

Efficacy of acibenzolar-S-methyl (Bion®) treatment of Australian commercial passionfruit, *Passiflora edulis* f. sp. *flavicarpa*, on resistance to *Passionfruit woodiness virus* (PWV) and activities of chitinase & β -1,3-glucanase

L. E. Parkinson · K. S. Crew · J. E. Thomas · E. K. Dann

Received: 11 December 2014 / Accepted: 5 March 2015 / Published online: 23 April 2015
© Australasian Plant Pathology Society Inc. 2015

Abstract This greenhouse study investigated the efficacy of acibenzolar-S-methyl (Bion®) treatment of lower leaves of passionfruit, (*Passiflora edulis* f. sp. *flavicarpa*), on *Passionfruit woodiness disease* and activities of two pathogenesis-related proteins, chitinase and β -1,3-glucanase after inoculation with passionfruit woodiness virus (PWV). All Bion® concentrations reduced disease symptoms, but the concentration of 0.025 g active ingredient (a.i.)/l was the most effective, reducing disease severity in systemic leaves by 23, 29 and 30 % compared with water-treated controls at 30, 40 and 50 days post inoculation (dpi) with PWV, respectively. Correspondingly, relative virus concentration as determined by DAS-ELISA in the upper, untreated leaves (new growth) above the site of inoculation at 50 dpi was reduced by 17 and 22 % in plants treated with 0.025 and 0.05 g a.i./l, respectively. Bion® treatment and subsequent inoculation with PWV increased chitinase and β -1,3-glucanase activities in the new leaves above the site of inoculation at 30 dpi with PWV. It was concluded that optimal protective Bion® treatment concentrations were 0.025 and 0.05 g a.i./l.

Keywords Systemic acquired resistance · SAR · PR proteins · ASM · Granadilla · Virus resistance

Introduction

The *Passiflora* genus has some 400 species, all but about 5 % of which originated in North and South America (Anderson et al. 2009). Two species are grown commercially in Australia, *P. edulis* and *P. quadrangularis* (Anderson et al. 2009). There are two recognized forms within *P. edulis*, viz. *P. edulis* f. *edulis* (purple passionfruit) and *P. edulis* f. *flavicarpa* (tropical golden passionfruit) which are selectively hybridized and cultivated in Australia for commercial production (Willingham et al. 2002; Anderson et al. 2009). Losses due to fungal diseases, for example fruit scab, Alternata spot, anthracnose, base rot and Septoria spot (caused by *Cladosporium oxysporum*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium solani* and *Septoria passifloricola*, respectively) (Willingham et al. 2002; Anderson et al. 2009) represent significant economic risks for passionfruit plantations. However, passionfruit woodiness disease caused by a number of distinct potyviruses is considered the most important and damaging disease of passionfruit crops (Cerqueira-Silva et al. 2014; Fischer and Rezende 2008; Parry et al. 2004).

In Australia, commercial passionfruit are infected with the potyviruses *Passionfruit woodiness virus* (PWV), *Passiflora virus Y* (PaVY), *Clover yellow vein virus* (CIYVV) (Parry et al. 2010) and the carlavirus *Passiflora latent virus* (PLV) (Anderson et al. 2009; Parry et al. 2010). Symptoms of passionfruit woodiness disease include severely distorted, puckered, crinkled leaves, with a mosaic, blisters or yellow

L. E. Parkinson
School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, QLD 4072, Australia

K. S. Crew
Department of Agriculture and Fisheries, Ecosciences Precinct, 41 Boggo Rd, Dutton Park, QLD 4102, Australia

J. E. Thomas · E. K. Dann (✉)
Queensland Alliance for Agriculture and Food Innovation, University of Queensland, Ecosciences Precinct, 41 Boggo Rd, Dutton Park, QLD 4102, Australia
e-mail: e.dann@uq.edu.au

lesions on the leaf surface, reduced plant development, and small, deformed, “woody” fruit, with abnormally thick, hard rind and reduced pulp (Anderson et al. 2009; Cerqueira-Silva et al. 2014; Fischer and Rezende 2008; Maciel et al. 2009). The potyviral causal agents are transmitted in a non-persistent manner by aphid vectors feeding (Fischer and Rezende 2008; Maciel et al. 2009). The viruses are also transmitted by mechanical inoculation, and on grafting and pruning tools used in propagation practices (Fischer and Rezende 2008; Anderson et al. 2009). Seed transmission of the above potyviruses in passionfruit has not been reported (Fischer and Rezende 2008; Anderson et al. 2009; Parry et al. 2010).

Current disease management and crop protection methods for passionfruit in Australia include growing commercial *P. edulis* f. sp. *flavicarpa* varieties from seed and use of *Fusarium* wilt-resistant seedling rootstocks on which to graft hybrid varieties (Willingham et al. 2002; Anderson et al. 2009). Passionfruit woodiness disease-tolerant plants were generated in Australia by pre-immunizing or cross-protecting plants with a mild strain of PWV (Simmonds 1959) to compete with a more virulent strain of the same viral species in the plant. However this method now appears ineffective (JN Parry and JE Thomas, unpublished), possibly due to the presence of new strains of PWV or additional potyvirus species have now emerged in Australia such as *Passiflora* virus Y (Parry et al. 2004; Coutts et al. 2011; Webster et al. 2007). Insecticide treatment is not effective as aphids rarely colonize passionfruit in Australia and it also encourages repeated aphid probing, thus increasing virus spread (Hooks et al. 2007). Virus control is therefore limited to good sanitation and removal of infected plants (Willingham et al. 2002; Anderson et al. 2009; Maciel et al. 2009) or complete replacement of significantly diseased orchards (Trevisan et al. 2006). Cerqueira-Silva et al. (2014) reported the current status on classical approaches to improve woodiness disease resistance in Brazil, which incorporate wild *Passiflora* species as potential sources of resistance. Although there is reported potential for producing resistant hybrids, it was nevertheless concluded that the control of the disease in passionfruit cultivation is still a major challenge (Cerqueira-Silva et al. 2014). Stimulating host response to viral attack by induced resistance could potentially improve passionfruit resistance to woodiness disease and could enhance woodiness disease management in cultivation.

