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Activity of benzothiadiazole on chrysanthemum yellows phytoplasma (‘*Candidatus* Phytoplasma asteris’) infection in daisy plants

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Abstract

A commercial preparation of the plant resistance elicitor benzothiadiazole (BTH) (Bion, Syngenta Crop Protection) was tested for its capacity to induce systemic resistance against chrysanthemum yellow phytoplasma (CYP) infection in the *Chrysanthemum carinatum* plant. Following one 2.4 mM BTH application, plants were exposed to CYP-infective *Macrosteles quadripunctulatus* leafhoppers. Symptom development and phytoplasma multiplication in the test plants were measured over time. BTH application delayed symptom development and phytoplasma multiplication in treated plants compared with the control ones. CYP titre and symptom severity were significantly lower for the first two weeks post-inoculation in treated plants compared with the control ones, suggesting that systemic acquired resistance (SAR) induced by BTH in *C. carinatum* is temporary. Higher concentrations of BTH resulted in phytotoxic effects involving the whitening of apical leaves. BTH application was ineffective in reducing the transmission efficiency of CYP by its leafhopper vector. Actually, in both single and group transmission tests, the proportion of infected plants was similar among BTH-treated and untreated plants. The survival of *M. quadripunctulatus* was unaffected by feeding on BTH-treated daisy plants. Moreover, when leafhoppers were allowed to choose between treated and untreated plants, they showed no preference. We conclude that SAR induced in daisy plants by BTH has no detrimental effects on the vector leafhopper. If the activity of BTH against phytoplasmas is confirmed also on other

phytoplasma/host–plant associations, BTH applications might be included in new, more environmentally friendly, integrated management strategies of phytoplasmoses.

Keywords

Benzothiadiazole, resistance elicitor, systemic acquired resistance, chrysanthemum yellows phytoplasma, *Macrosteles quadripunctulatus*

Introduction

Phytoplasmas are wall-less pathogenic bacteria belonging to the class Mollicutes that are associated with economically important diseases of fruit trees (pome fruit, stone fruit, coconut and grapes among others), vegetables (lettuce, potato, tomato, etc.), ornamentals (herbaceous, trees and shrubs) and weeds (Seemüller et al., 1998; Bertaccini et al., 2007). They are phloem-limited pathogens that cause several different symptoms such as yellowing, stunting, proliferation, virescence, phyllody and general decline. Phytoplasmas are transmitted in a persistent, propagative manner by Hemipteran phloem-sucking insects in the Cicadellidae, Cixiidae, Derbidae, Delphacidae and Psyllidae families (Weintraub and Beanland, 2006).

The control of phytoplasma diseases is difficult and relies mainly on insecticide treatments against vector insects and the planting of healthy propagation material. Insecticides can be effective in suppressing the vector population but only neonicotinoids actively prevent the transmission from infectious insects visiting the crop (Saracco et al., 2008). Large scale use of insecticides (in preventing the spread of phytoplasma diseases) has a negative impact on non-target arthropods, especially mites (Waetermeulen et al., 1999) and, possibly, on pollinators (Vorwohl, 1977), and represents a potential threat to human health. Planting phytoplasma-free stocks is advisable, but control of vector populations is still required to prevent transmission during the vegetative season (Morone et al., 2007). Traditional vector exclusion methods, such as the use of insect-proof nets,

though very effective if properly secured in place (Walsh et al., 2006), can be applied in protected crops but not open field conditions.

Systemic acquired resistance (SAR) is an inducible resistance mechanism in plants that provides resistance against plant pathogens. The treatment of plants with various agents (e.g., virulent or avirulent pathogens, nonpathogens, cell wall fragments, plant extracts and synthetic chemicals) can lead to the induction of resistance to subsequent pathogen attack. SAR by plant activators provided interesting results in the control of a broad spectrum of pathogens, such as bacteria, fungi and viruses (Oostendorp et al., 2001; Vallad and Goodman, 2004). In the vast majority of cases, SAR depends on the early increase of the endogenously synthesized signal salicylic acid and it is correlated with the immediate expression of a specific set of genes which includes those coding for pathogenesis-related-proteins (PR) (Pieterse and Van Loon, 2007). Several PR proteins (e.g. β-1,3 glucanases, chitinases, osmotin) possess anti-microbial activity and are thought to contribute to the state of resistance together with other PR (e.g. hydroxyproline rich glycoproteins, callose) that are important in cell wall structure. Among the synthetic inducers of plant disease resistance, acibenzolar-S-methyl, a benzothiadiazole (BTH), is a well-studied compound (Schurter et al., 1987; Friedrich et al., 1996) that has extensively been shown to induce SAR in many crops, such as monocots, solanaceous, leguminous and fruit trees (Gorlach et al., 1996; Buonaurio et al., 2002; Dann and Deverall, 1995; Ishii et al., 1999).

