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Effect of Acibenzolar-S-methyl (ASM) pre-treatment in inducing resistance against *Pythium aphanidermatum* infection in *Curcuma longa*

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ABSTRACT

In vitro experiments were carried out to test the efficacy of plant activator Acibenzolar-S-methyl (ASM, a benzothiadiazole derivative; trade name Bion 50WG) against rhizome rot disease of turmeric (*Curcuma longa* L.) caused by *Pythium aphanidermatum*. The plant activator was applied as a liquid rhizome pre-treatment followed by inoculation with *P. aphanidermatum*. Cell death, activities of pathogenesis related (PR) proteins such as cysteine protease (EC 3.4.22), peroxidases (EC 1.11.1.7) both soluble and ionically bound (IB), trypsin inhibitor (EC 3.4.21.1) and chymotrypsin inhibitor (EC 3.4.21.4) were monitored. Rhizome pre-treatment was effective in controlling *P. aphanidermatum* infection. Anatomical observation of turmeric rhizomes indicated the presence of calcium oxalate deposits in infected tissue and an accumulation of starch grains in response to infection by *P. aphanidermatum*. Pathogen infection also induced new basic polypeptides corresponding to 18.0 and 41.0 kDa. Induction of protease, protease inhibitors, soluble and ionically bound peroxidase activity was observed after ASM pre-treatment and *P. aphanidermatum* infection. ASM treatment also enhanced activities of proteases and peroxidase in rhizomes already infected with *P. aphanidermatum*. Increases in enzyme activities and protease inhibitors occurred much more rapidly and were enhanced in *P. aphanidermatum* infected rhizomes that were previously treated with ASM suggesting that increased activities of peroxidases and protease inhibitors may play a key role in restricting the development of disease symptoms on the rhizomes infected with *P. aphanidermatum* as evidenced by a reduction in cell death. Hence, pretreatment with ASM suppress the *P. aphanidermatum* induced oxidative damage through higher accumulation of peroxidases and induced defense through activities of protease inhibitors thereby, protected turmeric rhizomes from rhizome rot disease.

1. Introduction

Induced resistance is a “state of enhanced defensive capacity” triggered by exposure of plants to virulent, avirulent or non-pathogenic microbes or artificially by various chemical agents such as salicylic acid (SA) and its functional analogue, Acibenzolar-S-methyl (ASM). Plants typically respond to pathogen infection by inducing defence responses, which are accompanied by the induction of defence genes. ASM is the most potent synthetic systemic acquired resistance (SAR) activator discovered to date (Kessmann et al., 1994) and elicits the same SAR pathway which includes the same pathogenesis related (PR) proteins as observed in SA (Friedrich et al., 1996). The plant PR proteins accumulate in response to infection or other signals related to plant defence responses and were initially identified in tobacco leaves exhibiting a hypersensitive reaction to Tobacco Mosaic Virus (TMV). They were divided into 11 groups based on studies with various plants (Hwang et al., 2003). There are limited research findings on induced resistance in seedlings and leaves of annual plants (Sticher et al., 1997). The plant activator ASM significantly enhanced resistance of tolerant tomato cultivars including “Neptune” and “FL 7514” against bacterial wilt and resulted in yield increase (Pradhanang et al., 2005). ASM has been more effective than rhizobacteria in reducing bacterial wilt incidence on susceptible cultivars at low soil populations of *Ralstonia solanacearum* (Anith et al., 2004). In general, ASM is known to induce cell wall degrading enzymes such as chitinases (EC 3.2.1.14) and β-1,3 glucanases (EC 3.2.1.39) (Suo and Leung, 2001) and antioxidant enzymes such as superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), ascorbate peroxidase (APX) (EC 1.11.1.11), associated with decreased leaf spot severity (Cavalcanti et al., 2007). The peroxidases (PODs) (EC 1.11.1.7) are widely distributed in higher plants (Van Huystee and Cairns, 1982) and implicated in a variety of functions, such as control of cell elongation (Ahmed et al., 1995), defense mechanisms (Bradley et al., 1992; Kolattukudy et al., 1992) and lignification (Blee

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et al., 2003). The free-radical intermediates produced by the oxidative activity of PODs are toxic to pathogens (Sutherland, 1991). Protease inhibitors (PIs) are one of the most important classes of defence proteins in plants (Koiwa et al., 1997). The induction of PIs is elicited by various biotic and abiotic stimuli including mechanical wounding, insect and pathogen attack and UV exposure in addition to various signal molecules and phytohormones. A chymotrypsin inhibitor from potato suppressed the growth and zoospore germination of *Phytophthora infestans* (Valueva et al., 1998). A previous study revealed that SA treatment of *Curcuma longa* rhizomes promotes resistance towards the development of rhizome rot disease (Radhakrishnan and Balasubramanian, 2009).

