γ-Aminobutyric acid induces resistance against *Penicillium expansum* by priming of defence responses in pear fruit

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ABSTRACT

The results from this study showed that treatment with γ-aminobutyric acid (GABA), at 100–1000 µg/ml, induced strong resistance against blue mould rot caused by *Penicillium expansum* in pear fruit. Moreover, the activities of five defence-related enzymes (including chitinase, β-1,3-glucanase, phenylalnine ammonialyase, peroxidase and polyphenol oxidase) and the expression of these corresponding genes were markedly and/or promptly enhanced in the treatment with GABA and inoculation with *P. expansum* compared with those that were treated with GABA or inoculated with pathogen alone. In addition, the treatment of pear with GABA had little adverse effect on the edible quality of the fruit. To the best of our knowledge, this is the first report that GABA can effectively reduce fungal disease of harvested fruit. Its mechanisms may be closely correlated with the induction of fruit resistance by priming activation and expression of defence-related enzymes and genes upon challenge with pathogen.

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1. Introduction

Pathogen infection is an important factor that affects fruit postharvest physiology and metabolism (Prusky et al., 2010). *Penicillium expansum*, the causal agent of blue mould, is a widespread fungal pathogen and causes considerable pome fruit losses, in apples, cherries, pears and peaches (Cao, Yang, Hua, & Zheng, 2011; Jurick et al., 2009; Malandrakis et al., 2013; Quaglia, Ederli, Pasqualini, & Zazzerini, 2011; Venturini, Oria, & Blanco, 2002). Besides the economic impact, *P. expansum* is also regarded as the major producer of patulin (4-hydroxy-4H-furo[3,2-c]pyran-2[6H]one), mycotoxin and secondary metabolites toxic to humans (Malandrakis et al., 2013). Moreover, the intense use of synthetic fungicides has led to the increasing resistance of fungal pathogens. Therefore, there is a growing need for eco-friendly alternative strategies to inhibit the fungal decay (Droby, Wisniewski, Macarisin, & Wilson, 2009).

Attempts to exploit induced resistance through the application of defence response elicitors are being pursued, to control postharvest diseases as a safer technology (Shoresh, Harman, & Mastouri, 2010). Several chemical elicitors have been reported to induce resistance to pathogens, e.g. salicylic acid (SA) and its functional analogue acibenzolar-S-methyl (ASM) and β-aminobutyric acid

(BABA) (Quaglia et al., 2011). A common feature of induced resistance caused by these elicitors is called priming (Conrath, Pieterse, & Mauch-Mani, 2002; Wang, Xu, Wang, Jin, & Zheng, 2013), which can cause a faster and stronger activation of defence response mechanisms after pathogen infection (Tonelli, Furlan, Taurian, Castro, & Fabra, 2011).

γ-Aminobutyric acid (GABA), a four carbon, non-protein free amino acid, is widespread in most prokaryotic and eukaryotic organisms (Bown, MacGregor, & Shelp, 2006). In plants, multiple signalling roles have been attributed to GABA, including involvement in pH regulation, nitrogen storage, plant development and defence against abiotic stresses, such as drought, oxidative stress, salinity and cold stress (Shelp et al., 2012). Experimental evidence shows that exogenous GABA can alleviate oxidative damage caused by H^+ and Al^{3+} toxicities in barley seedlings (Song, Xu, Wang, Wang, & Tao, 2010) and reduce chilling injury of peach fruit by inducing proline accumulation, enhancing the enzymatic antioxidant system and maintaining energy status (Shang, Cao, Yang, Cai, & Zheng, 2011; Yang, Cao, Yang, Cai, & Zheng, 2011). However, there is little information about the effect of GABA on the postharvest fungal diseases of fruit.

The aim of this research was to assess the effect of GABA on induction of resistance to blue mould rot caused by *P. expansum* in pear fruit and the possible action mechanisms involved.

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2. Materials and methods

2.1. Fruit, pathogen and GABA

Pear fruit (*Pyrus pyrifolia* Nakai. cultivar "Shuijing") were sorted on the basis of size, colour, maturity and absence of physical injury or infection. The fruit surfaces were disinfected in 0.1% sodium hypochlorite solution for 2 min, thoroughly washed in tap water and air-dried at 25 °C prior to use.

An isolate of *P. expansum* obtained from rotted pear fruit was maintained on potato dextrose agar (PDA) medium (containing the extract from 200 g of potato, 20 g of glucose and 20 g of agar in 1 l of distilled water) for 7 days at 25 °C. Sterile distilled water was used to flood the surface of the PDA culture and conidia were scraped by a sterile loop. The spore concentrations obtained were determined using a hemacytometer and adjusted with sterile distilled water as required.

GABA was purchased from Sangon Biotech (Shanghai, China). The stock solution of GABA was diluted to a series (1, 10, 100 and 1000 μg/ml).

