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Research article

Salicylic acid seed priming instigates defense mechanism by inducing PR-Proteins in *Solanum melongena* L. upon infection with *Verticillium dahliae* Kleb.

H.M. Mahesh [a], M. Murali [a], M. Anup Chandra Pal [b], Prasad Melvin [b], M.S. Sharada [a, *]

[a] Department of Studies in Botany, University of Mysore, Manasasgangotri, Mysuru 570 006, Karnataka, India
[b] Department of Studies in Biotechnology, University of Mysore, Manasasgangotri, Mysuru 570 006, Karnataka, India

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ABSTRACT

Salicylic acid (SA) is a hormone connected with various cellular functions including the fight against invading pathogens. Priming of seeds pre-sowing is a very simple method to the farmers' to produce better growth, yield and manage the pathogens. The present study was aimed to determine the growth and disease resistance ability in brinjal seeds primed with different concentrations (0.25, 0.5, 0.75 and 1.0 mM) of SA under greenhouse conditions. Priming of seeds with SA significantly increased seed germination and seedling vigor with a maximum of 84% and 859.18, respectively at 0.5 mM concentration. Seed priming with SA also reduced *Verticillium* wilt incidence to 39.25% (at 0.5 mM) under greenhouse conditions and also enhanced the vegetative growth parameters of the plant compared to control. The induced resistance obtained with SA was in line with higher expression of PR-protein (β-1,3-glucanase and chitinase) related defense enzymes. Further, an increase of 1.7, 2.9, 2.1, 2.5 and 2-fold increase in gene expression of IAA27, MPK1, GPX, chitinase and β-1,3-glucanase, respectively were observed in SA primed challenge inoculated seedlings than non-primed susceptible inoculated controls. The higher expression of IAA27, MPK1, GPX, chitinase and β-1,3-glucanase correlates with the plant growth promoting and disease protection studies as these genes are vital for increasing plant growth and inducing resistance during host-pathogen interaction. Enhanced activation of defense-related activities in plants upon priming with SA suggests that it alters plant physiology which in turn is useful for production and protection of brinjal.

1. Introduction

Plants are known to respond to any physical, chemical or biological stresses during their life cycle (Agrios, 2005). Plant reacts irrespective of the stress involved, resulting in activation of several biochemical and physiological changes in the host to withstand the stress involved including the expression of pathogenesis-related (PR) proteins (Bowles, 1990; Kombrink and Somssich, 1997; Murali et al., 2013). These PR-proteins are known to express coordinately after fungal infection in many plant species during induction of resistance (Mauch et al., 1988; Grant and Lamb, 2006) and are important in crop plants where resistant varieties to pathogens are not readily available. Among the PR-proteins, PR-2 (β-1,3-glucanase) and PR-3 (chitinase) are an important class of proteins, which act alone or in combination during fungal infection. These PR-proteins are known to hydrolyze chitin and β-1,3-glucans in the cell wall of fungal pathogens (Kombrink and Somssich, 1997), activate downstream defense signaling components and also comprise antimicrobial properties (Mauch et al., 1988). Apart from these, β-1,3-glucanase and chitinase enzymes are known to form some elicitors during infection, which in turn elicit other PR-proteins like phytoalexins to combat diseases (Klarzynski et al., 2000; Edreva, 2004).

The phytohormone auxin (IAA) is one of the most essential plant hormones that modulate many key pathways during plant growth and development processes. Auxin is known to control cell division, expansion and differentiation which is also initiated upon stress/defense responses (Vanneste and Friml, 2009; Ghanashyam and Jain, 2009). Auxin regulates these developmental processes by

* Corresponding author.
E-mail addresses: mahy.hsn666@gmail.com, mssharada.botany@gmail.com (M.S. Sharada).

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the interaction of three main multigene family coding for the TIR1/AFB receptors, the Aux/IAA repressors and the auxin response factor (ARF) transcription factors. Auxin regulates IAA27 and it has been reported to be involved in plant vegetative and reproductive growth signifying the importance of IAA27 in the plant growth and development (Bassa et al., 2012). Mitogen-activated protein kinase (MAPK/MPK) cascades are highly conserved signalling modules that transduce signals from cell surface to specific targets inside the cell that are involved in plant development as well as in response to biotic and abiotic stresses (Xu and Zhang, 2015). It has been reported that plant MAPK/MPKs are involved in immunity and stress responses as well as in plant growth and development (Melvin et al., 2015; Xu and Zhang, 2015; Jia et al., 2016). The reactive oxygen species (ROS) level in plants are known to increase under biotic/abiotic stress conditions, which mainly plays a role in the hypersensitive response. To avoid the excessive damage caused by ROS in the cell, ROS scavenging enzyme glutathione peroxidase (GPX) plays a crucial role in pathways such as antioxidant and secondary metabolite metabolism, maintaining redox homeostasis, stress adaptation and photosynthesis/respiration. This also supports the functional role of these enzymes in H_2O_2 scavenging, thereby implicating their importance in plant defense (Ozyigit et al., 2016). Hence, IAA27, MAPK and GPX genes could be used as a marker gene for the analysis of plant growth and immunity.

