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β-AMINOBUTYRIC ACID INDUCES DISEASE RESISTANCE AGAINST *BOTRYTIS CINEREA* IN GRAPE BERRIES BY A CELLULAR PRIMING MECHANISM

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The present study was performed to investigate the effect of β-aminobutyric acid (BABA) treatment on defence activation in grape berries and to analyse its cellular mechanism. The results implied that BABA treatment at an effective concentration of 20 mM significantly inhibited gray mould rot caused by *Botrytis cinerea* in grape berries by inducing resistance. Accordingly, 20 mM BABA triggered a priming defence in grape suspension cells, since only the BABA-treated cells exhibited an accelerated ability for augmenting defence responses upon the pathogen inoculation. The primed cellular reactions were related to an early H_2O_2 burst, prompt accumulation of stilbene phytoalexins and activation of *PR* genes. Thus, we assume that 20 mM BABA can induce resistance to *B. cinerea* infection in intact grape berries perhaps via intercellular priming defence. Moreover, the BABA-induced priming defence is verified, because no negative effects on cell growth, anthocyanin synthesis, and quality impairment in either grape cells or intact berries were observed under low pathogenic pressure.

Keywords: grape, cells, β-aminobutyric acid (BABA), priming, *Botrytis cinerea*

In the grapevine, one of the most significant postharvest diseases is gray mould infection by the necrotrophic fungi *Botrytis cinerea* that can proliferate rapidly under a wide range of temperature and humidity conditions, thus leading to severe quality deterioration in grape berries and other berry fruit. At a time when synthetic pesticide use is being curtailed in grape fruit production, alternative measures for controlling postharvest disease have been in strong demand. Among the recent new strategies, one sustainable method is to activate broad-spectrum resistance against pathogenic infections in the plant itself via the application of eco-compatible natural inducing elicitors (Romanazzi et al., 2016).

Currently, the representative mode of induced resistance is commonly referred to as the defence of SAR (systemic acquired resistance), which can be activated directly by various biotic or abiotic elicitations and mediated by signal conduction that relies on the endogenous generation of salicylic acid and the functional regulatory *NPR* protein (Gozzo & Faoro, 2013). However, in cases of ISR (induced systemic resistance) via non-pathogenic rhizobacteria in *Arabidopsis*, the defence is based not on direct activation but on quicker and stronger induction once the plant has been subjected to a high disease pressure, which can be considered a typical priming response (Conrath et al., 2015). Since induced resistance can cause an undesirable trade-off of impairment on normal plant growth because of the metabolic or energy investments in costly defence processes, priming indeed entails fewer costs relative

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to direct resistance, which could be attributed to saving energy in the priming response under pathogen-free conditions (VAN HULTEN et al., 2006). The inducer β-aminobutyric acid (BABA), which is known as a non-protein amino acid without remarkable antimicrobial properties at relatively low doses, triggers highly efficient resistance in terms of enhanced transcripts of pathogenesis-related (*PR*) genes, which results in the provision of broad-spectrum protection against various microbes in plants (COHEN et al., 2016). BABA has also been applied to enhance resistance against different fungal infections in agronomic fruit, including apple (ZHANG et al., 2011) and strawberries (WANG et al., 2016). Nevertheless, the molecular mechanisms underpinning the BABA-induced defence in horticultural crops are not yet fully understood.

Compared with intact plants, cultured suspension cells are generally useful materials for researching several aspects of plant biology, since the suspension confers a source of homogeneous cells that minimize interference factors, such as slow plant growth, seasonal or environmental variations, and pathogen or herbivore attack (BELCHÍ-NAVARRO et al., 2013). The application of these simplified cellular systems, fungal cell wall fragments, or inducing chemicals to grape cell suspensions promotes the expression levels of a series of defence genes closely related to the biosynthesis of *PR* proteins and/or phytoalexins (MARTINEZ-ESTESO et al., 2009). In our previous study, a BABA treatment at 20 mM effectively reduced postharvest decay of grape fruit. Therefore, the present study aimed to investigate the ability of the 20 mM BABA treatment to induce resistance against *B. cinerea* infection in grape berries and clearly elucidate the mode of action in a model cellular system.