Systemic acquired resistance (SAR) is the plant defence response activated after initial pathogen attack (Vallad and Goodman 2004) resulting in reduced expression of disease after subsequent “challenge” inoculation with the same or another pathogen (Hammerschmidt 2007). Pathogenesis-related (PR) proteins are among the defences activated locally and at distal parts of the plant

(Van Loon and Van Strien 1999). Synthetic non-antimicrobial chemicals such as benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (i.e. acibenzolar-S-methyl or ASM) have been developed and found to also activate induced resistance and PR proteins in various dicots such as cotton (Whan et al. 2009) and pea (Dann and Deverall 2000). ASM is currently sold and used as a plant defence activator in disease management programs in many countries, including Australia, where it is commercially known as Bion[®], as a seed dressing for cotton to protect emerging seedlings from soil-borne pathogens *Fusarium oxysporum* f. sp. *vasinfectum* and *Thielaviopsis basicola*.

Studies on passionfruit in Australia and Brazil found that Bion[®] was effective in reducing disease symptoms caused by the fungal pathogen, *Cladosporium oxysporum*, which causes passionfruit scab (Willingham et al. 2002), and the bacterial pathogen, *Xanthomonas axonopodis* pv. *passiflorae* (Boro et al. 2011). As a foliar spray alone, Bion[®] reduced *Xanthomonas* sp. symptoms by up to 67 % and as a combination of seed treatment and foliar spray, symptom reduction increased to 90 % (Boro et al. 2011). Boro et al. (2011) also found that the concentration of Bion[®] can affect its efficacy, with lower concentrations (0.0125 g a.i./l) performing significantly better. Willingham et al. (2002) found that combining fungicide treatment with Bion[®] reduced passionfruit scab in the field significantly more than fungicide treatment alone.

PR proteins are useful molecular markers of SAR as their accumulation is directly correlated to the level of SAR in uninfected tissue (Smith-Becker et al. 2003; Ryals et al. 1996). β -1,3-glucanase and chitinase are glycosyl hydrolases which respectively catalyse cleavage of the (1,3)- β -D-glucosidic linkages in (1,3)- β -glucans (Gupta et al. 2013) and degradation of the unbranched linear chain of β -1,4-linked N-acetyl D-glucosamine residues which make up chitin (Das et al. 2012). Thus, induction of the PR proteins β -1,3-glucanase and chitinase results in the breakdown of glucans and chitin in the fungal cell wall (Gupta et al. 2013; Das et al. 2012). β -1,3-glucanase and chitinase activity is conserved in a broad spectrum of host plant species and these proteins have been used as effective markers in various research regarding host response to pathogen attack and the efficacy of plant inducing chemicals in activating SAR (Whan et al. 2009; Smith-Becker et al. 2003; Dann and Deverall 2000; Colson-Hanks and Deverall 2000). The efficacy of Bion[®] in reducing passionfruit woodiness disease caused by PWV and enhancing activities of PR proteins in passionfruit has not been investigated.

The specific aims of this study were to determine whether i) Bion[®] would enhance resistance to PWV in *P. edulis* f. sp. *flavicarpa* and ii) whether there is an associated increase in activity of the PR proteins, β -1,3-glucanase and chitinase.

Materials and methods

Preparation of plants, application of Bion[®] and inoculation with PWV

P. edulis f. sp. *flavicarpa* (Department of Agriculture and Fisheries (DAF) Queensland, “DPI rootstock”) were raised from seed under shade house conditions (uncontrolled temperatures and 12.0–13.5 h photoperiod) for approximately three months until at least 40 cm tall with at least eight developed true leaves. Selected plants were transferred to greenhouse conditions (27 °C day/16 °C night and 12.5–13.5 h photoperiod).

Bion[®] (50 % w/w a.i. acibenzolar-S-methyl, ASM, in wettable granule formulation) was kindly provided for testing by Ken McKee (Syngenta Australia Pty. Ltd, Brisbane, Australia). Four concentrations of Bion[®] (0.0125, 0.025, 0.5 and 0.1 g a.i./l) were prepared in deionised water immediately prior to treatment.

French bean (*Phaseolus vulgaris* cv. Bountiful) was used as a maintenance host for PWV (DAF Queensland, Plant Virology Isolate #386, GenBank accession AY461662). This isolate was originally obtained from symptomatic passionfruit at Bundaberg, Queensland (Parry et al 2004), stored as freeze-dried leaf tissue and periodically passaged through passionfruit and French bean. Systemic symptomatic leaf tissue was ground with a mortar and pestle with 0.1 M potassium phosphate buffer (pH 7.0), with a small quantity of celite. The upper three leaves of passionfruit, *P. edulis* f. sp. *flavicarpa*, were mechanically inoculated by wiping the ground inoculum onto the adaxial side of each leaf. The mechanically inoculated leaves were misted with water to remove excess celite, then maintained in the greenhouse (27 °C day/16 °C night, natural day length).

