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Plant growth regulator-mediated anti-herbivore responses of cabbage (*Brassica oleracea*) against cabbage looper *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae)

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ABSTRACT

Plant elicitors can be biological or chemical-derived stimulators of jasmonic acid (JA) or salicylic acid (SA) pathways shown to prime the defenses in many crops. Examples of chemical elicitors of the JA and SA pathways include methyl-jasmonate and 1,2,3-benzothiadiazole-7-carbothioate (BTH or the commercial plant activator Actigard 50WG, respectively). The use of specific elicitors has been observed to affect the normal interaction between JA and SA pathways causing one to be upregulated and the other to be suppressed, often, but not always, at the expense of the plant's herbivore or pathogen defenses. The objective of this study was to determine whether insects feeding on Brassica crops might be negatively affected by SA inducible defenses combined with an inhibitor of detoxification and anti-oxidant enzymes that regulate the insect response to the plant's defenses. The relative growth rate of cabbage looper *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) fed induced cabbage *Brassica oleraceae* leaves with the inhibitor, quercetin, was significantly less than those fed control cabbage with and without the inhibitor. The reduced growth was related to the reduction of glutathione S-transferases (GSTs) by the combination of quercetin and increased levels of indole glucosinolates in the cabbage treated with BTH at 2.6× the recommended application rate. These findings may offer a novel combination of elicitor and synergist that can provide protection from plant disease and herbivores in cabbage and other Brassica crops.

1. Introduction

Chemical pesticides remain the mainstay of crop protection, despite many examples of negative impacts to the environment and human health. Biological control and transgenic crops are a component of integrated pest management (IPM) along with chemical pesticides, but transgenic crops are not always acceptable alternatives [1]. A recent innovation in crop protection is the use of induced resistance by stimulating the plant immune system with synthetic elicitors that mimic a pest attack [1]. The priming of plant defenses is the process of putting these mechanisms on "standby" mode, a lower level and one less costly than full expression in response to a pathogen [2]. Elicitors can also be used to directly activate or stimulate the plant's defenses [3].

The typical plant defense response against pathogens and herbivores is through the salicylate and jasmonate pathways, respectively. The jasmonic acid (JA) pathway is one of the most important for all plant's systemic responses [4]. In Solanaceae plants, up-regulation of jasmonates due to herbivore damage can lead to inducible defense proteins such as proteinase inhibitors (PIs), polyphenol oxidase (PPO), glandular trichome density, and secondary metabolites including glycoalkaloids, acyl sugars, polyphenolic compounds and mono- and sesquiterpenes volatiles [5–12]. These changes can in turn lower nutritional value of plant tissue and affect herbivore survivorship and growth [13,14], as was the case for cabbage looper *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) larvae fed on JA over-expressing mutant 35S:Proysystemin (Prosys) tomato compared to those fed on wild-type tomato [15]. Recent findings with tomato mutants that over-express *TomLoxD*, a gene that regulates the induction of JA biosynthesis, found increased expression of wound-induced defense-related genes and enhanced resistance to arthropod herbivores and microbial pathogens [12]. Although plants like the Prosys tomato effectively reduce herbivory [15], there are fitness costs associated with maintaining continuous levels of elevated defenses proteins, while the *TomLoxD* over-expressing plants had enhanced expression of defense-related genes only observed after insect damage and pathogen infection [12].

Another approach to elevate defense proteins against herbivores only when necessary is to spray wild-type plants with a chemical elicitor, for example JA or methyl jasmonate, so that plants elevate inducible proteins and defense chemicals that protect plants from insects [3,16]. A similar induced systemic resistance (ISR) can also be induced by exposure of the roots to specific strains of plant growth-promoting rhizobacteria, unrelated to pathogens [17]. In contrast, the induced

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defense response to pathogens is termed systemic acquired resistance (SAR), and is initiated by localized necrotic lesions [18]. SAR is dependent on salicylic acid (SA) signaling, leading to systemic expression of genes encoding pathogenesis related (PR) proteins. SA acts as a "negative modulator" by repressing the JA response and is associated with the response to biotrophic pathogens and in some cases insects, typically phloem-feeding aphids and spider mites [19]. SAR plants are primed to respond to subsequent infections by the expression of an oxidative burst, cell wall alterations at the infection site and phytoalexin production [18]. SAR can be induced by microbes, including bacterial derived compounds such as lipopolysaccharides produced by *Pseudomonas syringae* and HrpN (harpin) produced by *Peronospora parasitica* [1]. SAR can also be induced by pathogen-derived molecules, and chemical inducers, like salicylic acid, 2,6-dichloroisonicotinic acid (DCINA), acibenzolar-*S*-methyl (ASM), or the synonym 1,2,3-benzothiadiazole-7-carbothioate (BTH), tiadinil (TDL), β-amino butyric acid (BABA) and others. A number of commercial products have been developed for preventative disease control by stimulating the systemic acquired resistance (SAR) through the salicylate pathway. For example, BTH, the active ingredient in Actigard 50WG Plant Activator (Syngenta) used in selected crops to protect from bacterial diseases by priming their antibacterial response prior to infection [17,20,21]. Actigard or BTH reduced both the severity of bacterial spot *Xanthomonas axonopodis* pv. *vesicatoria* and bacterial speck *Pseudomonas syringae* pv. *tomato* diseases of field tomato in five Northeastern U.S. and Canadian locations using 35 g a.i./ha rates [21]. Similarly, a survey of BTH use in monocots (wheat, maize), dicots (tobacco, tomato, pepper), legumes (bean, soybean), cotton, spinach and tree fruits documented suppression of several typical diseases, including downy and powdery mildew, blue mold, leaf spot, white mold, fireblight and rust [17].

