxide (H_2O_2) separate or with the fungicide difenoconazle (F): AB + F, 2,4-D + F, and H_2O_2 + F after the inoculation with *Alternaria solani* (*A. solani*).

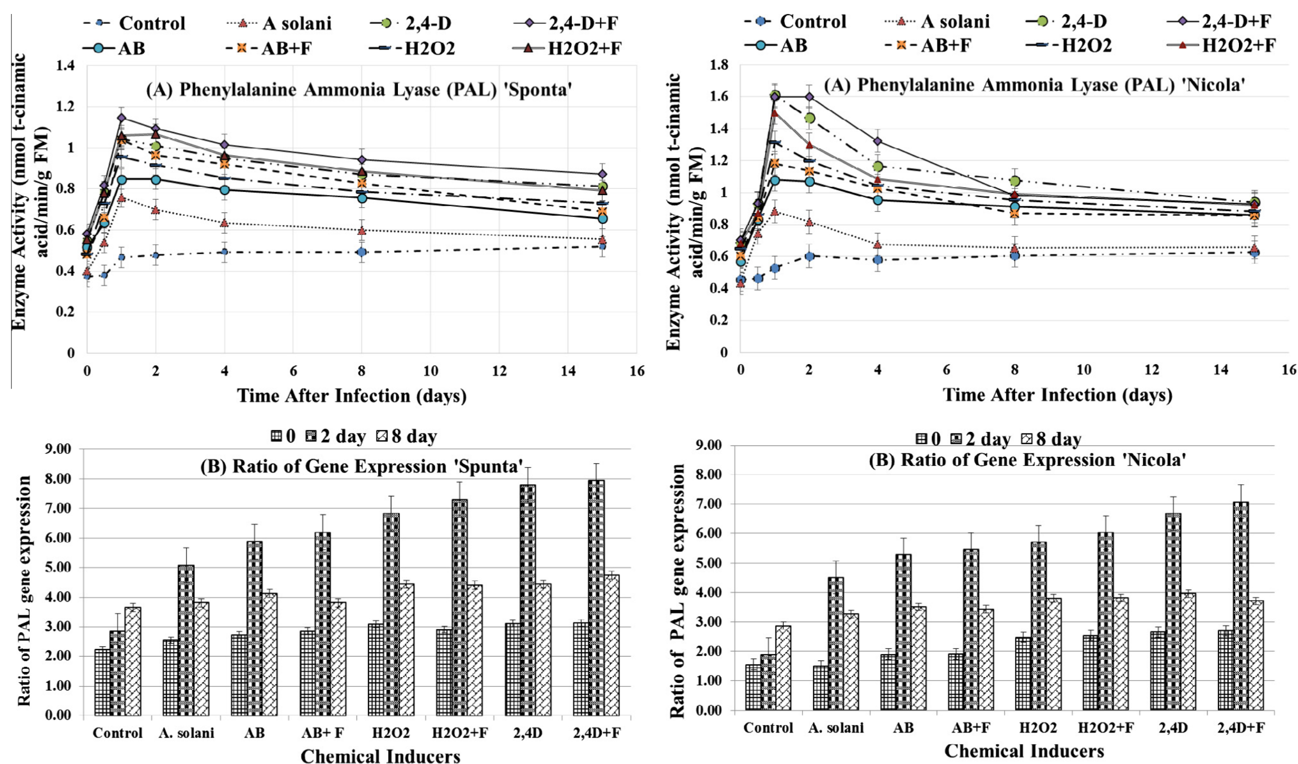


Fig. 2 Specific enzyme activity and ratio of gene expression of phenyl ammonia lyase (PAL; μmol t-cinamic acid min^{-1} g^{-1} FM) of potato cultivars Spunta and Nicola after the application of Abssisic acid (AB), 2,4-dichlorophenoxy acetic acid (2,4-D), and hydrogen peroxide (H_2O_2) separate or with the fungicide difenoconazle (F): AB + F, 2,4-D + F, and H_2O_2 + F after the inoculation with *Alternaria solani* (*A. solani*).

