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Original article

Reactive oxygen species accumulation and homeostasis are involved in plant immunity to an opportunistic fungal pathogen

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ABSTRACT

Alternaria blight is a major and destructive disease of potato worldwide. In recent years, *A. tenuissima* is recognized as the most prevalent species of this phytopathogenic fungus in potato fields of Asian countries, which causes high yield losses every year. Any potato cultivar with complete resistance to this disease is not recognized, so far. Therefore, screening resistance levels of potatoes and identification of plant defense mechanisms against this fungus might be important for designing novel and effective disease management strategies for controlling the disease. In this research, the role of reactive oxygen species, antioxidants, lignin and phenolics in potato basal resistance to *A. tenuissima* was compared in the partially resistant Ramus and susceptible Bamba cultivars. Priming O_2^- and H_2O_2 production and enhanced activity of peroxidase (POX) and catalase (CAT) during interaction with *A. tenuissima* were observed in Ramus cultivar. Application of ROS generating systems and scavengers revealed critical role of O_2^- and H_2O_2 in potato defense, which was associated with lignification and phenolics production. More OH^- and lipid peroxidation in the susceptible Bamba compared to Ramus cultivar showed their negative effects on resistance. Priming the POX and CAT activity, in correlation with upregulation of the corresponding genes was observed in Ramus. The POX and CAT inhibitors increased disease progress, which was related with decreased lignification. This assay demonstrated not only POX-dependency of lignification, but also its dependence on CAT. However, POX had more importance than CAT in potato defense and in lignification. These findings highlight the function of ROS accumulation and homeostasis in potato resistance against *A. tenuissima*.

1. Introduction

Early blight, caused by *Alternaria solani*, is a major disease of potato worldwide. Another important species of this opportunistic and necrotrophic fungus obtained from potato growing regions in Iran (Hajipour-Jarchelou et al., 2013; Taheri-Ardestani et al., 2010) and China (Zheng et al., 2014) is *A. tenuissima*, which causes Alternaria blight (Zheng et al., 2014). Primarily, *A. tenuissima* attacks older or stressed leaves and causes leaf necrosis with concentric rings which expand, fuse and appear as larger necrotic lesions on leaves. Under favorable conditions and without disease management, it can cause extensive disease symptoms on the stems and defoliation which increase the possibility of potato tuber infection and huge yield loss. The disease not only can occur on potato, but also on tomato and other plants belonging to Solanaceae.

The disease epidemics may decrease potato yields around 30–90% (Christ and Maczuga, 1989; Shtienberg et al., 1990). Also this disease reduces potato tuber quality. Synthetic fungicides used against the disease are expensive and often ineffective. So, introduction of potato genotypes with high levels of resistance to Alternaria blight could be an effective disease management strategy for reducing destructive effect of this wide spread disease. Up to now, monogenic traits responsible for complete resistance against *Alternaria* spp. have not been found in potato. Resistance to this disease is associated with plant age since higher level of susceptibility is observed in older plants (Pelletier and Fry, 1989).

Resistance mechanisms including numerous biochemical and cytomolecular changes occur in plant response to pathogens. Defense signaling is complex and comprises mechanisms which distinguish a range of environmental stimuli, from basal resistance to different pathogens, as well as signaling resistance to specific pathogens (Chen et al., 2013).

Generation of reactive oxygen species (ROS; such as hydrogen peroxide: H_2O_2, superoxide: O_2^-, and hydroxyl radical: OH^-), known as oxidative burst, is one of the earliest plant defense responses to various biotic or abiotic stresses. The ROS can enhance hypersensitivity response (HR) or play an important role as second messengers in

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resistance mechanisms leading to upregulation of defense-related genes and interface with other signaling molecules (Chen et al., 2013). For many years, it had been assumed that various ROS accumulate sequentially from O_2^- as the primary origin. Today, we know that different ROS can be produced independently by different sources, which seems reasonable because ROS accumulation must be under control of antioxidative systems to avoid toxicity (Hückelhoven and Kogel, 2003). All aerobic organisms have developed antioxidant systems for controlling ROS accumulation (which is to maintain redox homeostasis), as well as to 'make use' of these highly reactive molecules in signal transduction, gene expression and cellular responses to biotic or abiotic stimuli (which is redox signaling). Derangement in redox homeostasis, caused by altered levels of ROS or antioxidants, can play a critical function in defense responses of plant tissues.

Although accumulation of high levels of ROS, which leads to HR and cell death, is efficient resistance strategy against biotrophic pathogens, it sometimes may not protect plants against infection by the necrotrophic pathogens (Glazebrook, 2005). On the contrary, Dita et al. (2007) verified that the number of infection sites exhibiting HR was higher in potato plants resistant against *A. solani* than in the susceptible genotype after inoculation with that pathogen. They also found a relationship between the number of sites with HR and the leaf age, being the number of the penetration sites with HR higher in leaves in the upper part of the plant, which are more resistant to the pathogen compared to the lower (older) parts of the plant. Plasma membrane-bound NADPH oxidases and cell wall peroxidases are considered as main sources of an oxidative burst in the apoplast via generating O_2^- and H_2O_2, respectively. Investigations of Kumar et al. (2007) revealed that a potato NADPH oxidase (StrbohA) is implicated in the wound healing and resistance to natural microbial infections of potato tubers (Kumar et al., 2007), which indicates the important role of O_2^- in potato defense responses against biotic and abiotic stresses.