The genus *C. longa* L. belongs to the family of Zingiberaceae, is a perennial herbaceous crop, well known for its antioxidant (Reddy and Lokesh, 1992), anti-inflammatory (Satoskar et al., 1986), anti-mutagenic, anti-carcinogenic (Nagabhushan and Bhide, 1992) and anti-angiogenic properties (Anupama et al., 2002). The dried rhizome of turmeric is one of the most important culinary spices in South-East Asia (Nageswara Rao et al., 1993). Rhizome rot disease caused by *Pythium aphanidermatum* (Edson) Fitzp. is the most destructive disease of turmeric (*C. longa* L.) and reduces its economic and commercial value (Ramakrishnan and Sowmini, 1954; Ushamalini et al., 2008), It is therefore necessary to search for effective combined methods of control of this pathogen including products that reinforce the natural defence mechanisms of the plant against an attack. The main objective of the present investigation was to study the structural changes of rhizomes infected with *P. aphanidermatum* and induction of PR proteins by ASM pre-treatment in turmeric.

2. Materials and methods

2.1. Plant materials

Rhizomes of *C. longa* (syn *C. domestica*) cultivars Erode Local (susceptible) and Erode #8 (tolerant) were obtained from a farmer's field at Erode, Tamilnadu, India. Rhizomes were thoroughly washed with running tap water three times followed by glass-distilled water, surface-sterilized by immersion in sodium hypochlorite 0.001% (v/v) for 15 min followed by three rinses in sterile distilled water.

2.2. Chemical treatment

Acibenzolar-S-methyl (ASM, trade name Bion 50 WG, Novartis, Switzerland) which is a water-dispersible granule form containing 50% the active ingredient, was dissolved in sterilized water. The rhizomes of cultivar Erode Local were dipped for 2 h in the ASM (2 mM) in a rotary shaker (50 rpm min^{-1}) solution, shade-dried and used for pathogen inoculation. Rhizomes dipped in sterile water served as control. For each treatment, approximately 30 rhizomes were placed in sterile petri dishes and each rhizome had three nodes.

2.3. Pathogen inoculation

A wound was made below the sub-epidermal surface of the rhizomes and an agar disk of *P. aphanidermatum* (5 mm diam.) grown at room temperature for 2 days on PDA was placed on the wound. The inoculated and non-inoculated rhizomes were placed in sterile petri dishes on sterile filter paper, kept in the dark at 21 °C and cell death observations were recorded at 48 h intervals. For ASM pretreated rhizomes pathogen inoculation was done immediately (0 h) and 24 h after elicitor treatment.

2.4. Cell death assay

Cell death analysis of *P. aphanidermatum* infected rhizomes was monitored by following the method of Levine et al. (1994). A known weight of rhizomes showing browning symptoms of both Erode Local and Erode #8 cultivars were ground with one ml of sterile glass-distilled water. For each sample, a 500 μl aliquot of sample was incubated with 0.05% Evans blue for 30 min and then washed extensively. The dye bound to dead cells was solubilized in 50% (v/v) methanol with 1% (w/v) SDS for 30 min at 50 °C and quantified by A_{600}.

2.5. Sectioning and staining

Susceptible rhizomes, non-inoculated or inoculated with *P. aphanidermatum* were fixed in FAA (formalin-5 mL + acetic acid − 5 mL + 70% ethyl alcohol − 90 mL) for 24 h. After fixation, the specimens were dehydrated with graded series of tertiary-butyl alcohol as per the schedule given by Sass (1940). Infiltration of the specimens was carried by gradual addition of paraffin wax (with melting point of 58–60 °C) until tertiary butyl alcohol (TBA) solution attained super saturation and then specimens were cast into paraffin blocks. The paraffin embedded specimens were sectioned with a rotary microtome. The thickness of the sections was 10–12 μm. Dewaxing of the sections was a customary procedure (Johansen, 1940). The sections were stained with toluidine blue following the method described by O'Brien et al. (1964). Since toluidine blue is a polychromatic stain, it is possible to study some cytochemical reactions. The dye rendered a pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage and blue to the protein bodies. Plant sections were also stained with safranin and Fast-green and IKI (for starch). Photographs were taken with a Nikon Labphot 2 Microscopic Unit. For normal observations, bright field imaging was used. For the study of crystals, starch grains and lignified cells polarized light were used. Since these structures have a birefringent property, they appear bright against a dark background under polarized light.

2.6. Protein extraction

The extraction of total proteins from turmeric rhizomes was made at 4 °C with sodium phosphate buffer (50 mM, pH 7.6) amended with polyvinylpolypyrrolidone (0.01% (W/V)), and ascorbic acid (0.1% W/V) and cysteine (0.001% (W/V)) in the ratio of 1:3. The homogenate was filtered through four layers of cheese cloth and centrifuged (Kubota KR-1500) at 8000 × g for 20 min. The clear supernatant was used as the enzyme source. For the extraction of soluble and ionically bound peroxidases, rhizomes were homogenized with sodium phosphate buffer (50 mM, pH 6.0). In samples showing discoloration, the tissue immediately surrounding (3 mm) an area of browning was sliced to avoid excessive dilution with an unaffected area. The resulting supernatant was used as a source of soluble peroxidases. The pellet was washed repeatedly by re-suspension and centrifugation until peroxidase activity could not be detected in the supernatant. To release ionically bound peroxidases, the pellet was suspended in sodium phosphate buffer (50 mM, pH 6.0) containing 1M NaCl. The suspension was stirred for 30 min on ice, centrifuged and the supernatant was used as a source of ionically bound peroxidases. For the extraction of basic PR proteins, pathogen infected turmeric rhizomes were extracted with citrate phosphate buffer (50 mM, pH 2.8)