1. Materials and methods

1.1. Grape, callus, and fungi

Organically grown grape berries of the cv. 'Kyoho' were harvested at the mature stage from a vineyard located in south-western China and then immediately transferred to our laboratory, where grape berries with a uniform maturity and size and free from visual blemishes, physical damage, and pathological infections were used (LIAO et al., 2018). Callus tissue was separated from sterilized skins of grape berries on 10-year-old *Vitis vinifera* plants by our previously described methods (WANG et al., 2015). The spore suspensions of the cultures of *B. cinerea* were generated by washing the spores from the sporulating edges of the 14-day-old cultures of the pathogen on potato dextrose agar (PDA) and then modifying the concentration to 5.0×10^5 spores/ml.

1.2. Intact fruit experiment

All selected berries were superficially sterilized with 75% (v/v) ethanol of medical grade, drained on filter papers, and wounded (2 mm deep by 1.5 mm wide) with dissecting needles at two symmetrical sites around the equatorial region. The wounded fruit were randomly divided into two equal groups of 1000 fruits each, after which a 20 µl aliquot of sterile water or a BABA solution at 20 mmol l^{-1} was pipetted into each wound site of each group of fruit. Then, the pathogenic inoculation was performed by pipetting 20 µl of *B. cinerea* suspension into all wounds. After air drying for 2 h at 22 °C, all treated grape berries were arranged in covered polyethylene boxes and stored at 22 °C and 90–95% RH for 5 d. Disease evaluations were conducted on days 1, 3, and 5 during storage. Quality parameters were measured at the

end of the storage, according to the method of LIAO and co-workers (2018). Each treatment consisted of three replications, and the entire experiment was conducted three times.

1.3. Cell experiment

1.3.1. Treatments. Five grams of fresh cells was transferred to 100 ml of B5 medium without hormones and incubated on a reciprocating shaker at 100 strokes/min in darkness at 22±1 °C after approximately 2–3 days of incubation (WANG et al., 2015), and then all flasks with active cells were randomly divided into four equal groups as follows: 1) Control, grape cell suspensions were incubated in B5 medium without any supplementation; 2) *B. cinerea*-challenge, aliquots (2 ml) of the prepared spore suspension of *B. cinerea* at 5.0×10^5 spores/ml were added to 100-ml cell cultures to obtain a 1.0×10^4 spores/ml pathogen suspension according to GRANADO and co-workers (1995); 3) BABA treatment, BABA powder (purity ≥99%, Sigma Co.) was dissolved in sterile distilled water and then added to the grape cell suspension to a final concentration of 20 mM; 4) BABA+*B. cinerea*: grape cells were elicited with the BABA 1 h prior to the *B. cinerea* challenge with 1.0×10^4 spores/ml. Afterwards, all cell cultures were incubated under the same conditions as mentioned above for 5 days. The cells were collected before the addition of elicitor (time 0) and daily for the assessment of cell weight and then quick-frozen at –80 °C until analysis. Each treatment was carried out in triplicate, and the entire experiment was performed three times.

1.3.2. Assay for growth. The fresh cell weight (FCW) and dry cell weight (DCW) were measured following the methods of WANG and co-workers (2015), and the results were expressed as grams per kilogram of culture.

1.3.3. Measurement of cellular H_2O_2 generation, defence-related enzyme activity, and individual phytoalexin and anthocyanin contents. The content of cellular H_2O_2 was measured according to the method of PATTERSON and co-workers (1984). Chitinase (EC 3.2.1.14) and β-1,3-glucanase (EC 3.2.1.6) activities were determined using the method of ABELES and co-workers (1971). Individual phytoalexins in the samples were identified and quantified by a HPLC method (AZIZ et al., 2006). The total anthocyanin content was measured via the pH differential method (CHENG & BREEN, 1991).

1.3.4. Analysis of PR gene expression. Frozen cell samples (5 g) were gently ground in liquid N_2. Total RNA was prepared with the RNAprep Pure Plant Kit (Tiangen, Shanghai, China) according to the manufacturer's instructions. A qRT-PCR assay was carried out using a GoTaq 2-step RT-qPCR System kit (Promega, USA) in a Thermal Cycler Dice™ Real Time System (Takara, Japan) with the specific primers of the *PR* genes (Table 1) based on the manufacturer's instructions. All qRT-PCR reactions were normalized by the threshold cycle value (C^T) compared with the internal reference gene of *Vv18S rRNA*, and the transcription level was calculated by the method of LIVAK and SCHMITTGEN (2001).