Bressan and Purcell (2005) reported a significant effect of BTH in reducing X-disease phytoplasma transmission to *Arabidopsis thaliana* (L.) Heynh. by the leafhopper *Colladonus montanus* (Van Duzee), whereas Romanazzi et al. (2009) noticed a higher recovery rate from bois noir phytoplasma in grapevines treated with BTH and other resistance elicitors. Conflicting information is available on the effect of BTH on phytophagous insects. In most cases, the salicylic acid pathway involved in SAR development has only negligible effects on phytophagous insects (Inbar et al., 2001; Bi et al., 1997), but for some phloem feeders reduced fecundity and increased mortality have been reported following feeding on BTH-treated plants (Bressan and Purcell, 2005; Boughton et al., 2006).

The aim of this study was to investigate the effect of benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), on the transmission of chrysanthemum yellows phytoplasma (CYP) to daisy plants under controlled conditions. The association of *Chrysanthemum carinatum* (Schousboe)–CYP–*Macrosteles quadripunctulatus* (Kirschbaum) represents an optimal model system because of i) the short incubation of the phytoplasma in the plant and short latency in the vector, ii) rapid and obvious symptom development, iii) extremely high transmission efficiency, iv) the polyvoltine vector that can be easily reared throughout the year and v) the availability of detection and quantification tools for the phytoplasma (Marzachì and Bosco, 2005). CYP is a strain of the '*Candidatus* Phytoplasma asteris' phytoplasma (16Sr-IB), which infects a variety of dicotyledonous plants and is transmitted with different efficiencies by several species of leafhoppers (Bosco et al., 2007). The activity of BTH on CYP disease was measured through the analysis of disease severity and the quantification of CYP cells in infected, treated and untreated plants, by quantitative real time PCR analysis (qRT-PCR).

Materials and methods

Plants and BTH treatments

C. carinatum plants were grown from seed, individually transplanted in 8 × 8 cm pots and maintained in a greenhouse (20–25°C, photoperiod L16:D8). One month after sowing (when they were 5–6 cm high), plants were sprayed with BTH solutions (Bion WG50, 50% active ingredient, a.i., Syngenta Crop Protection) diluted with sterile water and applied as a foliar treatment to a run-off point. Control plants were sprayed with water. After the treatment, plants were maintained in a greenhouse under the conditions described above. Three different concentrations of BTH were evaluated in preliminary assays, 1.2, 2.4 and 4.8 mM, to determine the concentration providing the best results. The preliminary experiment was carried out twice. Three further experiments were then performed using the 2.4 mM concentration only. In each experiment, 10 plants per treatment were included: BTH-treated plants not exposed to CYP infection (BTH), untreated CYP-inoculated

plants (CYP), control plants untreated and unexposed to CYP (C) and BTH-treated and CYP-infected plants (BTH-CYP).

Phytoplasma and vector insect

The '*Candidatus* Phytoplasma asteris' (strain 16Sr-IB), originally isolated from *Argyranthemum frutescens* (L.) Schultz-Bip plants in Liguria (Italy) (Conti and Mela, 1987), was maintained on the daisy (*C. carinatum*) by vector transmission.

Healthy colonies of *M. quadripunctulatus* were reared on potted oat plants inside Plexiglas and nylon cages in growth chambers at 25°C (photoperiod L16:D8) and checked by nested PCR using the R16F2/R2 followed by R16F1/R1 (I) primers (Lee et al., 1993, 1994) to verify phytoplasma absence.