Experiments were undertaken to determine the effect of Bion[®] treatment on PWV disease symptom severity, relative PWV virus titre and PR protein activity. Treatments of 0 g a.i./l (water only), 0.0125, 0.025, 0.05 and 0.1 g a.i./l Bion[®] were each applied to the adaxial and abaxial sides of the leaves, of 20 plants with a hand-held atomiser, except for the upper three leaves which were left for later inoculation. Five days after treatment, the upper three (untreated) leaves were mechanically inoculated with PWV. The inoculated leaves were sampled at 15 days post inoculation (dpi) for PR protein activity. The upper, non-treated leaves (new growth above the inoculation site) were assessed for virus symptom severity at 30, 40 and 50 dpi, and were sampled at 30 dpi for PR protein activity and 50 dpi for virus titre determination. Leaf samples, 0.05 g fresh weight (FW), from PWV-inoculated plants were obtained using 4 mm diameter leaf punches with a cork borer. The leaf samples from two plants per treatment group were pooled to provide 10 replicate samples per treatment. All leaf samples were stored at -80 °C. These samples were included in a

double antibody sandwich (DAS) ELISA for assessment of PWV in tissue, and defence enzyme assays. Three replicate experiments were conducted, for disease assessment of symptoms and for PR protein enzyme assays. Samples from two of the experiments were used for quantifying PWV titre in leaves.

The data from each replicate experiment was pooled, after confirming that there was no significant treatment by experiment interaction, and the appropriate statistical analyses were performed on the pooled means using the statistical analysis software, GenStat (14th edition, VSN International Ltd) as described in the listed experiments below.

Assessment of PWV symptoms in inoculated *P. edulis* f. sp. *flavicarpa*

The symptoms used to identify PWV were yellow lesions, blisters, mottling, leaf curling or crinkling and mosaic, and assessment was performed on upper, non-treated leaf tissue (new growth above the inoculation site) at three time points (30, 40 and 50 days) post inoculation. A disease severity rating scale was devised (Table 1 and Fig. 1).

Data were subjected to repeated measurements analysis of variance (ANOVA), pairwise tests between the means were carried out and Fischer’s Least Significant Difference (LSD) ranked the means. The statistical analyses were carried out in GenStat (14th edition, VSN International Ltd).

Double Antibody Sandwich ELISA on PWV-infected *P. edulis* f. sp. *flavicarpa*

DAS-ELISA was performed to measure the virus titre in systemic leaves of Bion[®]-treated, PWV-infected plants. The antigens from the test samples and controls (0.05 g FW), were extracted with 0.5 ml extraction buffer (containing 0.05 M tri sodium citrate pH 8.0, 0.5 mM EDTA, 0.05 % v/v Tween20,

Table 1 Disease score scale describing the symptom severity of PWV-inoculated plants

Score	Symptoms
1	Leaves with less than 5 yellow lesions.
2	First signs of disease, obvious mottling or up to 25 % of the leaf covered in yellow lesions.
3	Minor leaf curling or clear crinkling of the leaf; first signs of green and dark green mosaic discoloration of the leaf; first signs of blistering on the leaf surface or up to 50 % covered in yellow lesions.
4	Clear leaf curling or crinkling; lesions and mosaic cover 50–75 % of the leaf or clear presence of blistering or bubbling on the leaf surface
5	Leaves are severely deformed and/or curled and crinkled; obvious green and dark green mosaic discoloration covering the majority of leaf; blistering of the leaf surface or yellow lesions greater than 75 % coverage.

Fig. 1 Leaf symptom severity of PWV-inoculated plants with corresponding disease score



1 % w/v skim milk powder and 0.5 % v/v monothioglycerol). Tissue lysis (*TissueLyser*, QIAGEN, Lvl 1/ 90–94 Tram Rd, Doncaster, Australia) was carried out at 30 shakes per second for two 1-min repetitions. The lysate was centrifuged for 5 min at 14,000 rpm and each supernatant was collected for immediate use as the antigen. Subsequent DAS-ELISA reactions were carried out in microtitre plates (Nunc MicroWell, Sigma-Aldrich Pty. Ltd, Sydney, Australia) essentially as described by Clark and Adams (1977). Reaction volumes were 50 μ l per well and reactions were carried out at room temperature (23 °C). Plates were coated with PWV IgG (2 μ g/ml) and the virus was detected with PWV-IgG-alkaline phosphatase conjugate (DAF Queensland) diluted to 1:1000.

A standard curve for virus concentration was created by preparing a two-fold dilution series (1/1 to 1/128) from a high-titred PWV-infected passionfruit sample, diluted in healthy passionfruit extract. A one-way ANOVA statistical analysis was performed on both the measured absorbance and the relative values of virus concentration (interpolated from the standard comparison curve). LSD pairwise tests were used to rank the means.