Many of the studies that have examined SAR have focused on plants in the Solanaceae, primarily tomato, to measure the effects of induced defenses. Tomato *Solanum* spp. has been the model plant for much of the evaluation of JA and SA induced defenses, where is has been shown that PPO is typically up-regulated by JA inducers [3] and peroxidase (PPD) can be altered by SA inducers [22]. More recent research has studied systemic responses in *Arabidopsis thaliana* as a model for other plants [4], and has direct implications for herbivore pest management in related crop species in the Brassicaceae. Brassicaceae species have been studied for the response to JA and SA induction, mainly through the conversion of glucosinolates (GS) to more toxic thiocyanate, isothiocyanate and nitrile defense compounds. For example, SA applied as a soil drench of roots was found to increase the level of phenylethyl GS in the leaves of *Brassica napus* [23], but SA induction by BTH did not alter indole GS [24]. In these cases, the other Brassica defenses such as PPO and PPD induced by SAR were not measured. With respect to anti-herbivore effects, SAR activation by BABA was observed to suppress the feeding of the cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphidae) and the cabbage moth *Pieris xylostella* L. (Lepidoptera: Pieridae) on *Arabidopsis* [25]. No follow-up studies on the mechanisms behind these effects were made, but it is likely that SAR induction increases both the toxic reactive oxygen species (ROS), such as hydrogen peroxide, as well as GS. Observations of cabbage looper, *T. ni*, fed leaf tissue with both elevated ROS and GS indicate increased metabolic (anti-oxidant and detoxification) enzymes including glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX) and glutathione reductase (GR) [26–28]. Inhibition of these enzymes would likely lead to reduced performance by those insects.

With this last point in mind, the objectives of this research were to measure the direct chemical changes in *B. oleracea* (cabbage) after treatment with the elicitor BTH and to determine whether the induced plant defenses can affect a generalist insect herbivore, the cabbage looper, in combination with an inhibitor of detoxification and anti-oxidant enzymes that regulate the insect response to the plant's defenses. In the latter case, a flavonoid, quercetin, known to negatively affect *T. ni* GST and GPOX was used as the enzyme inhibitor.

2. Materials and methods

2.1. Chemicals

Actigard™ 50WG Plant Growth Regulator (50% BTH) was provided by Syngenta Canada Inc. (Guelph, ON, Canada). Quercetin was purchased from ChromaDex (Irvine CA).

2.2. Insects and plants

Cabbage looper, *T. ni*, were reared at the London Research and Development Centre (LoRDC), Agriculture and Agri-Food Canada (AAFC), London ON, Canada, for >15 years. Insects for the *T. ni* colony were originally obtained as egg masses from the Forest Pest Management Centre, Natural Resources Canada, Sault Ste. Marie ON, Canada, and has been refreshed several times over the past 10 years when required. *Trichoplusia ni* larva were fed a modified wheat germ-based artificial diet [29] and held at 25 ± 1 °C, 50 ± 5% RH and 16:8 h light:dark. Cabbage *B. oleracea* var. Golden Acre were grown in the greenhouse at LoRDC in Promix and watered and fertilized as required. Plants were grown under greenhouse daylight conditions with supplemental evening lighting (350 μEinstein/m2/s) and temperature (22 ± 1 °C). Plant leaves were selected for use in bioassays after 4–6 weeks post-germination.

2.3. Induced plant bioassays

Actigard (BTH) was dissolved in reversed osmosis (RO) water at the recommended application rate (RAR) or 1× BTH (the low BTH treatment) for cabbage, 1 oz./20 gal or 375 mg/L as instructed by Syngenta Crop Protection, Inc. The 2.6× RAR (the high BTH treatment or 2.6× BTH) or 975 mg/L for cabbage, was the concentration applied in a previous study [30] that documented negative effects of induced defenses to insects at 1.85 g a.i./3.8 L (0.97 g/L). Cabbage plants were randomly selected for BTH treatments and each set of 4 plants were sprayed with approximately 50 mL of BTH solution, or R.O water only for the control treatment. Plants treated with BTH at the two rates or no-BTH were kept separate to avoid possible interaction between induced and non-induced plants. Evidence from the chemical analyses of GS levels from plants kept in separate growth cabinets indicated these were consistent regardless of where the plants were held.

The enzyme inhibitor, quercetin, was dissolved in acetone within a concentration range of 0.001, 0.01, 0.1, 1, 10 and 100 mg/mL. Three young leaves were selected, either the top 3rd or 4th leaf from mixed plants (4 per BTH treatment), cut at the stem and sprayed on both sides with 5 mL of the inhibitor using a Potter Spray Tower (Burkard Scientific, Uxbridge UK). An individual trial consisted of BTH/no BTH treatments in combination with the inhibitor at one or more of the 3 concentrations plus an acetone control. Each treatment combination had 3 replicate leaves and each trial tested a minimum of 40 insects over all treatments. After leaves were dried for a few minutes, they were placed in a 15 cm dia petri dish with a moistened filter paper disc underneath. Pre-weighed (1–2 mg) 2nd instar *T. ni* larvae were then placed on each leaf and allowed to feed for 4 days. After 2 days each leaf was replaced with a freshly sprayed leaf of the same treatment combination. The 4 day insect relative growth rate (RGR) based on the change in fresh weight was calculated as [(final weight − initial weight) / (initial weight × number of days)] [3].

2.4. Leaf chemistry

2.4.1. Cabbage leaf peroxidase (PPD) and polyphenol oxidase (PPO) assays

An increase in polyphenol oxidase is a marker of jasmonate-induced response following herbivore damage or JA spray [3]. The levels of PPO in leaves of 1× and 2.6× BTH-treated cabbage plants were measured 4–5 days after treatment (DAT) with water sprayed cabbage providing the

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Table 1
Effect of plant elicitor BTH at 1× and 2.6× the recommended Actigard application rate on cabbage polyphenol oxidase (PPO) and peroxidase (PPD) activity. Multi-factorial ANOVA analysis of polyphenol oxidase (PPO) activity and peroxidase (PPD) activity in cabbage plants exposed to BTH treatments in 3–4 separate trials.