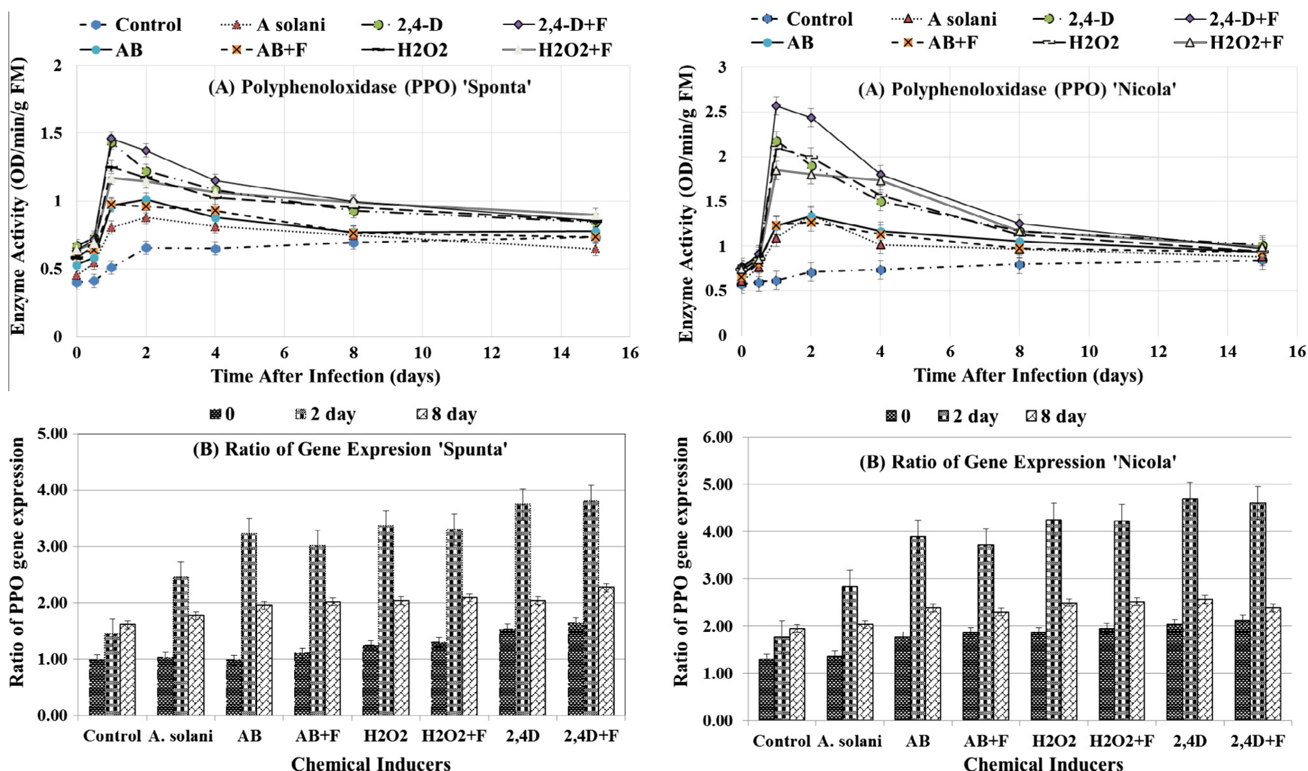


Fig. 3 Specific enzyme activity and ratio of gene expression of polyphenoloxidase (PPO; OD/min/g FM) of potato cultivars Spunta and Nicola after the application of Abssisic acid (AB), 2,4-dichlorophenoxy acetic acid (2,4-D), and hydrogen peroxide (H_2O_2) separate or with the fungicide difenoconazle (F): AB + F, 2,4-D + F, and H_2O_2 + F after the inoculation with *Alternaria solani* (*A. solani*).