Assays for the measurement of β -1,3-glucanase and chitinase activity

The PR protein enzyme assays were performed with mechanically inoculated leaves sampled at 15 dpi and systemic leaves sampled at 30 dpi, of Bion[®]-treated inoculated plants at Bion[®] treatment concentrations of 0.05, 0.1 g a.i./l and water-treated controls. Each Bion[®] treatment group contained 10 plants, where the leaf extracts of each plant were pooled into three test replicates comprising of three or four plants for each Bion[®] concentration.

Enzyme assays were conducted according to Dann and Deverall (2000) with the following modification to suit passionfruit extracts. At the completion of the β -1,3-glucanase assay, the tubes were centrifuged for 5 min at 10,000 g; 0.3 ml of the supernatant was added to the wells of a microtitre plate and the optical density was read at 595 nm. For the chitinase assay, the incubation times for equilibration and the reaction initiation times were 10 min for each step, at

37 °C; the tubes were centrifuged at 10,000 g for 5 min at the completion of the assay, and the absorbance was read at 540 nm.

Total protein was measured for each sample by the Bradford protein assay method (Bio-Rad 2013), and enzyme activity was presented as change in absorbance per mg protein per minute (Δ Abs_{nm}/ mg protein/ min).

A repeated measures ANOVA was performed on the enzyme activities and LSD pairwise tests ranked the means.

Results

PWV symptom severity

The effect of Bion[®] treatment on the severity of virus symptoms was assessed at 30, 40 and 50 days after inoculation with PWV (Fig. 2). The data were appropriately pooled across the three experiments for analysis (Fig. 2) because statistical analyses demonstrated that there was not a significant treatment by experiment interaction. There were significant differences in the disease severity scores among treatments ($P < 0.001$) and over time ($P < 0.001$).

At 30 days after inoculation with PWV, plants which had been treated with Bion[®] at 0.0125 g a.i./l were not significantly different in appearance to those treated with water (Fig. 2), however, disease symptoms were significantly less severe in plants treated with the other concentrations of Bion[®] (Fig. 2). This corresponded to reductions in symptom severity (lower disease scores) compared with water controls of 23, 23.5 and 36 % for 0.025, 0.05 and 0.1 g a.i./l Bion[®] treatments, respectively.

At 40 days after PWV inoculation, all Bion[®] concentrations significantly reduced disease severity scores in passionfruit leaves compared with water-treated plants. Treatment at 0.0125, 0.05 and 0.1 g a.i./l showed statistically similar disease score reductions, however the least severe symptoms (lowest disease score) was from plants treated with 0.025 g a.i./l Bion[®], representing a reduction in severity of symptoms of 29 % compared with water controls (Fig. 2).

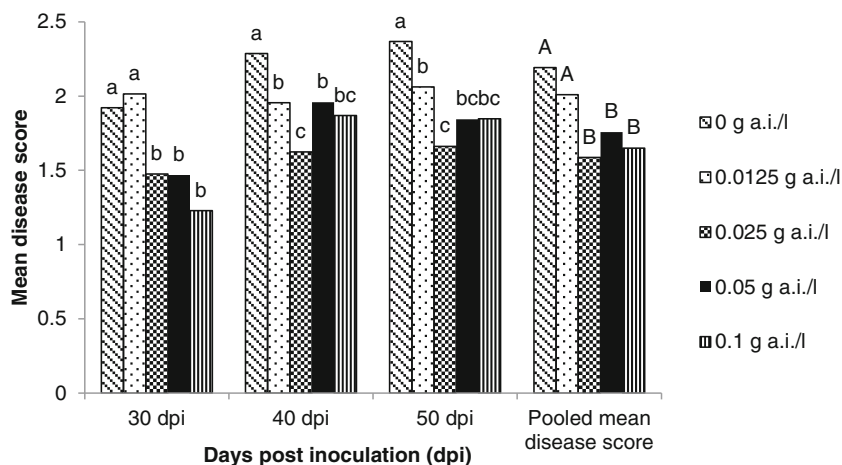


Fig. 2 The effect of Bion[®] treatment of passionfruit leaves and subsequent inoculation with PWV on severity of passionfruit woodiness disease symptoms. At each time point, means where columns are surmounted by the same lowercase letter are not significantly different at $P < 0.001$. Disease scores from all time periods for each Bion[®] concentration were pooled; means with the same uppercase letter are

not significantly different at $P < 0.001$. The lower leaves of three trials of *P. edulis* f. sp. *flavicarpa* ($n = 20$) were treated with Bion[®] concentrations of 0.0125, 0.025, 0.05 and 0.1 g a.i./l, and the control group with water. Five days later the upper three leaves were inoculated with PWV and the systemic leaves were scored for disease severity based on a scale of 1 to 5, after 30, 40 and 50 days post inoculation (dpi)

At 50 days after PWV inoculation, all Bion[®] concentrations significantly reduced disease severity scores in passionfruit leaves. Treatment at 0.025, 0.05 and 0.1 g a.i./l showed statistically similar disease scores reduced by 30, 22 and 22 % compared to water controls.

Pooled mean disease scores of each Bion[®] concentration across time revealed a significant difference between the concentrations ($P < 0.001$) (Fig. 2). Plants treated with 0.025 g a.i./l Bion[®] had the least severe PWV symptoms, but the values were not significantly lower than for plants treated with 0.05 or 0.1 g a.i./l Bion[®].