Uncontrolled ROS accumulation leads to cell death, which may enhance plant susceptibility to pathogens (Torres et al., 2006). So, ROS accumulation and removal are controlled in plant-pathogen interactions. Enzymatic antioxidants such as peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) are involved in scavenging H_2O_2 in living cells (Barna et al., 2012).

The POXs are α-helical heme-containing proteins with various functions. They might be involved in H_2O_2 production (peroxidation) or in oxidizing various molecules mainly of phenolic nature. In addition to the enzymatic H_2O_2 scavenging system, phenolics are strong non-enzymatic antioxidants due to availability of their phenolic hydrogen (Nikraftar et al., 2013). Some phenolics, such as monolignols, are constituents of lignin. They are oxidized and polymerized by POX using H_2O_2, which leads to lignification (O'Brien et al., 2012).

The CAT is a tetrameric heme protein with four polypeptide chains, which has α-helix and β-sheet domains. It is found in all aerobic organisms and functions in converting hydrogen peroxide to water and oxygen (Chelikani et al., 2004). Both CATs and POXs are involved in protecting living cells and tissues against toxic effects of ROS, including oxidative damage of DNA, proteins, and lipids (Halliwell and Gutteridge, 1989; Novo and Parola, 2008). Whereas the activity or gene expression of POXs and CATs at different stress conditions have been investigated in various plant species, their activity and transcription during potato- *A. tenuissima* interaction have not been documented till now. Analyzing the role of these antioxidant enzymes in plant-pathogen interactions might be helpful in breeding programs and designing novel and powerful disease management strategies.

To our knowledge, there is not any information on the role of ROS in potato basal resistance against *A. tenuissima* so far. Furthermore, the comparative role of enzymatic antioxidants such as POX and CAT in potato defense to this pathogen is not investigated to date. Thus, the objectives of this study were to (i) identify sources of potato resistance to Alternaria blight, (ii) determine the role of oxidative burst, POX, CAT, lipid peroxidation, lignin, soluble and insoluble phenolics in potato basal resistance to *A. tenuissima*, and (iii) which antioxidative enzyme (CAT or POX) had prevailing function in basal resistance and lignification as a key resistance marker in our pathosystem.

2. Materials and methods

2.1. Potato genotypes and plant growth conditions

Two potato (*Solanum tuberosum* L.) genotypes, including the partially resistant Ramus and the susceptible Bamba cultivars were used in this study. Potato seeds were treated with thiabendazole and then planted in 30 cm-diameter plastic pots filled with autoclaved commercial potting soil and grown in greenhouse (30 ± 4 °C; 16/8 h light/dark photoperiod).

2.2. Fungal inoculum preparation

The AT5 isolate of *A. tenuissima*, obtained from culture collection of Ferdowsi University of Mashhad, was used to investigate ROS-related defense responses in potato against this pathogen. *A. tenuissima* was grown at 28 °C with alternate cycles of 12 h light/12 h darkness on potato dextrose agar (PDA) for short-term storage and on potato carrot agar (PCA) for spore production. Inoculation with *A. tenuissima* was done using spore suspension of each isolate (10^6 spores/ml concentration) containing 0.05% (v/v) Tween 20.

2.3. Inoculation of intact potato plants

In the greenhouse experiments, virulence assays were performed by spraying the spore suspension on six-week-old potato plants until runoff. Control plants were sprayed using 0.05% Tween 20. Then, the plants were kept in greenhouse for 5 days. After development of the disease symptoms, the pathogen was re-isolated from infected plants. The leaves of intact plants were collected at various time points after *A. tenuissima* infection and used for measuring lignin, phenolics, POX and CAT activity, and also gene expression analysis.

2.4. Leaf disc bioassay

In the leaf disc bioassay, 2-cm diameter discs were prepared from the first outer leaves of 6-week-old potato plants. Inoculating the discs was performed using spore suspension (Taheri et al., 2014) and the inoculated leaf discs were kept in laboratory with 22 ± 2 °C temperature and 12 h/12 h of light/dark photoperiod. Symptom development was evaluated at 5 dpi. The intensity of disease symptoms was graded into five classes based on the leaf area infected and disease index (DI) was calculated (Taheri and Tarighi, 2010).

2.5. Histochemical detection of H_2O_2 and O_2^-

The 3,3-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997) was used to compare H_2O_2 production in the leaf discs of partially resistant and susceptible potato cultivars at various time points after *A. tenuissima* infection. Polymerization of the DAB molecule at the site of H_2O_2 generation and peroxidase activity results in a reddish brown polymer that was macroscopically visible and it could be visualized by microscopy. After DAB staining, the leaf discs were decolorized in ethanol: glycerol (9:1) at 70 °C for 3–4 h. After cooling, the leaves mounted in 50% (v/v) glycerol and then were evaluated under microscope (Olympus BX41).

Accumulation of O_2^- in potato leaves was detected using nitro-blue tetrazolium (NBT) staining as described by Dong et al. (2009). When NBT reacted with O_2^- a dark blue insoluble foramazan compound is produced. Decolorizing the leaf discs and microscopic analysis were done as mentioned before. Intensity of DAB and NBT staining in each sample was quantified using Image J software (http://rsb.info.nih.gov/