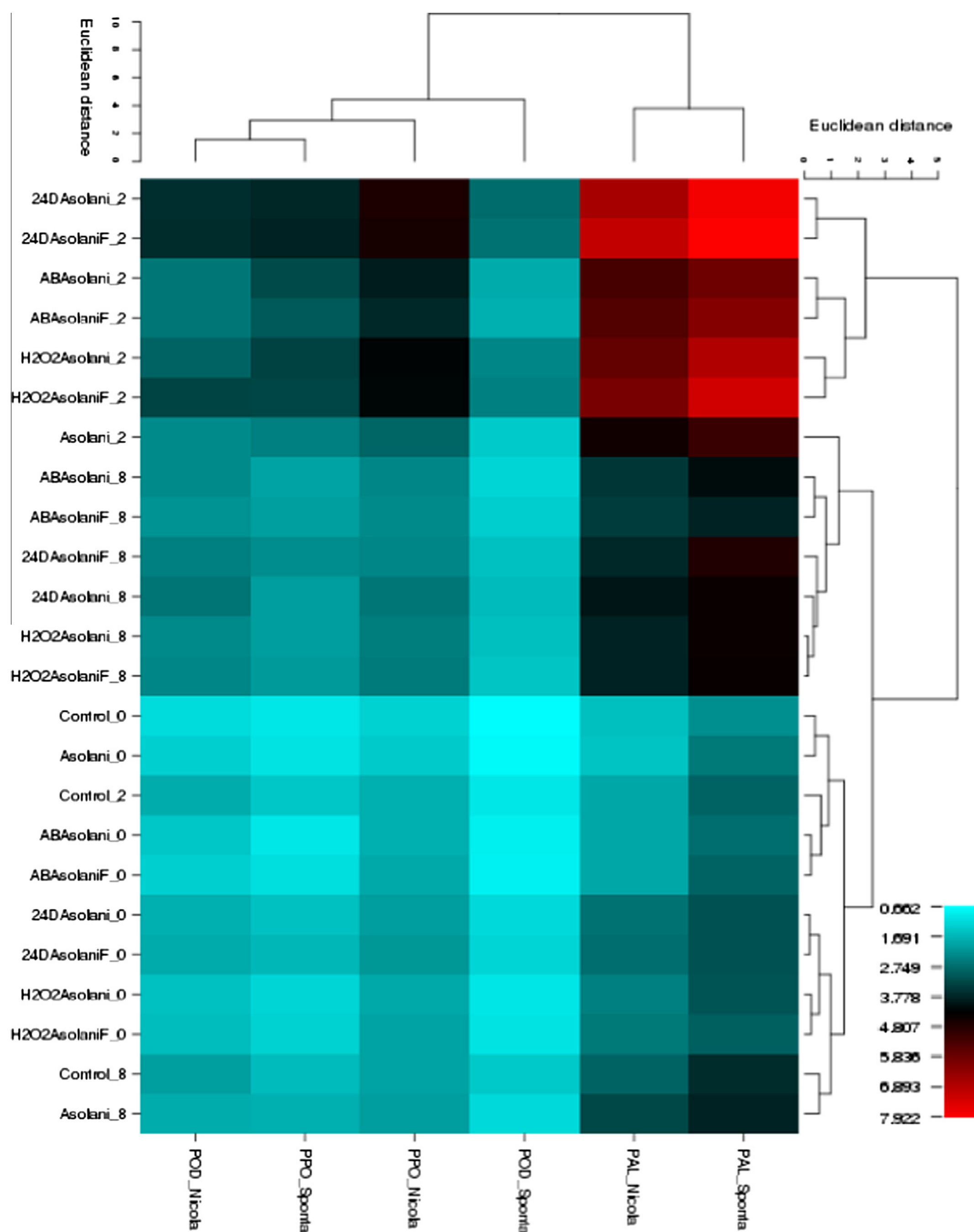


Fig. 4 Heat map for proteins up-regulated in potato leaves submitted to chemical inducers. Data are presented as log values of relative regulation from red (high-expression) to turquoise (less-expression) compared to control (untreated) and *A. solani*-treated control. Modulated proteins were categorized into two groups, POD and PPO in one group and PAL in the second one (upper dendrogram). More than 7-fold increases were noticed after the 2,4-D and difenoconazle treatment. Map was generated using the OpenHeatMap website, http://www.openheatmap.com.