Virus titre in upper leaves of Bion[®]-treated and PWV inoculated plants

The absorbance and relative virus titre in systemic leaves of Bion[®]-treated infected plants at 50 dpi was assessed by DAS-ELISA (Fig. 3). Statistical analyses indicated that there was not a significant treatment by experiment interaction. Therefore the data were appropriately pooled across the two experiments for analysis (Fig. 3).

Virus titre by absorbance in all Bion[®]-treated plants was significantly reduced ($P < 0.001$) compared to the water-treated controls, however there was no significant difference in virus titre among the different concentrations of Bion[®]. Bion[®] treatment reduced absorbance by 9–13 % compared with water controls. Virus titre by relative concentration was also significantly reduced ($P < 0.001$) in the Bion[®]-treated leaves compared to the water treated controls, and Bion[®] at 0.05 g a.i./l resulted in significantly lower relative virus concentration in leaves compared with all other treatments except 0.025 g a.i./l Bion[®] (Fig. 3). Bion[®] treatment reduced relative virus titre by approximately 14, 17, 22 and 15 % at Bion[®]

concentrations of 0.0125, 0.025, 0.05 and 0.1 g a.i./l respectively, compared to the water treated controls.

Enzyme activity after Bion[®] treatment and PWV inoculation

Chitinase and β -1,3-glucanase assays measured the change in enzyme activity among three treatment groups (Bion[®] at 0.05, 0.1 g a.i./l and water-treated controls) in

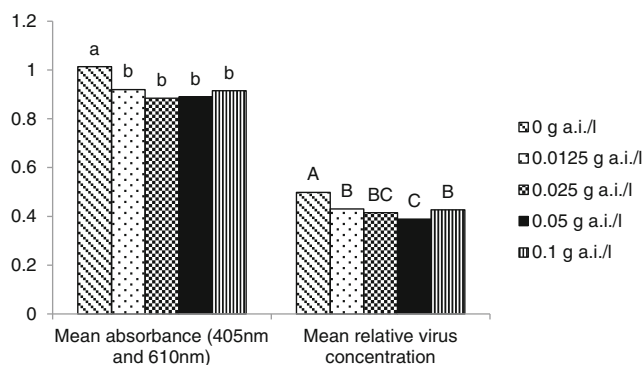


Fig. 3 The effect of Bion[®] treatment of passionfruit leaves and subsequent inoculation with PWV on DAS-ELISA absorbance and relative virus concentration. Mean absorbance where columns are surmounted by the same lowercase letter are not significantly different at $P < 0.001$; mean relative virus concentration with the same uppercase letter are not significantly different at $P < 0.001$. Data from two separate experiments were combined, and the relative PWV virus concentration was calculated, in new leaf tissue above the site of inoculation of Bion[®]-treated *P. edulis* f. sp. *flavicarpa* harvested at 50 dpi ($n = 10$). Relative virus concentration as a proportion relative to the Bion 0 g a.i./l treatment being 1.0 was calculated from the linear equation of a standard curve generated from the absorbance of a two-fold dilution series (1/1 to 1/128) from high-titred PWV-infected passionfruit leaf sample diluted in healthy passionfruit extract. The absorbance values of each Bion[®] treated sample were extrapolated from the standard curve

PWV-inoculated leaves at 15 dpi and in new leaves above the inoculation site at 30 dpi. The data were pooled across the three trials (Fig. 4) since statistical analyses demonstrated that there was not a significant treatment by experiment interaction.

Chitinase activity was similar across the tested Bion[®] concentrations in inoculated leaves at 15 dpi (Fig. 4). Chitinase activity was significantly higher at 30 dpi in new leaves (above the site of inoculation) of plants treated with 0.05 g a.i./l Bion[®] ($P < 0.05$) compared with those from plants treated with 0.1 g a.i./l Bion[®] or water controls (Fig. 4).

At 15 dpi there was significantly higher β -1,3-glucanase activity in inoculated leaves of plants treated with 0.1 g a.i./l Bion[®] compared to the water controls. At 30 dpi, Bion[®] at 0.05 g a.i./l resulted in significantly higher β -1,3-glucanase activity in systemic leaves than both the control and 0.1 g a.i./l treatments, representing a 1.7 and 1.4 fold increase in activity respectively (Fig. 4).

Discussion

Passionfruit woodiness disease symptoms and relative concentration of PWV particles in new leaves above the inoculation site were reduced in Bion[®]-treated *P. edulis* f. sp. *flavicarpa* plants. This is the first conclusive report demonstrating Bion[®]-mediated activation of resistance to PWV in passionfruit. This is consistent with current literature of induced resistance by Bion[®] in passionfruit to fungal and bacterial diseases (Willingham et al. 2002; Boro et al. 2011) and in several other plant-pathogen

systems (for example, Heil 2007; Walters et al. 2007). Other examples of Bion[®] (ASM) inducing resistance to viruses include significantly reducing the incidences of ELISA detection and virus titre of *Glycine mosaic comovirus* (GMV) in pea (Dann and Deverall 2001); activation of PR proteins following inoculation with *Tobacco mosaic virus* (TMV) in tobacco plants pre-treated with Bion[®], (Friedrich et al. 1996); and reduced number and size of local lesions in *Nicotiana glutinosa* pre-treated with ASM and challenge inoculated with TMV and *Tomato mosaic tobamovirus* (ToMV) by 67 and 79 % respectively (Madhusudhan et al. 2008). Madhusudhan et al. (2008) also demonstrated that pre-treatment with ASM reduced ToMV movement to distal leaves.