Experiment	Factor	*df*	F	P
PPO activity	Trial	3	0.09	0.9628
	Treatment	2	6.55	0.0039
	Trial × Treatment	6	4.78	0.0012
	Error	34	–	–
PPD activity	Trial	2	3.85	0.0368
	Treatment	2	2.07	0.1499
	Trial × Treatment	4	0.26	0.9019
	Error	22	–	–

control leaves. The top 3rd and 4th leaves from all plants were collected and frozen immediately in liquid nitrogen and then stored at −80 °C until the assay was performed. Leaves (0.3 g) were homogenized in 1.25 mL of 0.1 M potassium phosphate buffer, pH 7, containing 7% (w/v) polyvinylpyrrolidone (PVP 40). A 1.5 mL volume of the homogenate was combined with 100 μL of 10% Triton X-100 and then the sample was centrifuged at 6000 ×*g* for 15 min to obtain the enzyme preparation from the supernatant. Enzyme extract, 10–100 μL, was added to 1 mL of 2.92 mM caffeic acid dissolved in 0.1 M potassium phosphate buffer, pH 8 [22]. The PPO assay measured the absorbance at 470 nm using a BioRad Smart Spec Plus spectrophotometer. PPD levels have been induced by salicylic acid (SA) [22] but typically not by methyl jasmonate (MJ) [6]. PPD activity in the leaf tissue was measured using similar steps up to the stage where the enzyme extract was combined with the substrate [6]. A 10–100 μL sample was added to 1 mL of 5 mM guaiacol dissolved in 0.1 M potassium phosphate buffer, pH 8, with 0.02 mM H_2O_2. PPD was measured by guaiacol conversion with the absorbance read at 470 nm as described above. The PPO and PPD activities are reported as Δabsorbance (optical density)/min/g fresh leaf weight.

2.4.2. LC-MS analyses of glucosinolates

High resolution mass spectrometry (HRMS) and $HRMS^2$ analysis was performed using heated electrospray ionization source (HESI) on a Thermo Q-Exactive Quadrupole Orbitrap Mass Spectrometer, coupled to an Agilent 1290 HPLC. A Zorbax Eclipse Plus RRHD C18 column (2.1 × 50 mm, 1.8 μm; Agilent) maintained at 35 °C was used for separation. Samples were injected at 2.0 μL volumes and the flow rate was 0.3 mL/min. Mobile phases were water with 0.1% formic acid (A), and acetonitrile with 0.1% formic acid (B). Mobile phase B was held at 0% for 30 s, before increasing to 100% over 3 min. Mobile phase B was held at 100% for 1.5 min, before returning to 0% over 30 s. The following conditions were used for HESI: capillary temperature, 320 °C; sheath gas, 20.00 units; auxiliary gas, 7.00 units; probe heater temperature, 300 °C; S-Lens RF level, 35.00. A capillary voltage was 3.6 kV and 3.5 kV for positive and negative mode respectively. All samples were analyzed in the scan range of 75–1125 *m/z* in both positive and negative mode at 140,000 resolution to enable separation of the ^{34}S and $^{13}Cx2$ isotopes, as well as an AGC target of 1e6 and a maximum injection time of 500 ms. For characterization of compounds, samples were also analyzed using data-dependent MS^2 which involved a full MS scan followed by MS^2 on the top 10 ions. The settings for the full MS scans were: resolution 35,000, AGC target 3e6, and max injection time 125 ms. The settings for the MS^2 scans were: resolution of 17,500, AGC target 1e5, maximum injection time 50 ms, isolation window 1.2 *m/z* and normalized collision energy 30. Glucosinolates were identified by high mass accuracy (<3 ppm) and when possible, comparison of ion fragmentation with published data [24].

2.5. Insect enzyme activity

The levels of anti-oxidant (CAT, GPOX and GR) and metabolic enzyme (GST) activity were measured in *T. ni* exposed to BTH-treated or untreated cabbage leaf in combination with the enzyme inhibitor. As was described previously in the induced plant bioassay section, 4–5 week old cabbage plants were sprayed with either water (control), 375 mg/L Actigard (1× BTH) or 975 mg/L Actigard (2.6× BTH) and kept separate for 4 days. Mid-sized 3rd and 4th leaves from treated plants were selected and sprayed on both sides with either 5 mL of acetone (control) or 10 ppm quercetin using the Potter Spray tower. Leaves were dried briefly and then transferred to a 140 mm dia petri dish with a moistened filter paper. A single, pre-weighed, early 5th instar *T. ni* larvae was transferred onto each leaf, the lid sealed and three replicates per treatment were held in a growth room at 25 °C, 50% RH, 16:8 L:D for a 24 h feeding period. Each trial used 3 replicate *T. ni* larvae per treatment, and each trial was repeated three times (N = 9 larvae per treatment). The anti-oxidant enzyme assays were initiated with *T. ni* larvae dissected on ice and the midgut removed and homogenized with a disposable pellet mixer in 50 mM phosphate buffer, pH 7.0. CAT was measured by change in absorbance of H_2O_2 at 240 nm [29,31]. GPOX and GR levels were assessed using a Sigma-Aldrich assay kit (Sigma Cat. # CGP1 and GRSA, respectively) to measure NADPH absorbance at 340 nm [31]. The methods measure the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPOX, which is then recycled back to GSH by glutathione reductase (GR) and NADPH. A decrease in NADPH absorbance at 340 nm directly measures GPOX and GR levels in the sample. The GST levels in *T. ni* were assessed by dissecting *T. ni* and removing the midgut and homogenizing in 500 μL of a pH 7.5 sodium phosphate buffer [15]. An additional 500 μL of buffer

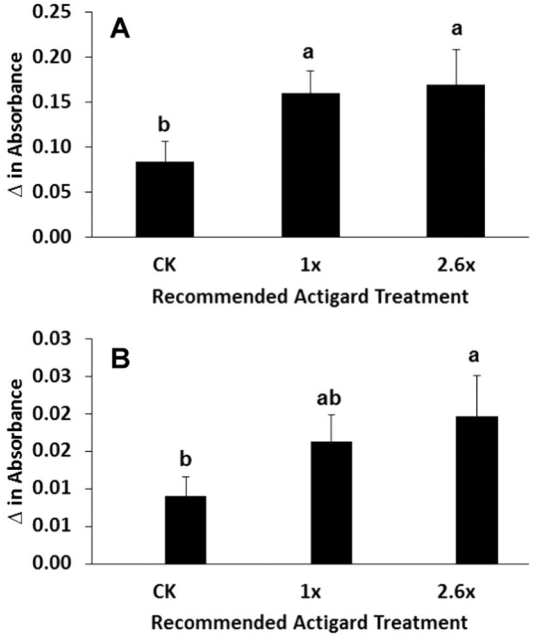

Fig. 1. Average polyphenyloxidase (PPO) (A) and peroxidase (PPD) ± S.E. levels (B) in cabbage leaves 4 DAT with water (CK) or BTH at 1× and 2.6× the recommended rate. Bar with different lower case letters indicates a significant difference (ANOVA PROC GLM, Tukey's HSD test, $P < 0.05$).