For transmission experiments, the third- to fifth-instar nymphs were fed for one week of the acquisition access period (AAP) on CYP-infected plants, transferred onto healthy oat plants for two weeks to complete the latency period (LP) and then transferred individually to 10 daisy plants, confined inside glass cylinders, for each elicitor and control treatment, for an inoculation access period (IAP) of three days. Males and females of *M. quadripunctulatus* were used in the transmission experiments since both genders proved to transmit CYP with 100% efficiency under the experimental conditions described above (Bosco et al., 2007). Test plants were exposed to vectors one week after BTH treatment (about five weeks after sowing). Insects were then removed and plants were drench-treated with the systemic insecticide thiamethoxam (Actara, Syngenta Crop Protection), 7 mg a.i. per plant, to kill nymphs hatched from eggs eventually laid by vector females. Test plants were maintained in the greenhouse for about one month. Daisy plants treated with water and exposed to vectors were used as transmission controls. Treated plants not exposed to vectors were used as treatment controls. Apical leaves of five daisy test plants were sampled at six, 11, 13 and 20 days after the end of the inoculation (dpi) from the same test plants. Total DNA was

extracted and CYP DNA quantified by qRT-PCR. The quantification of phytoplasma cells was not performed in the two preliminary experiments where only symptom severity was evaluated.

Choice test

In a choice experiment, eight healthy daisy plants (5–6 cm high) were sprayed to the run-off point with 2.4 mM BTH and eight healthy daisy plants were sprayed with water as a control. After one week, treated and untreated plants were set interspersed inside a Plexiglas and nylon cage (80 × 80 × 60 cm). Following an AAP of seven days on untreated source plants and an LP on oat as described above, 10 infective adult vectors were introduced into the cage for an IAP of three days; vectors were free to fly and feed on all the plants. The cage was maintained in a climatic chamber at 25°C (photoperiod L16:D8). At the end of the IAP, insects were counted and removed. Test plants were treated with insecticide and transferred to the greenhouse for about one month or until the appearance of symptoms. Infection of the plants was established on the basis of symptoms. The experiment was performed twice.

No-choice test

Sixteen daisy plants (5–6 cm high) were treated with 2.4 mM BTH to the run-off point and 16 plants were treated with water as a control. After one week, BTH-treated and control plants were separately placed inside two Plexiglas and nylon cages (80 × 80 × 60 cm). Following an AAP on CYP source plants of seven days and an LP on oat as described, 10 infective adult vectors were introduced into each cage for an IAP of three days on the test plants; vectors were free to fly and feed on all plants. The cages were maintained in a climatic chamber at 25°C (photoperiod L16:D8). At the end of the IAP, insects were counted and removed. Plants were treated with insecticides and transferred to the greenhouse for about one month. Infection of the plants was established on the basis of symptoms. The experiment was performed twice.

Symptom evaluation

The severity of the symptoms of test plants was evaluated three times a week between 11 and 32 dpi, and plants were classified into five classes of severity: 0 = no symptoms, 1 = yellowing of the apex, 2 = yellowing and distortion of the apex, 3 = apex growth stunt, 4 = severe yellowing and dwarfing of the whole plant and 5 = plant death.

Phytoplasma concentration: DNA extraction and qRT-PCR

The activity of BTH on CYP was quantified by assessing phytoplasma titre in five plants from each treatment. Total DNA was extracted from CYP-infected daisy leaves (0.1 g) using the PureLink Plant Total DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and eluted in 50 μL of kit elution buffer. Total DNA was extracted from healthy daisy plants using the same protocol. The concentration of DNA extracts from CYP-infected daisies and healthy controls was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Diluted samples (1 ng/μL in sterile double-distilled water) were analysed in triplicate in qRT-PCR assays. CYP DNA in each sample was measured as the number of CYP cells per ng of plant DNA (Marzachì and Bosco, 2005).

The absolute quantification of CYP was achieved by comparing the CTs of the samples with those of three dilutions of a plasmid, pOP74, containing the phytoplasma 16S rDNA target sequence. One femtogram of pOP74 contained 194 molecules of plasmid, with each containing a single copy of the CYP 16S rDNA gene. Because this gene is present in two copies in phytoplasma genomes, one femtogram of pOP74 corresponded to 97 CYP cells (Marzachì and Bosco, 2005). The absolute quantification of daisy plant DNA was achieved by comparing the CTs of the samples with those of four dilutions of healthy daisy DNA. All samples were run on triplicate.