Nicola and Spunta were increased significantly after 2,4-D, H_2O_2 and AB treatments compared to control, while the application of difenoconazole after those treatments slightly increased the PAL gene expression. The greatest boost in gene expression of PAL was noticed after 2 d of inoculation and decreased to reach the lowest level after 8 d of infection but was significantly greater than control. PAL gene expression was greater in Nicola (resistance) compared with in Spunta (susceptible). Results also showed that 2,4-D was the most effective in inducing defense responses followed by H_2O_2 and AB through the increased level of PAL gene expression in both Nicola and Spunta cultivars (Fig. 4). PAL was reported to increase resistance against pathogens through the catalysis of formation of lignin and synthesis of phytoalexins and phenolics (Ramamoorthy et al., 2002). Also, results reported herein were in agreement with Song et al. (2015), they stated that

PAL activity was increased in tomato plants-treated with soil-borne arbuscular mycorrhizal fungi and challenged with *A. solani*.

Enzyme activity and gene expression of polyphenoloxidase (PPO)

Activity of PPO enzyme was increased up to more than 2-folds after treating potato plants with the defense inducers (Fig. 3A). Cultivar Spunta, treated with 2,4-D or fungicide and 2,4-D and challenged with *A. solani*, showed the greatest activity of PPO (OD min^{-1} g^{-1} FM) with more than 3-folds of increase compared with untreated plants. For Nicola, the activity of PPO enzyme was greater than that of Spunta. Also, PPO enzyme activity was increased more than 5-folds compared to control. The protective effects of chemical inducers lasted up to 15 d of infection.

Levels of gene expression of PPO in Nicola and Spunta after the application of 2,4-D, H_2O_2, and AB and inoculated with *A. solani* are presented in Fig. 3B. PPO gene expression was up-regulated after the application of all treatments compared to control or *A. solani* inoculated plants. The 2,4-D treatment was the most effective in the initiation of the defense responses followed by H_2O_2 and AB compared with the control (Fig. 4). Results also indicated that the maximum up-regulation in the expression of PPO gene was noticed after 2 d of infection in Nicola and Spunta. Treating potato plants with difenoconazole after the chemical inducers slightly elevated expression of PPO in both tested cultivars.

Polyphenoloxidase enzyme catalyzes the production of quinones, which play important functions in plants, including antipathogenic effects (Mayer and Harel, 1991; Li and Steffens, 2002; Thipyapong et al., 2007). It was reported to be increased by at least of 10-folds after infection with *Pseudomonas syringae* in transgenic tomato plants (Li and Steffens, 2002 and Li and Steffens, 2010). PPO activity is often dormant until disruption occurs by wounding, senescence, or attack by pathogens; then, it's been released from the thylakoid to interact with mono and/or *o*-diphenolic substrates (Steffens et al., 1994). Afterward, PPOs protect plants through quinone-mediated covalent modification and/or anti-microbial toxicity of H_2O_2 in the place where the infection takes place (Thipyapong et al., 2007).

Conclusions

Several chemical inducers have been reported to trigger the systemic resistance in plants after infection with pathogens. Current study reported that repeated treatments of low doses of AB, 2,4-D, and H_2O_2 enhanced the acquired resistance of Nicola and Spunta cultivars to infection with *A. solani*. The percentages of disease severity of all inducers' treatments varied from 2% to 19% for Nicola and 5–32% for Spunta compared with 43 and 61% for both cultivars challenged with *A. solani*, respectively. The 2,4-D was the most effective treatment followed by H_2O_2 and AB. Data also indicated that the spray with the fungicide (difenoconazole as a single application) with elicitors slightly decreased the disease severity compared with the elicitors alone. PAL gene expression was significantly and rapidly up-regulated after treating potato plants with 2,4-D followed by H_2O_2 with or without the fungicide compared to AB treatment (Fig. 4). Application of the fungicide along with inducers significantly increased both enzymatic activity and gene expression of POD, PAL, and PPO. Induced resistance reported herein highlighted the promising role of repetitive treatments of 2,4-D and H_2O_2 against *A. solani* on potato plants. Also, it's the first study (to the best of our knowledge) to report on the use of H_2O_2 as chemical inducer against potato fungal diseases. Additionally, incorporation of such treatments in a management program with synthetic fungicides might have significant reduction in the amount of fungicides required. The Early Blight disease is very destructive to potato, especially at the early stage of growth. So, the involvement of such cheap and non-toxic concentrations of used compounds would reduce the management costs and environmental pollution.

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