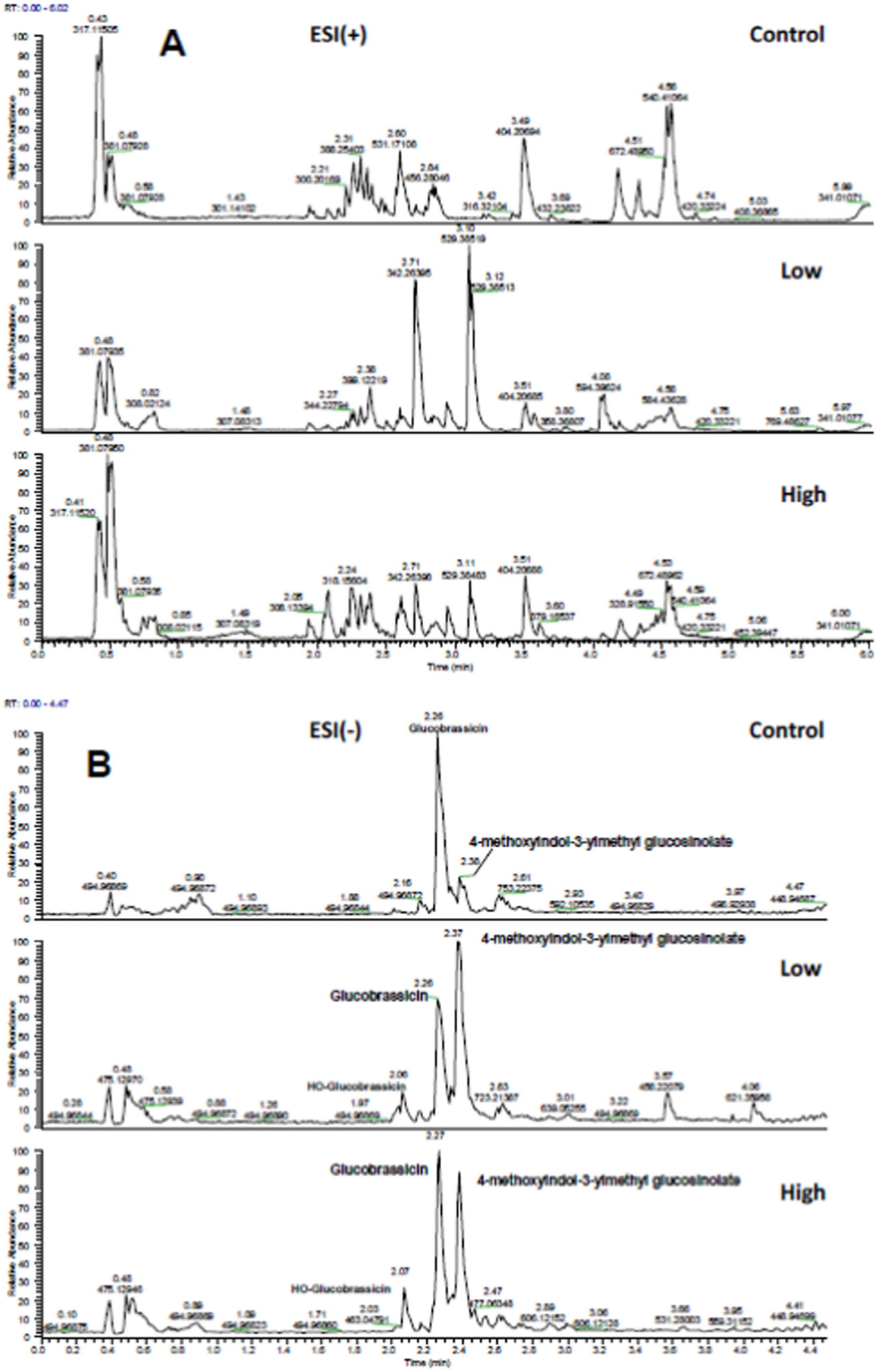

Fig. 2. LC-MS chromatographs ESI + (A) and ESI- (B) of cabbage leaf indole glucosinolate profiles 4 DAT with water (control), low (1× BTH) Actigard and high Actigard (2.6× BTH).

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was then combined before centrifugation at 10,000 RPM. The supernatant (25 μL) was combined with 150 mM 1-chloro- 2,4- dinitrobenzene (CDNB) (1 μL) or 1,2-dichloro-4-nitrobenzene (DCNB) (1 μL), 15 mM reduced glutathione in pH 7.0 potassium phosphate buffer buffer (50 μL). Three replicates were prepared from each midgut sample in a 96 well plate on ice. The readings were measured at 340 nm for 10 min with 30 s intervals at 25 °C using a microplate reader (BioTek Fisher Scientific) set on the kinetic setting. The initial and final readings were taken at 3 and 8 min after the initiation of the plate readings. Blank wells contained phosphate buffer in place of the sample volume and CDNB or DCNB for each GST assay, respectively. Three individual *T. ni* larvae per treatment from each of the three trials were analyzed. The anti-oxidant and GST activity was standardized to the protein content in the homogenized tissue samples [15] and the protein concentration in the homogenized insect tissue samples was determined using bovine serum albumin (BSA) as the reference protein [32]. The total number of insect midgut analyzed was 3 replicates per trial for each enzyme measurement (N = 9 midgut per enzyme measurement).