Data analysis

Symptom evaluation. At each rating date, the means and standard errors of severity class were calculated for treated and control plants and pairwise *t* tests were performed to compare symptom severity between treatments, according to Alexander et al. (1993). Results from the transmission experiments (positive/negative plants) were analysed using the χ^2 test.

Phytoplasma concentration. For the analyses, the concentration of CYP in each plant was expressed as the difference between the logarithm concentration of CYP and that of daisy plant DNAs. Finally, CYP concentration was expressed as cell number per ng of plant DNA. After comparing the phytoplasma titre measured at each sampling date in the three quantification experiments, data were pooled since they did not differ significantly among experiments. To compare phytoplasma titre measured at different dpi in BTH-treated and control plants, a two-way ANOVA for date and treatment was performed. The ANOVA, *t* test and χ^2 test were performed using Jandel SigmaPlot 11.0 (Systat Software, Inc, San Jose, CA, USA).

Results

Transmission results and symptom severity

Following inoculation with CYP-infective leafhoppers, 16 out of 20 plants treated with 1.2 mM BTH, 14 out of 20 plants treated with 2.4 mM BTH and 19 out of 20 plants treated with 4.8 mM BTH became infected. Among the untreated control plants, 16 out of 20 became infected. The proportion of infected/uninfected plants between BTH-treated and untreated plants did not differ significantly. Moreover, the 4.8 mM BTH treatment caused phytotoxic effects on some plants, which exhibited an unusual whitening of the apical leaves and inhibited plant growth compared with the controls. These effects lasted from 14 dpi until the end of the experiment. Symptom severity recorded from 11 to 32 dpi on plants treated with different BTH concentrations and untreated infected control plants is shown in Fig. 1. Consequently, in the following experiments the 2.4 mM BTH treatment was chosen. Twenty-five out of 30 2.4 mM BTH-treated plants and 29 out of 30 untreated plants became infected in the remaining experiments. Overall, following inoculation

with CYP-infective leafhoppers, 39 out of 50 plants treated with 2.4 mM BTH and 45 out of 50 untreated plants became infected, but the difference was not significant ($\chi^2 = 1.860$; $P = 0.173$). The results of the five experiment repeats with 2.4 mM BTH are summarised in Fig. 2. Symptom severity recorded from 11 to 32 dpi on 50 plants treated with 2.4 mM BTH and the 50 untreated infected control plants is shown in Fig. 3. Overall, test plants treated with 2.4 mM BTH showed lower symptom severity, but this difference was significant only at 11, 14 and 17 dpi ($P = 0.013$, $P = 0.001$ and $P = 0.005$, respectively) (Fig. 3).

In one experiment, two 2.4 mM BTH-treated plants showed clear symptoms until 31 dpi (class 3 symptoms), but afterwards the symptoms completely disappeared. The recovery of these plants was checked by nested PCR using the R16F2/R2 followed by R16F1/R1 (I) primers (Lee et al., 1993, 1994) and they proved to be phytoplasma-negative.

Choice test

When infective leafhoppers were allowed to feed freely on 2.4 mM BTH-treated and control plants, 14 out of 32 BTH-treated plants and 12 out of 32 control plants became infected; however, the difference was not significant. Eight and nine live insects were counted at the end of the two repeats of the experiment.

No-choice test

When infective leafhoppers were allowed to feed separately on 16 BTH-treated or control plants, 21 out of 32 control plants and 23 out of 32 BTH-treated plants became infected; however, the difference was not significant. Eight and nine live insects were counted at the end of the two repeats of the experiment for the treated plants, and seven and nine live insects were counted at the end of the two repeats of the experiment for the untreated plants.

CYP titre

Phytoplasma titre in plants treated with 2.4 mM BTH was significantly lower than that of control plants ($P<0.001$). Phytoplasma titre significantly increased over time from 6 to 20 dpi ($P<0.001$) in treated and control plants. There was no significant interaction between date and treatment. Mean numbers of CYP cells per ng of plant DNA in treated and control plants are shown in Table 1.

