Induction of resistance in pepper against *Xanthomonas euvesicatoria* by β -aminobutyric acid

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Abstract *Xanthomonas euvesicatoria* is one of the most destructive pathogens of pepper that results in serious yield loss worldwide. Previous studies confirmed that β -aminobutyric acid (BABA) induces resistance in plants against a variety of pathogens. A promoted resistance of pepper induced by BABA to *X. euvesicatoria* was demonstrated in this study. Additionally, induction of catalase and pathogenesis-related gene 1 during the BABA-induced resistance was confirmed. Our data provides useful information for the development of disease management programs.

Keywords β -aminobutyric acid · Catalase · Pepper · Pathogenesis-related gene 1 · *Xanthomonas euvesicatoria*

In recent years, the possibility of induced resistance in plants against different pathogens using chemical inducers has opened up new alternatives for management of plant diseases. β -aminobutyric acid (BABA) is a non-protein amino acid compound that has been shown to mediate induction of resistance in some plant species versus a broad range of plant pathogens including fungi, nematodes, viruses and bacteria (Justyna and Ewa 2013; Baccelli and Mauch-Mani 2016). Plants employ various strategies to defend against pathogens. Production and accumulation of pathogenesis-related (PR) proteins in plants are important defense mechanisms in response to pathogen invasion. PR proteins are divided into 17 groups according to their properties and functions (Ebrahim

et al. 2011). The role of PR genes in induced resistance by BABA was investigated earlier (Zimmerli et al. 2001; Cohen 2002). Reactive oxygen species (ROS) are produced in plants upon pathogen attack. On the other hand, plants produce antioxidant enzymes such as catalase (CAT) to protect themselves versus direct toxic effects of ROS (Mittler et al. 2004). Accumulation of antioxidant enzymes during enhanced resistance of plants against different stresses induced by BABA has been previously demonstrated (Sahebani and Hadavi 2009; Hossain et al. 2012).

Bacterial spot caused by *Xanthomonas euvesicatoria* is an economically important disease of pepper worldwide (Kousik and Ritchie 1996a). Although BABA has been widely used to enhance resistance against a variety of plant pathogens, to date, there are no studies about application of BABA-mediated induced resistance to *X. euvesicatoria* in pepper. Therefore, the objective of this study was to investigate the efficacy of BABA on disease severity of bacterial spot in pepper plants. Moreover, to extend our understanding about the probable mechanisms involving in induced resistance, the expression of *PR-1* and *CAT* was investigated during enhanced resistance of pepper against *X. euvesicatoria*.

The pathogen used for this experiment (*X. euvesicatoria* Xeu3)- deposited in International Collection of Microorganisms from Plants (ICMP), accession number: ICMP 22075 - was originally isolated from pepper in East Azerbaijan (north-western Iran) in 2013 (Osdaghi et al. 2016). To survey the effect of BABA on the bacterial growth in vitro, an overnight of culture with a density of 10^9 CFU/ml was diluted 1000 times in the same medium (nutrient broth) complemented with BABA to the final concentration of 200 μ l. The media were placed on a shaker (200 rpm) at 28°C. A control treatment not containing BABA was prepared under the same conditions. The bacterial concentration was measured by optical density (OD₆₀₀) after 5 and 10 h. Five

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replicates were used for each treatment. Pepper seeds (*Capsicum annuum*) were sown in quartz sand in 10-cm plastic pots in a growth chamber. BABA at concentration of 200 μ M was sprayed onto the leaves of five-week-old plants. Distilled water was sprayed onto the seedlings as a control. For inoculum production, the bacterium was grown overnight on nutrient agar at 28°C. Bacterial suspensions were adjusted to a concentration of about 10^8 CFU/ml in sterile distilled water. Plants were treated with BABA or water 24 h prior to spraying the bacterial inoculum (150 μ l) onto each leaf. Plants were incubated at 28°C with 16-h light daily and 70% relative humidity. Symptoms of the disease were scored at 7 and 14 day intervals post pathogen inoculation (dpi) using the following scale: 0 = no lesions observed, 1 = less than 1% leaf area diseased, 2 = 1 to 10% leaf area diseased, 3 = 11 to 20% leaf area diseased, 4 = 21 to 35% leaf area diseased, 5 = 36 to 50% leaf area diseased, 6 = 51 to 65% leaf area diseased, 7 = 66 to 80% leaf area diseased, 8 = 81 to 99% leaf area diseased, and 9 = complete defoliation (Kousik and Ritchie 1996b). After assessment, the leaves were harvested at 12, 24, 48 and 72 h post pathogen inoculation (hpi) and RNA isolation was performed using a RNA extraction kit (DENArist, Iran), according to manufacturer's protocol. RNA quality and quantity was determined by agarose gel electrophoresis and spectrophotometry. Synthesis of cDNA was carried out on total RNA using a commercial kit (Fermentas, Lithuania) following the manufacturer's instructions. Real-Time PCR experiments were performed using RealQ PCR 2 $\times$ Master Mix (Ampliqon, Denmark). Primers used in this study are listed in Table 1. The PCR mixture contained 10 μ l (2 $\times$) SYBR green Master Mix, 100 ng of cDNA, 0.5 μ l of 10 μ M of each forward and reverse primers with a total volume of 20 μ l which was adjusted with water. Thermal cycling conditions included 95°C for 5 min, 35 cycles of 95°C for 1 min, 65°C for 2 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. Relative gene expression was measured using the comparative $2^{-\Delta\Delta C_t}$ according to the method described by Livak and Schmittgen (2001). Seven plants were used for greenhouse experiments per treatment and the experiments were repeated twice. Four replicates were used for real-time assay per each sample. Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC, USA) and a probability of $p < 0.05$ was considered significant. The means were separated by Duncan's multiple range tests.