2.6. Statistical analysis

Differences in the cabbage leaf PPD and PPO between the control and BTH-treated plants and the average RGR between the combination of BTH cabbage leaves and inhibitor treatments were determined by multi-factorial analysis of variance (ANOVA) (Proc GLM) ANOVA) (Proc GLM) with Tukey's Studentized Range (HSD) Test. The mean fold-changes in *T. ni* CAT, GPOX, GR, GST levels were determined by one-way ANOVA (Proc GLM) with Tukey's Studentized Range (HSD) Test. All analyses were performed using the Statistical Analysis System (SAS Institute 2008).

3. Results

3.1. Plant chemistry

The effect of the plant elicitor applied at the recommended rate (1× BTH) significantly altered PPO but not PPD levels in cabbage leaves (Table 1). Four days after treatment the average PPO activity for 3 trials was significantly greater ($P < 0.05$) with the 1× and 2.6× BTH treatments compared to the control leaves (Fig. 1A), but there was no difference in PPO between the 1× and 2.6× BTH-treated plants (d.f. = 5,40; F = 1.71; P = 0.1549). In contrast, only the 2.6× BTH treatment significantly (d.f. = 4,26; F = 3.34; P = 0.0246) increased the PPD levels in cabbage compared to the control leaves (Fig. 1B), but the 1× BTH treatment was no different ($P > 0.05$).

Five glucosinolates were identified by high mass accuracy (<3 ppm) and three unknown sulfur containing compounds with differential expression across test groups were detected in positive mode (Fig. 2). Absolute identification of the unknown compounds will require authentic standards. Indole glucosinolates (GSs) identified in the un-induced (control) and BTH activated cabbage leaves were hydroxy-glucobrassicin (OH-indol-3-ylmethyl-GS), 1-methoxy-3-indoylmethyl-GS and 4-methoxy-3-indoylmethyl-GS. All three compounds were present in the methanol extracts of all cabbage leaves, but hydroxy-glucobrassicin and 4-methoxy-3-indoylmethyl-GS were higher in the BTH treated leaves (Table 2, Fig. 3). Other unidentified compounds with a ^{34}S isotope and *m/z* of 210.9997 $[C_8H_6ON_2S_2 + H]^+$ and 195.0224 $[C_8H_6O_2N_2S + H]^+$ were observed to be present in higher concentrations only in cabbage leaf treated with the higher (2.6×) BTH treatment (Fig. 3). Other GS compounds that decreased in concentration with increasing BTH application rate included glucobrassicin and glucoiberin (3-methylsulfinylpropyl-GS) (data not shown).

Table 2
Glucosinolate compounds identified in cabbage leaf extracts by LC-MS.

	RT	Experimental *m/z*	Theoretical *m/z*	Mass error (ppm)
Negative mode				
4-methoxyindol-3-ylmethyl glucosinolate	2.38	477.0636	477.0643	−1.5
1-methoxyindol-3-ylmethyl glucosinolate	2.52	477.0636	477.0643	−1.5
glucobrassicin	2.26	447.0531	447.0537	−1.4
glucoiberin or related	0.67	422.0246	422.0255	−1.8
OH-glucobrassicin	2.04	463.0480	463.0487	−1.4
Positive mode				
Unknown # 1	0.48	381.0795	–	–
Unknown # 2	3.44	195.0225	–	–
Unknown # 3	3.73	210.9997	–	–

3.2. Effect of plant activator and inhibitor on insect growth

The effect of BTH and BTH combined with the inhibitor, quercetin, had a significant effect on the *T. ni* 4 day RGR (Table 3). The combination of 2.6× BTH induced cabbage with 10 ppm quercetin significantly reduced (d.f. = 3,19; F = 3.21; P = 0.0462) the RGR compared to the *T. ni* that fed on cabbage treated with 2.6× BTH alone or the control plants with and without quercetin (Fig. 4A). In contrast, the RGR of *T. ni* larvae fed 2.6× BTH cabbage was significantly greater (d.f. = 3,19; F = 4.65; P = 0.0134) than control cabbage with or without quercetin, but not 1× BTH cabbage alone ($P > 0.05$)(Fig. 4B).

3.3. Insect anti-oxidant and metabolic enzyme activity

The response of *T. ni* to cabbage treated with BTH, inhibitor or both, was no different with respect to catalase (CAT) activity (d.f. = 5,12; F = 1.86; P = 0.176), and *T. ni* that fed on untreated cabbage had the same CAT as those that fed on 1× and 2.6× BTH cabbage when both were combined with quercetin (Fig. 5A). The glutathione reductase (GR) levels were also not significantly different (d.f. = 5,11; F = 1.62; P = 0.235) across treatments, including *T. ni* that fed on the 1× BTH cabbage alone versus the 1× BTH and quercetin combination ($P < 0.05$) (Fig. 5B).

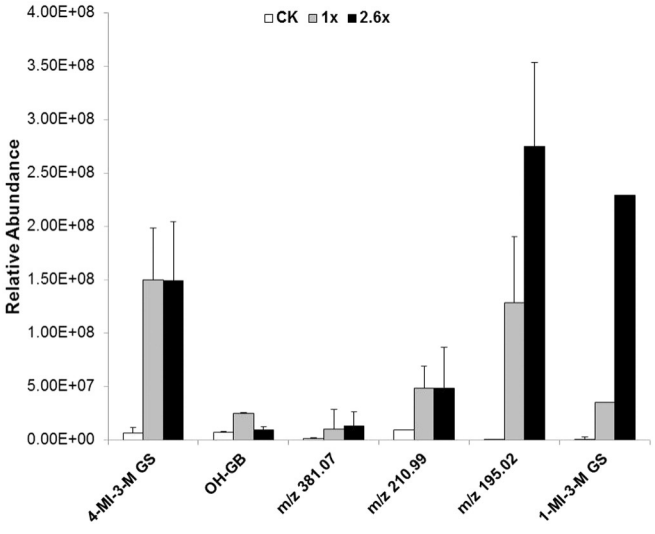

Fig. 3. Indole glucosinolate (GS) concentrations in cabbage leaf extracts 4 days post-treatment with water (control), 1× RAR Actigard and 2.6× Actigard. Average relative abundance ± S.D. for 4-methoxyindol-3-ylmethyl (4-MI-3-M) GS; hydroxyl-glucobrassicin (OH-GB); unidentified GS with *m/z* 381.0795; unidentified GS with *m/z* 210.99969; unidentified GS with *m/z* 195.02242; 1-methoxyindol-3-ylmethyl (1-MI-3-M) GS.