Systemic acquired resistance in Bion[®]-treated monocots is reported to be profoundly long lasting, however SAR in dicots is reported to be short-lived (Loebenstein 2009). In a study by Mejía et al. (2009) Bion[®] was applied to tomatoes prior to inoculation with *Tomato mosaic virus* and then twice more at 20 day intervals. They found that multiple applications of Bion[®] after pathogen inoculation reduced viral symptoms by 50 %. The significant reduction in disease symptoms in Mejía et al. (2009) study is consistent with the findings of this study, and it is likely that multiple Bion[®] applications may increase the longevity and reduction of disease symptoms in the passionfruit-PWV interaction.

The concentration of Bion[®] applied to lower leaves affected the severity of disease, and relative PWV concentration in new leaves above the site of inoculation. The greatest reduction in symptom severity occurred in plants treated with 0.025 g a.i./l Bion[®]. These plants also had the lowest virus titre. The concentration correlations in this study were

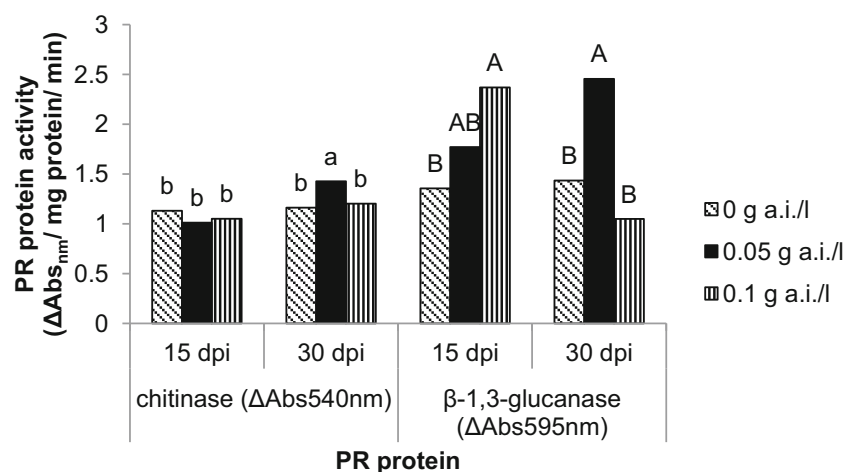


Fig. 4 The effect of Bion[®] treatment of passionfruit leaves and subsequent inoculation with PWV on chitinase and β -1,3-glucanase activity in leaves at 15 dpi and 30 dpi. For each PR protein, means where columns are surmounted by the same letter are not significantly different at $P < 0.001$. Mean chitinase and β -1,3-glucanase

activities in leaf tissue of three trials of *P. edulis* f. sp. *flavicarpa* treated with Bion[®] concentrations 0.05 and 0.1 g a.i./l, and the control group with water, five days prior to PWV inoculation ($n=3$). These were harvested at 15 dpi (inoculated leaves) and 30 dpi (new leaves above the site of inoculation)

consistent with other studies. Boro et al. (2011) demonstrated reduced *Xanthomonas axonopodis* pv. *passiflorae* symptoms in Bion[®]-treated passionfruit and found that the lowest concentration tested, (0.0125 g a.i./l) reduced the bacterial symptoms significantly more than the higher concentration tested (0.025 g a.i./l). This demonstrates an importance in optimizing Bion[®] treatment for each host-pathogen interaction. Lower concentrations may be more effective due to lower phytotoxicity and increased fitness to pathogen infection (Heil 2007). Studies on bacterial and fungal diseases of tobacco by Cole (1999) found that phytotoxicity occurred at Bion[®] concentrations of 0.1 g a.i./l. Balajoo et al. (2012) inoculated Bion[®]-treated *Cydonia oblonga* with *Erwinia amylovora* and found phytotoxicity at concentrations of 0.1 and 0.2 g a.i./l. Heil (2007) listed multiple studies providing evidence for plant developmental and fitness trade-offs when using high Bion[®] concentrations; but none of those effects at low concentrations. These studies demonstrated that lower Bion[®] concentrations were effective in reducing disease symptoms, which is consistent with the findings of this study. Potentially, high Bion[®] concentrations (0.1 g a.i./l) reduce fitness in passionfruit.

Activities of defence related enzymes chitinase and β -1,3-glucanase were increased in Bion[®]-treated and PWV-inoculated plants. β -1,3-glucanase but not chitinase activity was elevated 15 days after PWV inoculation. At 30 dpi plants treated with Bion[®] at 0.05 g a.i./l had significantly higher activities of both enzymes, compared with untreated controls or Bion[®] at 0.1 g a.i./l. Studies of the effects of acibenzolar-S-methyl (ASM) in tobacco against *Tomato spotted wilt virus* (Mandal et al. 2008) and in cantaloupe against *Cucumber mosaic virus* (Smith-Becker et al. 2003), found an increase for PR protein gene, PR-3, other classes of PR proteins such as PR-1 and PR-5 (Mandal et al. 2008) and for chitinase (Smith-Becker et al. 2003). Both studies found an effective reduction in systemic spread of the viruses. Mandal et al. (2008) also confirmed phytotoxicity (foliar spotting and stunting of plants) at 4.0 g a.i./7000 plants and effective protection with minimal phytotoxicity at 1.0–2.0 g a.i./7000 plants. This is consistent with the current study, where lower Bion[®] concentrations of 0.025 and 0.05 g a.i./l were most effective at reducing disease severity and PWV virus levels and increasing defence enzyme activity in systemic leaves.