1.4. Data analysis

Data from one independent experiment were analysed and expressed as the means ± SE (standard error) of triplicate assays, since the results of the three experiments were similar. Duncan's multiple range test was applied to compare the means at the 0.05 significance level using SPSS software (ver. 14.0).

Table 1. Sequence of primers used for real-time quantitative PCR in grapes

Gene	NCBI or Genoscope *Vitis* accession number	Primer (Forward/ Reverse)
VvNPR1.1	GSVIVT00016536001	ATAGTGCGGTCTGCCATCTTTAC /GGAGTCCATTAGCACTCCTTTG
VvPR1	GSVIVT00038575001	GGAGTCCATTAGCACTCCTTTG /CATAATTCTGGGCGTAGGCAG
VvPR2	AY137377	TGCTGTTTACTCGGCACTTG /CTGGGGATTTCCTGTTCTCA
VvChit4c	AJ27790	TCGAATGCGATGGTGGAAA /TCCCCTGTCGAAACACCAAG
Vv18S-rRNA	AF321266	GCTTTGCCGTTGCTCTGATGAT /TTTGCCGATGGTGTAGGTTCCT

2. Results and discussion

2.1. Effects of BABA elicitation on B. cinerea *infection and quality in grape berries*

The BABA treatment at 20 mM led to a significant inhibitory effect on *B. cinerea* infection in grape berries over the whole storage period. After 5 days of storage at 22 °C, the percentage of infected berries and the diameter of disease lesions in the BABA-treated grape berries were 65.7% and 24.6% lower than those in the controls, respectively (Fig. 1). Since BABA had no direct inhibitory effect on spore germination and germ tube elongation of the pathogen *B. cinerea* in vitro (EL-METWALLY et al., 2014), the BABA treatment suppressed the development of gray mould rot in grape berries by directly inhibiting fungal growth and indirectly inducing host resistance. Additionally, the BABA treatment alone significantly reduced the weight loss and maintained higher TSS, anthocyanin content, and DPPH radical scavenging activity among all treatments (Table 2), which indicated that BABA could maintain the overall quality of grape berries.

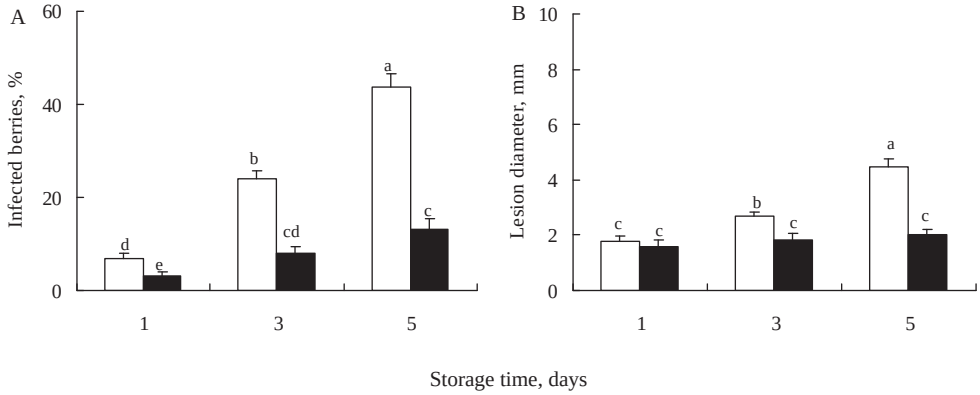

Fig. 1. Effects of 20 mM BABA treatment on the percentage of infected berries (A) and lesion diameter (B) in grape berries inoculated with *B. cinerea* during the storage. Different letters above the bars indicate statistically significant differences.
□: Control; ■: BABA treatment

Table 2. Effects of 20 mM BABA treatment and *B. cinerea* inoculation on postharvest quality in grape berries