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Table 3
Synergism of quercetin and plant elicitor BTH (1× and 2.6× recommended Actigard application rate). Multi-factorial ANOVA analysis of cabbage looper relative growth rate (RGR) on cabbage plants in four treatments (control, BTH, quercetin and BTH × quercetin) with 3 BTH and 2 quercetin levels.

Experiment	Factor	*df*	F	P
RGR of *T. ni*	BTH	3	8.27	0.0015
	Quercetin	1	0.25	0.6257
	BTH × Quercetin	3	3.47	0.0412
	Error	16	–	–

The *T. ni* glutathione peroxidase (GPOX) activity was increased post-feeding on cabbage leaves exposed to the 1× BTH and quercetin combination compared to all other treatment (d.f. = 5,12; F = 4.09; P = 0.0212) with the exception of 2.6× BTH and no quercetin (Fig. 5C). Glutathione S-transferase (GST) activity was significantly greater (d.f. = 5,12; F = 4.09; P = 0.0212) in *T. ni* that fed on the 1× BTH cabbage with quercetin compared to those that fed on 1× BTH cabbage alone but was no different than those fed on the other cabbage treatments (Fig. 5D).

4. Discussion

Actigard™ 50WG (BTH) is registered for the crop protection from certain phytopathogens and acts by inducing SA pathway defenses. Even though there was promise for comparable elicitor products to activate the JA pathway [33], no chemical products, with the exception of methyl jasmonate and JA, have been applied on a large scale. Certain plant growth-promoting rhizobacteria (PGPR) will activate ISR [20] and induction of the SA pathway can have negative effects on herbivores, as observed with leaf miner and bean beetle reduced performance on induced plants [30,34], likely due to the increased level of common defenses regulated by both JA and SA pathways. Generally, the effect of SAR to insects is mixed, and often with no effect [35–37] but sometimes it can lead to increased insect performance, partially explained by the induction of salicylate response at the expense of the jasmonate pathway [3]. The prospect of more plant activators or SAR inducing products being used for disease management indicates there should be further research on the potential impact to herbivores on those crops. Our approach was to study the mechanism of insect response to SAR up-regulated defenses to illuminate new strategies to manage insect pests.

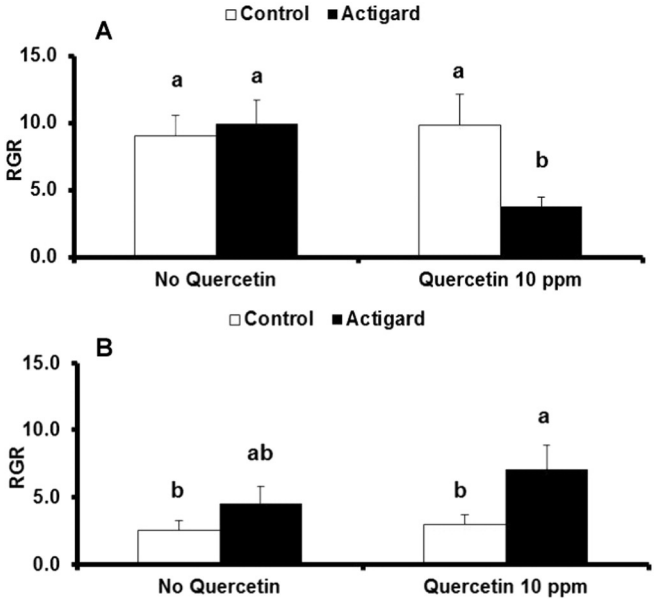

Fig. 4. The relative growth rate (RGR) ± S.E. of *T. ni* larvae fed cabbage treated with 2.6× BTH and 10 ppm quercetin (A) or cabbage treated with 1× BTH and 10 ppm quercetin (B). Bar with different lower case letters indicates a significant difference (ANOVA PROC GLM, Tukey's HSD test, $P < 0.05$).

This investigation has focused on the synergism of combining an enzyme inhibitor with induced plants to negatively affect herbivores. *Trichoplusia ni* larvae that fed on BTH-induced cabbage leaves demonstrated that insect performance was not affected when SA pathway responses are elevated. However, when a high BTH (2.6×) concentration was applied, the *T. ni* fed cabbage leaves treated with quercetin had significantly reduced relative growth rate (RGR) compared to *T. ni* fed on untreated control leaves. It is known that quercetin acts as a pro-oxidant and metabolic enzyme inhibitor, so in this combination with induced leaves there are a number of explanations for the observed anti-herbivore effect. Quercetin acts as a pro-oxidant and in *T. ni* may stimulate increased levels of anti-oxidants such as superoxide dismutase (SOD) as well as the activities of catalase, glutathione peroxidase and glutathione reductase (CAT, GPOX and GR, respectively) [27]. In the present study, no changes in CAT and GPOX were observed for insects that fed on cabbage leaves induced by 1× BTH alone, but GR activity increased in the presence of quercetin, suggesting that the insect enzymes were responding to quercetin and the induced plant defenses in combination (Fig. 5C). In contrast, feeding on 2.6× BTH treated cabbage did not affect the insect GR levels compared with the 1× BTH treatment, perhaps because of the presence of additional defenses (PPD and GS) activated only by the higher BTH concentration.