Discussion

BTH has been largely employed to activate SAR for the control of plant disease caused by several pathogenic fungi, bacteria and viruses. However, little is known about its activity towards phytoplasma diseases and, to our knowledge, only two reports are available on the activity of this chemical on phytoplasmas: one towards X-disease phytoplasma transmission to *A. thaliana* studied under controlled conditions (Bressan and Purcell, 2005), and the other on grapevine bois noir phytoplasma (Stolbur phytoplasma or '*Candidatus* Phytoplasma solani') under field conditions (Romanazzi et al., 2009). Our study demonstrated for the first time that phytoplasma multiplication and symptom development are partially hampered in the host plant by BTH treatment. CYP symptom development and multiplication were both delayed 13–17 dpi under our experimental conditions. Later, BTH elicitation against phytoplasma infection seemed to be lost. This suggests that BTH elicitation against CYP is temporary and tends to disappear over time. In our experiments, we treated test plants only once, one week before insect transmission, and we cannot exclude that a further application might have delayed phytoplasma multiplication and symptom severity later in the infection process. Actually, Buonaurio et al. (2002) found that, under field conditions, the highest efficacy of this elicitor against *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye was obtained by spraying pepper plants 6–7 times every 8–12 days. YuHong et al. (2006) reported that the induced resistance against downy mildew in cucumber plants persisted for 15 days. It is known that BTH-elicited resistance lasts longer in monocots than dicots (Oostendorp et al., 2001; Vallad and Goodman, 2004), and that the temporary activity of BTH we recorded in CYP-infected daisy

277 plants is consistent with this. Interestingly, BTH application was effective in delaying CYP
278 multiplication and symptom development but not in reducing transmission efficiency of CYP by its
279 leafhopper vector. Actually, in both single and group transmission tests, the proportion of infected
280 plants was similar among BTH-treated and untreated plants. This result is consistent with the fact
281 that the survival of *M. quadripunctulatus* was unaffected by the feeding period on daisy BTH-
282 treated plants. On the contrary, the reduced survival of *C. montanus* on *A. thaliana* BTH-treated
283 plants suggests that SAR in this plant might have some detrimental effect on this leafhopper,
284 inhibiting vector feeding and thereby phytoplasma transmission efficiency (Bressan and Purcell,
285 2005). Considering that the different pathways might not be similarly regulated in all systems
286 (Maleck and Dietrich, 1999), it seems that the systemic response induced by Bion on insect
287 herbivores could vary among plant and insect species. Our results are, therefore, consistent with
288 other studies reporting that SAR had a slight or negligible effect on phytophagous insects such as
289 some cotton pests or the silverleaf whitefly (Inbar et al., 2001; Bi et al., 1997). Other studies
290 noticed some resistance against the silverleaf whitefly induced by BTH. The resistance consists in
291 nymph number reduction and in a delay in insect development (Nombela et al., 2005; Muniz et al.,
292 2009).
293 We recorded lower phytoplasma multiplication in BTH-elicited plants. Similarly, SAR induced by
294 BTH or plant growth-promoting rhizobacteria was found to suppress the replication of cucumber
295 mosaic virus (Anfoka, 2000, Raupach et al., 1995). Moreover, our study also showed that BTH-
296 treated plants had less severe symptoms. Therefore, we suggest that the lower phytoplasma titre in
297 the elicited plants is directly correlated to the delay in symptom expression. These results suggest
298 that BTH could induce resistance with an indirect or direct mechanism. An indirect activity, through
299 the activation of SAR, is more likely to occur because BTH is known to induce the expression of
300 SAR genes (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). Until now, Bion is
301 known to activate a plant's defence system only and not directly on the pathogen. A direct effect on
302 phytoplasma multiplication cannot be excluded *a priori*. Because the chemical persistence of BTH