Treatments	TSS, %	TA, %	pH value	Weight loss, %	Total anthocyanins content, μmol C3G/kg FW	DPPH radicals scavenging activity, %
Control	11.15±0.58b	0.93±0.05a	3.68±0.02a	0.94±0.09b	102.18±8.17c	40.71±2.98c
B. cinerea inoculation	9.63±0.63c	0.79±0.02a	3.81±0.06a	1.41±0.12a	97.42±6.36c	32.62±1.93d
BABA treatment	14.73±0.41a	0.97±0.07a	3.62±0.01a	0.72±0.08c	157.49±11.15a	53.29±3.91a
BABA+*B. cinerea* inoculation	13.75±0.34a	0.96±0.04a	3.73±0.05a	0.99±0.15b	134.11±10.89b	48.85±2.84b

Values in a column followed by a different letter are significantly different according to Duncan's multiple range test at $P<0.05$ level.

2.2. Effects of BABA elicitation on cellular H_2O_2 content in grape cells

The earliest stages of the defensive response of plant cells to pathogens include the overproduction of ROS, which functions as a molecule messenger in the signal transduction pathway and leads to a diverse set of defence processes, including activation of defensive genes and biosynthesis of defensive compounds (O'BRIEN et al., 2012). In this study, treatment with 20 mM BABA alone failed to induce H_2O_2 generation and other defensive responses in grape cells. Interestingly, in response to *B. cinerea* supplementation, H_2O_2 underwent an augmented burst in the BABA-treated grape cells (Fig. 2) along with the elevated expression of *PR* genes and synthesis of individual phytoalexins (Figs 3–5). These results support a priming defence mechanism in BABA-treated grape cells, and the triggering of BABA-mediated defence responses by pathogen challenge may have been related to fortified H_2O_2 accumulation.

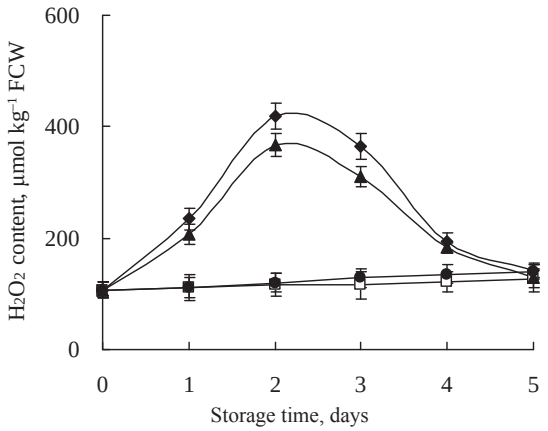

Fig. 2. Effects of 20 mM BABA treatment and *B. cinerea* challenge on cellular H_2O_2 generation in grape suspension cells during storage

—□—: Control; —●—: BABA treatment; —▲—: *B. cinerea* challenge; —◆—: BABA+*B. cinerea*