Plants exhibit a broad range of defense responses which may be pre-existing or induce protection upon pathogen infection. For activation of induced defense in plants, several abiotic and biotic factors have been employed which are in general termed as elicitors (Choi and Hwang, 2011; Altinok and Dikilitas, 2014; Abhayashree et al., 2016). Numerous abiotic elicitors have been employed in various crop plants to induce resistance in plants upon pathogen attack including salicylic acid (SA) (Ahmad et al., 2013; Awang et al., 2013; Kuzniak et al., 2013; Raut and Borkar, 2014). SA is a hormone signaling molecule associated with diverse cellular functions during defense against invading pathogens. Exogenous application of SA are known to induce hypersensitive response at the site of infection, increase phenolic content (leads to production of ROS), provide antioxidant protection, activate defense-related enzymes and its gene expression which finally leads to induction of resistance in plants against wide range of pathogens (Buzi et al., 2004; Grant and Lamb, 2006; Loake and Grant, 2007; Xu et al., 2008; Torres, 2009; Yun and Chen, 2011; Zhang et al., 2016).

Brinjal (*Solanum melongena* L.) is an important vegetable crop grown in tropical and temperate regions of the world and India stands second largest producer next to China. The brinjal varieties which are currently in use are highly susceptible to pathogens as well as abiotic stresses which have resulted in significant yield loss (Wally and Punja, 2010; Ceasar and Ignacimuthu, 2012). One of the major biotic constraints to brinjal is wilt disease caused by various fungal species including *Verticillium* which results in an enormous loss in crop yield annually. *Verticillium* wilt caused by soil-borne fungi *V. dahliae* is one of the most destructive species in *Verticillium* and is known to infect a broad range of crop plants (Bhat and Subbarao, 1999). *Verticillium* wilt is hard to control due to the continued viability of resting spores (nearly 15 years), wide host range (200 hosts) and the inability of fungicides to affect once the pathogen enters the vascular system (Fradin and Thomma, 2006). Interestingly, no resistant cultivars of brinjal against *Verticillium* wilt exist (Zhou et al., 2012; Singh et al., 2014; Liu et al., 2015). Presently the methods used to control *Verticillium* wilt by crop rotation is found ineffective, fungicides like benomyl and carbendazim have been futile due to development of resistant strains (Talboys, 1984), while modern plant activators like acibenzolar-S-methyl (ASM) are found to be the only source reporting induction of resistance in many solanaceous crop plants including brinjal against *Verticillium* wilt (Bubici et al., 2006). Hence, the present work was aimed to elucidate the mechanism of induction of resistance at molecular level offered by SA against *Verticillium* wilt of brinjal by analyzing the transcript accumulation patterns of PR-proteins (chitinase and β-1,3-glucanse) along with IAA27, MPK1 and GPX in response to pathogen infection.

2. Materials and methods

2.1. Collection of samples

Seeds of brinjal cultivar (cv.) Local M5 susceptible (unpublished data) to *Verticillium* wilt were obtained from Srirangapatna, Mandya, Karnataka, India and used throughout the study.

2.2. Isolation and molecular characterization of Verticillium dahliae

Brinjal plants displaying typical symptoms of *Verticillium* wilt (root and stem) were collected from brinjal growing regions of Karnataka, India (Fig. 1). In brief, infected plant material was surface sterilized with 2% sodium hypochlorite solution and repeatedly washed with sterile distilled water (SDW). Moisture was absorbed on sterile filter paper and the infected plant materials were placed on potato dextrose agar (PDA) medium supplemented with chloramphenicol (100 mg L^{-1}). All plates were incubated at 25 ± 2 °C for seven days and fungal colonies showing typical symptoms of *V. dahliae* were picked using sterile inoculation needle and subcultured on PDA. The fungal isolate was confirmed based on morphology, conidiophores, conidia and presence of microsclerotia (Hawksworth and Talboys, 1970). Further, genomic DNA was extracted from the lyophilized fungal mat of (*Verticillium dahliae*) by the cetyltrimethylammonium bromide (CTAB) method. The nuclear ribosomal DNA and internally transcribed spacer (ITS) region were amplified using ITS1 (5′- TCCGTAGGTGAACCTGCG-3′) and ITS4 (5- TCCTCCGCTTATTGATATG-3′) primers (White et al., 1990). The amplicon was sequenced using ITS1 and ITS4 primers in two different sequencing reactions. A contiguous sequence out of two sequences was generated using CAP3 sequence assembly programme (Huang and Madan, 1999) and submitted to the GenBank nucleotide collection with NCBI Accession No. KY775342.

2.3. Preparation of inoculum

V. dahliae pure cultures grown on PDA (10–15 days) were flooded with 5–10 ml of SDW for each plate and spores were dislodged by shaking or by using a sterile brush. The spore suspension was passed through double layer cheesecloth. The concentration of the spore suspension was adjusted to 1×10^7 spores mL^{-1} using Haemocytometer and used as standard inoculum throughout the study (Zhou et al., 2012).

2.4. Seed priming with salicylic acid (SA)

Aqueous solutions of salicylic acid (Sigma-Aldrich) were prepared at different concentrations viz., 0.25, 0.5, 0.75 and 1.0 mM. The surface sterilized brinjal seeds were primed with various concentrations of SA by placing 400 seeds in 50 ml for 3 h in a rotary shaker at 25 ± 2 °C. The SA primed seeds were aseptically air-dried and used throughout the study. Seeds treated with SDW served as control.