The relationship that may explain the reduced growth of *T. ni* larvae fed on cabbage leaves of BTH-induced plants in combination with quercetin is the interaction between elevated GS levels and insect enzyme activity. The leaf chemistry in the BTH-treated cabbage was significantly increased compared to the control plants, but in the case of PPO and PPD, there was no difference between the lower and higher BTH treatments (Fig. 1A and B). However, the level of several GS was significantly higher in the 2.6× BTH treated leaves (Fig. 3), a fact which likely reduced the amount of GST activity measured in the *T. ni* that fed on the 2.6× BTH leaves combined with quercetin compared to those that fed on the un-induced leaves and quercetin (Fig. 5D). The *T. ni* that fed on 2.6× BTH leaves could not fully metabolize the greater amount of indole GS present as the phenotypic plasticity of the *T. ni* GR and GST response was exceeded, in part by the dual challenge of higher GS levels and GST inhibition by the flavonoid. Without sufficient enzymes to detoxify the elevated GS, there was reduced nutritional assimilation and greater fitness costs, manifested through the reduced growth rate over the 4 day period. Therefore, these results suggest that it may be the difference in GS that affects the detoxification enzymes rather than the PPO or PPD levels in the induced cabbage leaves (Table 4).

In the present study, hydroxy-glucobrassicin and 4-methoxy-3-indoylmethyl-GS were the GS that increased in concentration in the BTH-induced cabbage leaves. The principal GS in cabbage leaves were 3-indolylmethyl-GS and 4-methoxy-3-indolylmethyl-GS, with lower concentrations of 2-hydroxybut-3-enyl-GS [38]. In contrast, sinigrin, gluconapin and progoitrin were found to be prominent, with 10–20-fold lower glucobrassicin levels on average in another cabbage GS study [39]. Analyses of GS in other Brassicaceae plants, for example Brussel sprouts, identified glucobrassicin with the highest leaf concentrations followed by the aliphatic-GS sinigrin, gluconapin and progoitrin [40].

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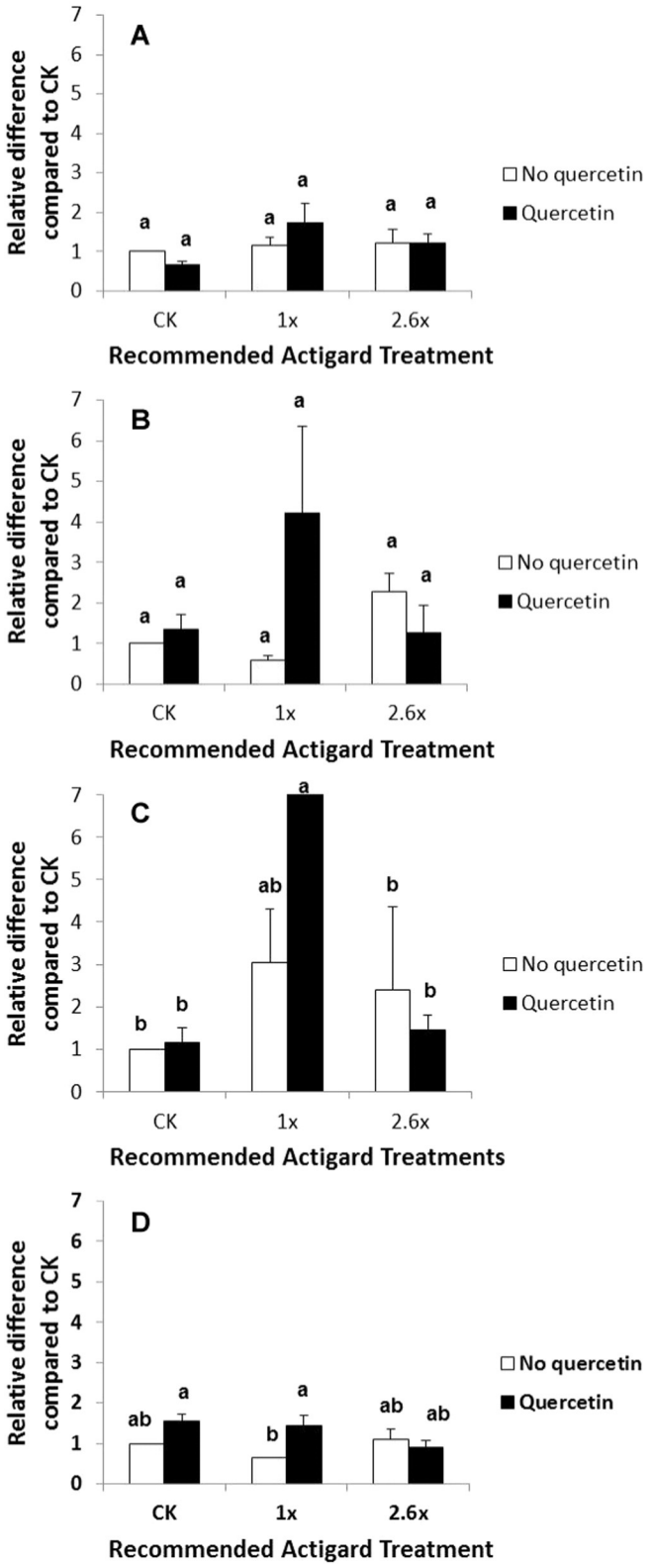

Fig. 5. Average fold-differences ± S.E. in *T. ni* catalase activity (A), glutathione reductase activity (B), glutathione peroxidase activity (C), and glutathione S-transferase activity (D) for insects that fed for 24 h on cabbage leaves from BTH-treated plants at 1× and 2.6× the recommended rate with/without quercetin compared to control leaves. Bar with different lower case letters indicates a significant difference (ANOVA PROC GLM, Tukey's HSD test, $P < 0.05$).

Table 4
Comparison of plant chemistry in BTH induced and un-induced cabbage leaves, insect enzyme activity and insect relative growth rate post feeding on 5 different plant-inhibitor combinations.