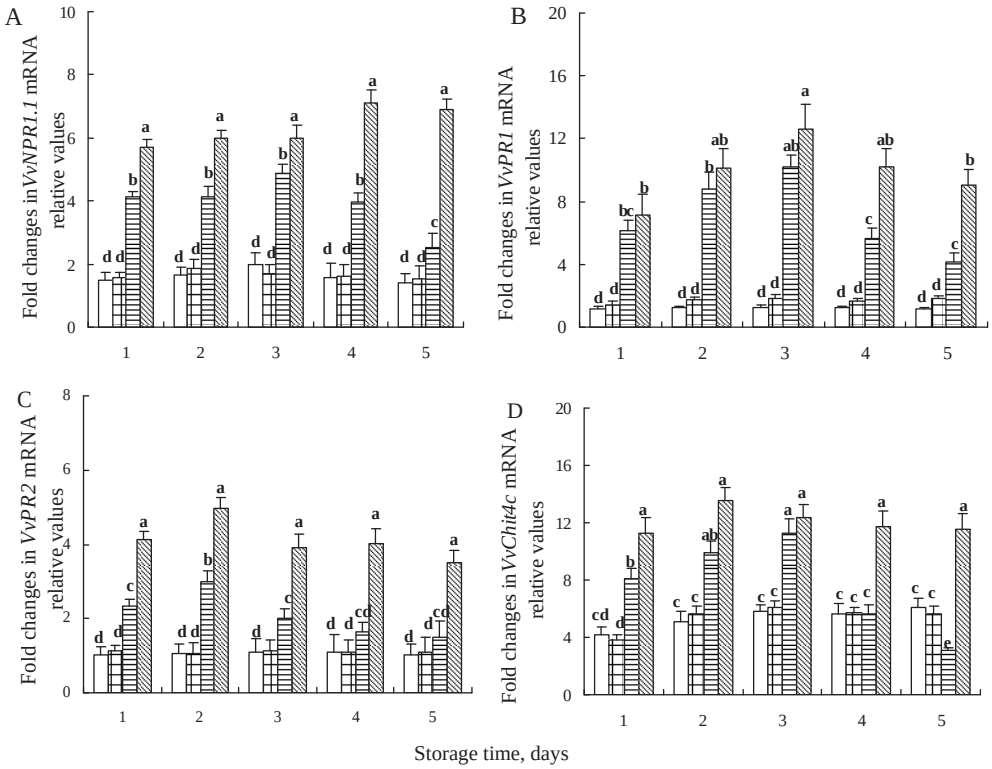

Fig. 3. Effects of 20 mM BABA treatment and *B. cinerea* challenge on the expression of representative regulatory gene *VvNPR1.1* (A) as well as defence-related genes such as *VvPR1* (B), *VvPR2* (C), and *VvChit4c* (D) in grape suspension cells during storage. Different letters above the bars indicate statistically significant differences between treatments.

□: Control; ▦: BABA treatment; ▥: *B. cinerea* challenge; ▧: BABA+*B. c*inerea

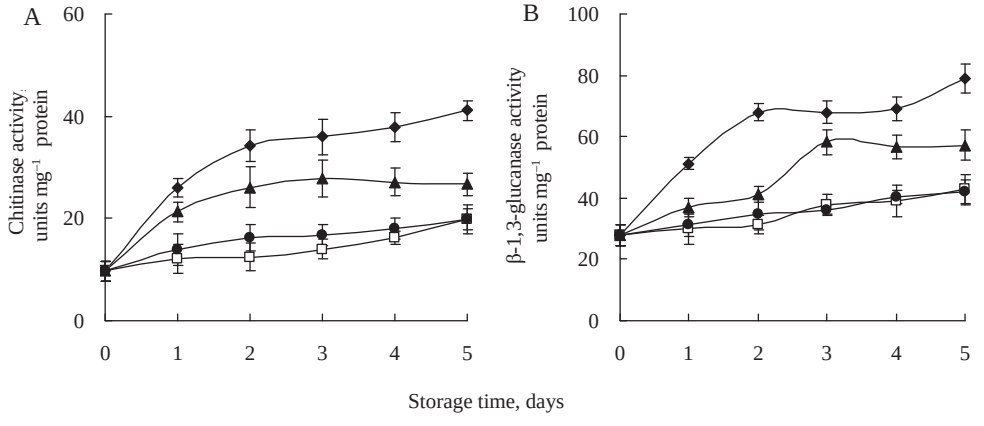

Fig. 4. Effects of 20 mM BABA treatment and *B. cinerea* challenge on the activities of chitinase (A) and β-1,3-glucanase (B) in grape suspension cells during storage

—□—: Control; —●—: BABA treatment; —▲—: *B. cinerea* challenge; —◆—: BABA+*B. cinerea*