The activities of PR proteins β -1,3-glucanase and chitinase were measured because they are useful markers of SAR induction (Loebenstein 2009; Smith-Becker et al. 2003); elevated PR protein activity is correlated with stronger SAR induction (Smith-Becker et al. 2003). The current study indicated an association between reduced virus titre and increased PR protein activity, however, there is no prior evidence suggesting that β -1,3-glucanase or chitinase have direct antiviral activity in virus-induced SAR (Loebenstein 2009). Little is known of how ASM induces SAR under virus attack, although previous

studies demonstrated that exogenous application of salicylic acid (SA), a plant defence hormone analogous to ASM (Smith-Becker et al. 2003), can trigger two anti-viral defence mechanisms; inhibition of viral replication at the site of inoculation or delaying movement of the virus out of the inoculation site to distal parts of the plant (Naylor et al. 1998; Murphy and Carr 2002). Viral replication at the inoculation site was inhibited in tobacco plants treated with SA and inoculated with Tobacco mosaic virus (TMV) (Chivasa et al. 1997), whereas after SA treatment and inoculation with Cucumber mosaic virus (CMV) (Naylor et al. 1998), movement of the virus out of the inoculation site was inhibited resulting in delayed CMV symptom development. These different host responses to viral attack were mediated by a salicylhydroxamic acid (SHAM)-sensitive pathway (Naylor et al. 1998), and may involve “Inhibitor of Virus Replication” (IVR) or IVR-like proteins (Loebenstein 2009), implicated in non-specific restriction of virus infection, replication and translocation. Further detailed studies with the passionfruit-PWV system is required to determine the mechanisms underlying Bion[®]-induced resistance.

Bion[®] does not have direct anti-microbial activity (Whan et al. 2009; Willingham et al. 2002) but it is shown to reduce PWV-susceptibility in this study. Prior research demonstrating that Bion[®] improves resistance in passionfruit to some fungal and bacterial diseases (Willingham et al. 2002; Boro et al. 2011) combined with these new findings that Bion[®] improves resistance in commercial passionfruit to *Passionfruit woodiness virus* in Australia, shows that the incorporation of Bion[®] in passionfruit disease management programs could provide a useful option for the industry.

Acknowledgments We wish to thank Kerri Chandra (Queensland DAF) for her valuable input in the statistical analysis of the data. Ken McKee, Syngenta Crop Protection Pty. Ltd., is acknowledged for supplying Bion[®].

References

- Anderson J, Pegg K, Thomas J (2009) Chapter 15: Passionfruit. In: Pearsley D, House S, Cooke T (eds) Diseases of fruit crops in Australia. CSIRO Publishing, Melbourne, pp 197–206
- Balajoo OM, Kesahavarzi M, Zahabi A, Danesh YR, Haghjuyan R (2012) Protective effect of acibenzolar-S-methyl on fireblight severity in quince and characterization of the *Erwinia amylovora* strains involved. *J Plant Pathol* 94:211–214
- Bio-Rad (2013) Quick Start[™] Bradford Protein Assay. Available: http://www.bio-rad.com/LifeScience/pdf/Bulletin_9004.pdf [Accessed 30 January 2013]
- Boro MC, Beriam LOS, Guzzo SD (2011) Induced resistance against *Xanthomonas axonopodis* pv. *passiflorae* in passion fruit plants. *Trop Plant Pathol* 36:74–80
- Cerqueira-Silva CBM, Conceicao LDHCS, Souza AP, Correa RX (2014) A history of passion fruit woodiness disease with emphasis on the