	BTH and quercetin treatment combinations				
Plant/insect response	Q[1]	1× BTH[2]	2.6× BTH[3]	1× BTH + Q[4]	2.6× BTH + Q[5]
Plant chemistry					
PPO[6]	−	+	+	−[7]	−
PPD[8]	−	N.D.	+	−	−
GS[9]	−	+	++	−	−
Insect enzymes					
CAT/GR/GST[10]	N.D.	N.D.	N.D.	N.D.	N.D.
GPOX[11]	N.D.	N.D.	N.D.	+	N.D.
Insect RGR[12]	N.D.	N.D.	N.D.	+	*

[1]Quercetin (10 ppm); [2]Recommended application rate (RAR) for BTH; [3]2.6-fold BTH RAR; [4]RAR BTH and quercetin combination; [5]2.6-fold BTH and quercetin combination; [6]Polyphenyloxidase (PPO) activity - 1× and 2.6× BTH leaves had significantly higher ($P < 0.05$) PPO (+) compared to un-induced leaves; [7]PPO and PPD levels in BTH and quercetin combinations were not measured; [8]Peroxidase (PPD) activity - 1× BTH leaves were not significantly ($P > 0.05$) different (N.D.), but 2.6× BTH leaves were significantly ($P < 0.05$) higher (+) compared to un-induced leaves; [9]Glucosinolate (GS) levels – indicate that the relative concentration of GS in the 1× BTH induced leaves was greater (+) and much greater in the 2.6× BTH induced leaves (++) compared to the un-induced leaves; [10]Catalase (CAT), Glutathione reductase (GR) and Glutathione S-transferase (GST) activity – no difference ($P > 0.05$) between treatments; [10]Glutathione peroxidase (GPOX) activity – significant increase ($P < 0.05$) in *T. ni* fed 1× BTH and quercetin but no difference ($P > 0.05$) between other treatments; [12]Relative growth rate for 4 days – *T. ni* fed 1× BTH cabbage and quercetin had significantly higher ($P < 0.05$) RGR (+) but *T. ni* fed 2.6× BTH cabbage and quercetin had significantly lower ($P < 0.05$) RGR (*) but no difference ($P > 0.05$) between other treatments.

The evidence for the role of GS in defense of JA and SA-activated plants is mixed. In support of the findings in the present study, SA applied as a soil drench increased significantly the levels of leaf GSs in oilseed rape *Brassica napus* L., an effect that was stronger than that observed with pathogen infection [23]. In contrast, herbivore damage was greater in plants deficient in GS while JA over-expressing *Arabidopsis thaliana* mutants were better defended, but analyses of damaged versus undamaged plants did not indicate GS levels were induced by feeding [41]. In another case, *Arabidopsis* plants sprayed with necrotrophic ascomycete *Botrytis* fungal conidia, known to induce the SA pathway, reduced the levels of aliphatic and indole GS [24]. Necrotrophic pathogens are reported to be responsible for the induction of SAR, but obviously do not always lead to increases in GS levels. However, the conclusions of those studies that examined the interaction of JA and SA pathway responses indicate that there is no distinct effect for herbivores and pathogens [3], or even between different types of pathogens [18].

The presence of higher indole GS concentrations and higher oxygen radicals in the SAR cabbage leaves plants would require the *T. ni* to activate both detoxification enzymes and anti-oxidants, respectively. In many insects that feed on GS-containing plants, glutathione S-transferase (GST) activity is thought to be responsible for the conjugation of isothiocyanates, including generalist lepidopterans *T. ni* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and aphids *Myzus persicae* and *Aulacorthum solani* (Hemiptera: Aphidae) [28]. In contrast, the GS specialist diamond back moth *Plutella xylostella* (Lepidoptera: Plutellidae) has a sulfatase in the gut to metabolize GS to desulfo-GS, which can then be more easily excreted and not act as a substrate for myrosinase, the enzyme that converts GS to more toxic isothiocyanates in the plant. The GS sulfatase has not been detected in *T. ni* or other noctuid species. Induced monooxygenase or glutathione levels play an important role in the insect response to plant toxins, and induced GST activity is a generalized detoxification response to plant allelochemicals [42–46]. Quercetin [47], and other flavonoids [48,49] have been found to inhibit GST activity in insects and to synergize the activity of insecticides [47,50]. The results of the present study suggest that the flavonoid quercetin may synergize the GS or isothiocyanate toxicity through inhibition of

GST activity in *T. ni*, thus reducing the performance of the insect on BTH-treated cabbage, a finding with potential pest management application.

Actigard has been registered for crop protection of tomato and tobacco from common plant diseases. This product, or other plant activators, in combination with natural products (quercetin or other flavanoids) that specifically target insect metabolism might provide a unique crop protection against herbivores and pathogens without a negative effect on other beneficial arthropods. It was predicted that elicitor cocktails would be used to induce several plant defenses that are regulated by SA, JA and ethylene and would suppress a complex of pests [17]. However, the use of SAR will require reliable disease forecasting so that the plant defenses will be activated at the most critical stage of development, otherwise the induced defenses will be ineffective. Combinations of elicitors or with elicitors applied after reduced application rates of fungicides have been shown to work effectively, in part because the fungicides have reduced the inoculum pressure to a level where the elicitors are more effective [51]. Synergy was also noted when BTH and PGPR products were applied to tomato, reducing both disease incidence and severity, in part through combined disease control and plant yield effects [20]. BTH and another elicitor, laminarin (a water-soluble B-1, 3glucan molecule), increased the attractiveness of herbivore-damaged maize seedlings to parasitic wasps, possibly through stimulating the plants by way of SA-JA pathway cross-talk, leading to fewer HIPVs being released [52].

In conclusion, it was confirmed that BTH treatments increased the levels of indole GS levels in cabbage. Although the actual concentration of GS was not determined, the BTH application did increase GS levels compared to those in un-induced cabbage. The findings of this study suggest that BTH and related elicitors can increase defenses for pathogens which are not necessarily at the expense of the herbivore defenses, and there is potential for synergism between the induced plant defenses and selected enzyme inhibitors. Future projects should include the testing of other plant activators and enzyme inhibitors in different plant-insect combinations.

Conflict of interest

To the best of the author's knowledge, there are no conflicts of interest with respect to the contents of the manuscript or the study completed.

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