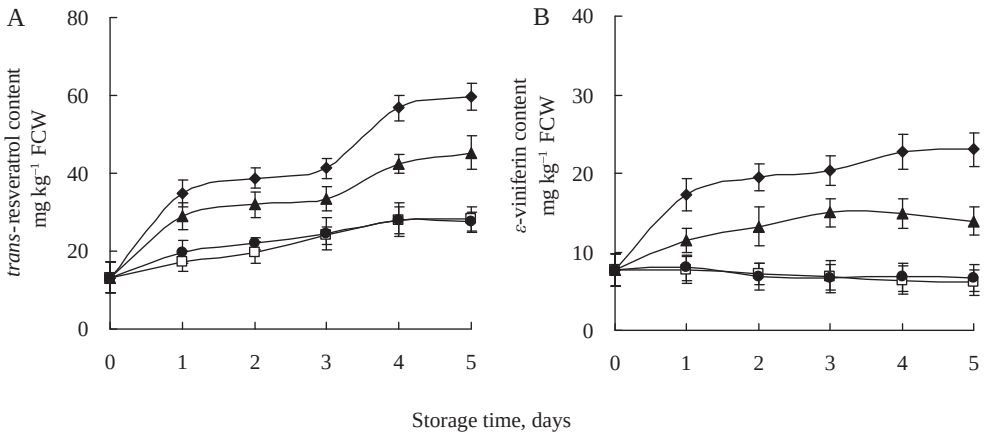

Fig. 5. Effects of 20 mM BABA treatment and *B. cinerea* challenge on the contents of *tran*-resveratrol (A) and ε-viniferin (B) in grape suspension cells during storage

—□—: Control; —●—: BABA treatment; —▲—: *B. cinerea* challenge; —◆—: BABA+*B. cinerea*

2.3. Effects of BABA elicitation on responses expressions of defence in grape cells

The *VvNPR1.1* gene in grapevine acts as a key defence regulator and is similar to the *Arabidopsis thaliana NPR1* gene, which has the potential to trigger the expression of *VvPR1*, *VvChit4c* (encoding chitinase), and *VvPR2* (encoding β-1.3-glucanase) genes to combat pathogens (LE HENANFF et al., 2011). The dominant stilbene phytoalexins in grapevine, including *trans*-resveratrol and ε-viniferin, present strong antimicrobial activity for protecting the plant from a broad spectrum of pathogens and, therefore, are considered crucial compounds involved in the induced resistance in grape fruit (AZIZ et al., 2006). BABA did not induce defence responses directly in the absence of the pathogen *B. cinerea*. However, the *B. cinerea*-challenged grape cells treated with BABA presented more prompt and stronger expression of the *VvNPR1*, *VvPR1*, *VvPR2,* and *VvChit4c* genes as well as an increase in the activities of chitinase and β-1.3-glucanase compared with those in cells challenged only with *B. cinerea* (Figs 3–4). Meanwhile, the BABA treatment did not induce a significant synthesis of any individual stilbene. After *B. cinerea* spore addition, the levels of *trans*-resveratrol and ε-viniferin were stimulated immediately and constantly up to the end of the incubation. BABA strongly accelerated the accumulation of individual phytoalexins and retained significantly higher contents over the incubation compared to cells challenged with *B. cinerea* only (Fig. 5). Hence, these results show that the BABA treatment induced defence responses in grape cells against *B. cinerea* through a priming mechanism, which accelerated the sensitization of the cells to express a basal defensive response upon subsequent pathogen attack. The present results were consistent with our previous results obtained for intact grape berry and strawberry experiments, which showed that lower concentrations of BABA triggered a priming defence mechanism in which the BABA-treated fruit expressed a strong defence response following pathogenic infection (WANG et al., 2016; LIAO et al., 2018). Because cellular defence activation can partially reflect the conditions of systemically resistant plant tissue (MARTINEZ-ESTESO et al., 2009), we postulated that 20 mM BABA exerted its inhibiting effect on gray mould rot in grape berries via cellular priming responses.

2.4. Effects of BABA elicitation on cell growth and total anthocyanin content in grape cells

From an ecological perspective, defence activation can entail costs that allocate limited resources toward the stimulation of elevated levels of defences from the plant's own primary metabolism, which may result in negative effects on plant fitness (HUOT et al., 2014). However, the priming defence is well documented as a more efficient type of induced resistance in several model plants, because the costs associated with the constitutive expression of the defensive response are limited or prevented under low levels of disease occurrence (VAN HULTEN et al., 2006). In this study, an augmented defence response activated by BABA treatment in grape cells was employed to manage pathogen invasion, although a significant reduction in cell weight and total anthocyanin content were simultaneously recorded, indicating that considerable losses were closely linked to the defence expression. In the absence of pathogen challenge, the BABA supply did not suppress the cell growth and secondary metabolite synthesis in grape cells (Fig. 6). Similarly, our current results also showed that the priming action induced by 20 mM BABA treatment significantly affected the balance of anthocyanin metabolism-related enzymes in grape berries during storage at 22 °C or 1 °C, which accelerated the rate of anthocyanin synthesis under low disease incidence