- current situation in Brazil and prospects for Brazilian passion fruit cultivation. *Eur J Plant Pathol* 139:255–264
- Chivasa S, Murphy A, Naylor M, Carr J (1997) Salicylic acid interferes with tobacco mosaic virus replication via a novel salicylhydroxamic acid-sensitive mechanism. *Plant Cell* 9:547–557
- Clark MF, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J Gen Virol* 34:475–483
- Cole DL (1999) The efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance, against bacterial and fungal diseases of tobacco. *Crop Prot* 18:267–273
- Colson-Hanks ES, Deverall BJ (2000) Effect of 2,6-dichloroisonicotinic acid, its formulation materials and benzothiadiazole on systemic resistance to *Alternaria* leaf spot in cotton. *Plant Pathol* 49:171–178
- Coutts B, Kehoe M, Webster C, Wylie S, Jones R (2011) Indigenous and introduced potyviruses of legumes and *Passiflora* spp. from Australia: biological properties and comparison of coat protein nucleotide sequences. *Arch Virol* 156:1757–1774
- Dann EK, Deverall BJ (2000) Activation of systemic disease resistance in pea by an avirulent bacterium or a benzothiadiazole, but not by a fungal leaf spot pathogen. *Plant Pathol* 49:324–332
- Dann EK, Deverall BJ (2001) Studies on systemic resistance to viruses in pea and faba bean. In: Proceedings of the thirteenth Biennial Plant Pathology Conference, Cairns, Australia, p 260
- Das SN, Neeraja Ch, Sarma PVS RN, Madhu Prakash J, Purushotham P, Kaur M, Dutta S, Podile AR (2012) Microbial chitinases for chitin waste management. In: Microorganisms in Environmental Management. Springer, Netherlands, p 135–150
- Fischer ICH, Rezende JAM (2008) Diseases of passion flower (*Passiflora* spp.). *Pest Technol* 2:1–19
- Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut Rella M, Meier B, Dincher S, Staub T, Uknes S, Métraux JP, Kessmann H, Ryals J (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J* 10:61–70
- Gupta P, Ravib I, Sharma V (2013) Induction of β -1,3-glucanase and chitinase activity in the defense response of *Eruca sativa* plants against the fungal pathogen *Alternaria brassicicola*. *J Plant Interact* 8:155–161
- Hammerschmidt R (2007) Chapter 1: 1.2.4. Cross protection. In: Walters D, Newton A, Lyon G (eds) Induced resistance for plant defence—a sustainable approach to crop protection. Blackwell Publishing Ltd, Oxford, pp 3–4
- Heil M (2007) Chapter 9: trade-offs associated with induced resistance. In: Walters D, Newton A, Lyon G (eds) Induced resistance for plant defence—a sustainable approach to crop protection. Blackwell Publishing Ltd, Oxford, pp 162–163, 159–160
- Hooks CR, Fereres A, Wang KH (2007) Using protector plants to guard crops from aphid-borne non-persistent viruses. *Soil Crop Man.* Available: <http://www.ctahr.hawaii.edu/oc/freepubs/pdf/SCM-18.pdf> [Accessed 13 March 2013]
- Loebenstein G (2009) Chapter 3—local lesions and induced resistance. *Adv Virus Res* 75:73–117
- Maciel SDC, Nakano DH, Rezende JAM, Vieira MLC (2009) Screening of *Passiflora* species for reaction to cowpea aphid-borne mosaic virus reveals an immune wild species. *Sci Agric* 66:414–418
- Madhusudhan KN, Deepak SA, Prakash HS, Agrawal GK, Jwa NS, Rakwal R (2008) Acibenzolar-S-methyl (ASM)-induced resistance against Tobamovirus involves induction of RNA dependent RNA polymerase (RdRp) and alternative oxidase (AOX) genes. *J Crop Sci Biotechnol* 11:127–134
- Mandal B, Mandal S, Csinos AS, Martinez N, Culbreath AK, Pappu HR (2008) Biological and molecular analyses of the acibenzolar-S-methyl-induced systemic acquired resistance in flue-cured tobacco against *tomato spotted wilt virus*. *Phytopathology* 98:196–204
- Mejía DA, Rodas EI, Patiño LF, González EP (2009) Effect of acibenzolar-S-methyl on virus infection progress caused by potyvirus in tree tomato. *Agron Colomb* 27:87–93
- Murphy AM, Carr JP (2002) Salicylic acid has cell-specific effects on *Tobacco mosaic virus* replication and cell-to-cell movement. *Plant Physiol* 128:552–563
- Naylor M, Murphy AM, Berry JO, Carr JP (1998) Salicylic acid can induce resistance to plant virus movement. *Mol Plant Microbe Interact* 11:860–868
- Parry JN, Davis RI, Thomas JE (2004) *Passiflora virus Y*, a novel virus infecting *Passiflora* spp. in Australia and the Indonesian province of Papua. *Australas Plant Pathol* 33:423–427
- Parry J, Parmenter K, Ballard E, Thomas J (2010) Passionfruit viruses in Eastern Australia. In: 9th Australasian Plant Virology Workshop, Melbourne, Australia, p 26
- Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner H-Y, Hunt MD (1996) Systemic acquired resistance. *Plant Cell* 8:1809–1819
- Simmonds JH (1959) Mild strain protection as a means of reducing losses from the Queensland woodiness virus in the passion vine. *Queensl J Agric Sci* 16:371–380
- Smith-Becker J, Keen NT, Becker JO (2003) Acibenzolar-S-methyl induces resistance to *Colletotrichum lagenarium* and cucumber mosaic virus in cantaloupe. *Crop Prot* 22:769–774
- Trevisan F, Mendes BMJ, Maciel SC, Vieira MLC, Rezende JAM (2006) Resistance to *Passion fruit woodiness virus* in transgenic passion-flower expressing the virus coat protein. *Plant Dis* 90:1026–1030
- Vallad GE, Goodman RM (2004) Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Sci* 44:1920–1934
- Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol* 55:85–97
- Walters D, Newton A, Lyon G (2007) Induced resistance for plant defence—a sustainable approach to plant protection. Blackwell Publishing Ltd, Oxford
- Webster CG, Coutts BA, Jones RAC, Jones MGK, Wylie SJ (2007) Virus impact at the interface of an ancient ecosystem and a recent agroecosystem: studies on three legume-infecting potyviruses in the southwest Australian floristic region. *Plant Pathol* 56:729–742
- Whan JA, Dann EK, Smith LJ, Aitken EAB (2009) Acibenzolar-S-methyl-induced alteration of defence gene expression and enzyme activity in cotton infected with *Fusarium oxysporum* f. sp. *vasinfectum*. *Physiol Mol Plant Pathol* 73:175–182
- Willingham SL, Pegg KG, Langdon PWB, Cooke AW, Peasley D, McLennan R (2002) Combinations of strobilurin fungicides and acibenzolar (Bion) to reduce scab on passionfruit caused by *Cladosporium oxysporum*. *Australas Plant Pathol* 31:333–336