No significant difference was found in bacterial growth in vitro between BABA and control treatments (Fig. 1). Our results showed that pre-treatment with BABA lead to significant decrease in bacterial spot disease severity at 7 and 14 dpi (Fig. 2). Pre-treatment with BABA increased expression of *PR-1* at all time points compared to the control. The highest expression was found at 12 hpi (Fig. 3). Moreover, a significant increase was observed in expression of *CAT* in BABA treatment compared to the control at 12, 24 and 48 hpi (Fig. 4).

Table 1 The primers used in this study

Target Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Reference
Pathogenesis-related gene 1	CTTTTGCTATTTCACTCAACACAGCCC	TGCTGGATTATTTTCCTTTTAAACACATGA	Mejía-Teniente et al. 2013
Catalase	GTCCATGAGCGTGGAAAGCCCCGAAT	CGCGATGCATGAAGTTTCATGGCACC	Mejía-Teniente et al. 2013
glyceraldehyde phosphate dehydrogenase (reference gene)	GGCCTTATGACTACAGTTTCACTCC	GATCAACCCACAGAGACATCCACAG	Mejía-Teniente et al. 2013

Fig. 1 Effect of BABA on growth of *X. euvesicatoria* in vitro. There was no significant difference in population of the bacterium in nutrient broth between BABA and control treatments at the both time points

Our results revealed that BABA does not have a direct toxic effect on *X. euvesicatoria*. The lack of antimicrobial activity of BABA against several bacterial pathogens like *Erwinia amylovora* (Hassan and Buchenauer 2007) and *Pectobacterium carotovorum* subsp. *carotovorum* (Safaie Farahani et al. 2016) has been reported. By contrast, toxic effects of BABA at high concentrations on some fungal pathogens such as *Penicillium italicum* (Tavallali et al. 2008), *Botrytis cinerea* (Fischer et al. 2009) and *Sclerotinia sclerotiorum* (Marcucci et al. 2010) have been observed. Our data confirmed that BABA increases resistance of pepper to *X. euvesicatoria*. Induction of some defense genes in primed plants by BABA upon pathogen invasion have been documented (Justyna and Ewa 2013; Baccelli and Mauch-Mani 2016). Expression of *CAT* and *PR-1* during BABA-induced resistance of pepper to *X. euvesicatoria* was demonstrated in this study. In Arabidopsis, BABA-induced resistance to *Pseudomonas syringae* pv. *tomato* by activating of *PR-1* (Zimmerli et al. 2000) which is consistent with this present study. Kamble and Bhargava (2007) showed the ability of BABA to protect brown mustard against *Alternaria brassicae*, which was also associated with increased expression of *PR-1*.

Fig. 2 Effects of BABA on disease score of bacterial spot of pepper at 7 and 14 dpi. Significant difference was found between plants treated with BABA and the control at the both time points

Fig. 3 Expression of *PR-1* in pepper plants treated with BABA compared to the control. Significant difference was found in expression of *PR-1* between BABA/*Xanthomonas euvesicatoria* and water/*Xanthomonas euvesicatoria* at all time points. The maximum expression was observed at 12 hpi

Zimmerli et al. (2001) showed that BABA increases expression of *PR-1* during induced resistance by BABA in Arabidopsis to *Botrytis cinerea*. In contrast, no accumulation of PR proteins was found in induced resistance by BABA in cauliflower, sunflower and lettuce against *Peronospora parasitica*, *Puccinia helianthi* and *Bremia lactucae*, respectively (Silue et al. 2002; Amzalek and Cohen 2007; Cohen et al. 2010). Hence, it seems that induction of PR genes is not the only mechanism of resistance induced by BABA. Additionally, expression of defense genes during BABA-induced resistance might depend on plant species, pathogen and their interaction. It can be speculated that pre-treatment with BABA leads to an oxidative burst of ROS upon pathogen attack. This leads to increased amounts of CAT which balances ROS and acts as a signal for stimulation of other defense responses such as systemic acquired resistance (Alvarez et al. 1998), phytoalexin production (Daudi et al. 2012) and callose deposition (O'Brien et al. 2012). Increased antioxidant enzyme activity can occur in tomato during promoted resistance

Fig. 4 Expression of *CAT* in pepper plants treated with BABA compared to the control. Significant difference was found in expression of *CAT* between BABA/*Xanthomonas euvesicatoria* and water/*Xanthomonas euvesicatoria* at 12, 24 and 48 hpi. The maximum expressions were found at 12 and 24 hpi

induced by BABA against *Meloidogyne javanica* (Sahebani and Hadavi 2009). CAT activity was increased in tomato by BABA following *Pseudomonas syringae* pv. *tomato* invasion (Baysal et al. 2007). In summary, the role of BABA for enhancing resistance of pepper against *X. euvesicatoria* was confirmed in this study. Our research has shown that the reduction in disease severity by BABA may be correlated with the activation of some defense genes such as *PR-1* and *CAT*.

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