2.2. Efficacy of GABA for control of blue mould rot caused by P. expansum in pear fruit

2.2.1. Effect of GABA at different concentrations on inhibition of blue mould rot in pear fruit

The fruit were wounded (5 mm wide by 3 mm deep) with the tip of a sterile border at five different sites. 30 μl of the aqueous preparation which contained GABA at 1, 10, 100 and 1000 μg/ml were injected into each wound. Wounds treated with the same amounts of sterile distilled water served as control. After 2 or 24 h, each wound was inoculated with 30 μl of a conidial suspension of *P. expansum* at 1×10^4 spores/ml. The fruit were then air-dried and incubated in the dark at 25 °C to maintain 90% relative humidity (RH). Each treatment included 3 replicates and each replicate consisted of 9 fruit.

2.2.2. Effect of GABA treatment time on induction of disease resistance against blue mould rot in pear fruit

Five wounds were made on each pear fruit as above and each wound was treated with 30 μl of GABA, at 100 μg/ml, or sterile distilled water as the control. After 0, 6, 12, 24 and 36 h, fruit were inoculated with 30 μl of the *P. expansum* suspension at 1×10^4 spores/ml in each wound. The fruits were then air-dried and incubated in the dark at 25 °C to retain about 90% RH. Each treatment included 3 replicates and each replicate consisted of 9 fruit.

2.2.3. Effect of GABA on blue mould rot at different spore concentrations in pear fruit

Six wounds were made on the surface of each pear fruit as described above and three of the wounds were treated with 30 μl of GABA at 100 μg/ml or with 30 μl of sterile water as control. After 24 h, 30 μl of 1×10^2, 1×10^4 or 1×10^6 spores/ml suspension of *P. expansum* were respectively inoculated into each wound. The fruits were then air-dried and incubated in the dark at 25 °C to retain about 90% RH. Each treatment included 3 replicates and each replicate consisted of 9 fruit.

2.2.4. Effect of GABA on blue mould rot development at low temperature in pear fruit

Pear fruit were wounded at two sites as above. Aliquots of 30 μl of GABA at 100 μg/ml and sterile water as a control, were pipetted onto each wound and 24 h later wounds were then inoculated with 30 μl of a 1×10^4 spores/ml suspension of *P. expansum*. The fruits were then air-dried and incubated in the dark at 4 °C to maintain 90% RH. Each treatment included 3 replicates and each replicate consisted of 9 fruit.

In the above four tests, the percentage of wounds showing rot symptoms was assessed on a daily basis. Disease incidence was defined as decayed fruit/total fruit × 100%. Each test was performed at least twice. The discussed data were from one individual experiment and representative of the two experiments with similar results.

2.3. Effect of GABA on germination and survival of P. expansum spores in vitro

The effect of GABA on spore germination of *P. expansum* was tested in potato dextrose broth (PDB) with various concentrations of GABA (0, 1, 10, 100, 1000 μg/ml). Aliquots of 100 μl of pathogen suspensions were put into 10 ml glass tubes containing 2 ml of PDB to obtain a final concentration of 1×10^6 spores/ml. All treated tubes were placed on a rotary shaker at 200 rpm at 25 °C. After 12 h of incubation, approximately 150–200 spores of the pathogen per replicate were measured for germination rate. Spores were considered germinated when germ tube lengths were equal to or greater than spore lengths. All treatments consisted of three replicates and the experiment was repeated twice.

Equal amounts of *P. expansum* spores were mixed with a solution of GABA in final concentrations of 0, 1, 10, 100, 1000 μg/ml, respectively and kept for 1 min. Then 100 μl of spore suspension were plated on PDA. The colonies per plate were counted after 72 h of incubation at 25 °C. There are three replicates per treatment with three plates per replicate and the experiment was conducted twice.

2.4. Effect of GABA on spore germination of P. expansum in pear fruit wounds

Two of the four wounded sites on each fruit were injected with 30 μl of sterile distilled water (as the control) and 30 μl of GABA, at 100 μg/ml, were placed in the other two wounds. After 2 or 24 h, each wound was inoculated with 30 μl of a conidial suspension of *P. expansum* at 1×10^7 spores/ml. After being air-dried, pear fruit were stored in enclosed plastic trays to maintain a high relative humidity at 25 °C. After 12 h postinoculation, 150–200 spores per replicate were examined microscopically to determine germination rate. Each treatment included 3 replicates and each replicate consisted of nine fruit for each time point.

2.5. Sample treatment for enzyme activity and gene expression analysis

The wounds were inoculated with 30 μl of the *P. expansum* suspension at 1×10^4 spores/ml 24 h after the treatment of GABA at 100 μg/ml. Tissue samples of pulp from the GABA or water-treated fruit, with or without inoculation, were collected at the same intervals (0, 12, 24, 36 and 48 h after the treatment) and immediately frozen in liquid N_2. They were then stored at −80 °C for further analysis of enzyme activity and gene expression.

2.6. Assay of enzyme activities in pear fruit

Frozen tissue samples (0.6 g) were ground in a mortar and pestle with different buffers to assay different enzymes: 1.2 ml of sodium acetate buffer (50 mM, pH 5.0), that contained 1% (w/v) polyvinyl-pyrrolidone (PVP) for chitinase and 5.4 ml for β-1,3-glucanase, 1.2 ml of 200 mM sodium borate buffer (pH 8.8) for PAL and 1.2 ml of sodium phosphate buffer (50 mM, pH 7.8) containing 1.33 mM EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP) for POD and PPO. The homogenate was centrifuged at 13,000g for 20 min