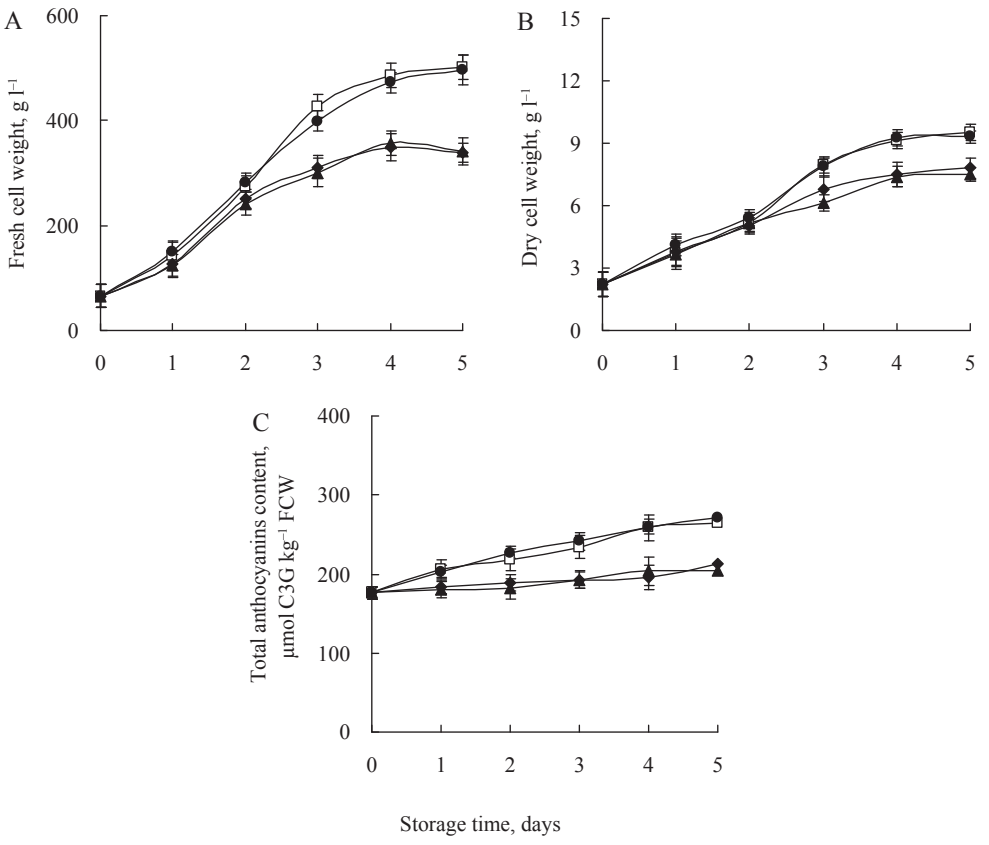

Fig. 6. Effects of 20 mM BABA treatment and *B. cinerea* challenge on the fresh cell weight (A), dry cell weight (B), and total anthocyanins content (C) in grape suspension cells during incubation in shake flasks
—□—: Control; —●—: BABA treatment; —▲—: *B. cinerea* challenge; —◆—: BABA+*B. cinerea*

(data not shown). Meanwhile, the BABA treatment maintained the overall quality, anthocyanin content, and antioxidant activity during the postharvest storage (Table 2). Thus, these results confirmed that BABA-induced priming might combine the advantages of augmented elicitation of defence responses and minor quality losses under pathogen-free conditions.

3. Conclusions

BABA at 20 mM could prime grape cells for augmented stimulation of the early oxidative burst, secretion of stilbene phytoalexins, and induction of *PR* genes and proteins when the fruit are exposed to a *B. cinerea* attack. Moreover, priming did not incur obvious impairment in cell growth or anthocyanin synthesis under pathogen-free conditions. Therefore, we suggest that the priming defence induced by the BABA treatment may be a promising strategy for activating cellular defence responses without apparent losses in secondary metabolite accumulation.

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