303 does not exceed five days (Buonaurio et al., 2002), whereas we recorded phytoplasma
304 multiplication and symptom expression for up to two weeks, an indirect mechanism of action is
305 suggested.
306 We observed that, under our conditions, BTH displayed some phytotoxic effects when applied at a
307 4.8 mM concentration. This is unsurprising given that several authors reported phytotoxic effects at
308 different concentrations in different crop species/cultivars (Iriti and Faoro, 2003; Amzalek and
309 Cohen, 2007; Perez et al., 2003; Bigirimana and Höfte, 2002).
310 Two daisy plants treated with 2.4 mM BTH and infected by CYP following insect transmission
311 completely recovered from the disease after one month. This finding, although not repetitive, is
312 peculiar because in our experience we have never observed such a recovery. This complete
313 recovery involved the disappearance of CY phytoplasmas, as demonstrated by nested PCR assays,
314 and was likely to be because of the BTH application. A similar recovery-inducing effect has been
315 reported for BTH-treated grapevines (Romanazzi et al., 2009).
316 The practical application of resistance elicitors towards phytoplasma diseases is in its infancy but,
317 given the difficulties in controlling phytoplasma diseases (relying mainly on insecticide treatments
318 against the vectors and roguing infected plants), the use of BTH is a promising new tool that could
319 be implemented in an integrated pest management strategy. Main limitations are represented by the
320 need of repeated applications and by the only partial tolerance that may be of low agronomical
321 interest, depending on the crop. In the light of avoiding repeated applications some biotic elicitors,
322 mycorrhizal fungi and rhizobacteria, seem to be promising (D'Amelio et al., 2007). Our results,
323 obtained on an experimental model pathosystem, should now be tentatively transferred to more
324 important crop systems (e.g., vineyards and orchards) before designing new integrated management
325 strategies that will eventually allow the reduction of insecticide applications for a more
326 environmentally friendly control of phytoplasmoses.
327
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Table 1. CYP concentration in BTH-treated and control daisy plants expressed as the mean ± standard error (SE) of cells per ng of plant DNA. Plants were sampled at six, 11, 13 and 20 dpi. n = sample size. Within rows, values followed by the same letter do not differ significantly ($P < 0.05$).

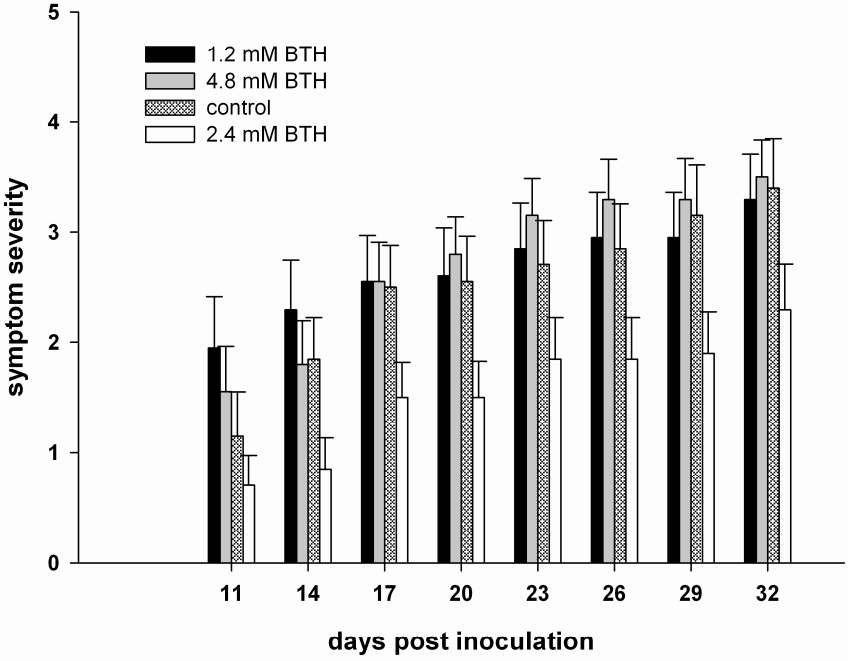
	CYP	**BTH + CYP**
dpi	**mean cells/ ng plant DNA ± SE (n)**	**mean cells/ ng plant DNA ± SE (n)**
6	$5.17 \times 10^3 \pm 1.05 \times 10^3$ (7) a	$2.43 \times 10^3 \pm 3.17 \times 10^2$ (7) b
11	$1.56 \times 10^5 \pm 2.5 \times 10^4$ (12) a	$8.45 \times 10^4 \pm 1.93 \times 10^4$ (10) b
13	$1.38 \times 10^5 \pm 2.53 \times 10^4$ (12) a	$6.88 \times 10^4 \pm 1.61 \times 10^4$ (11) b
20	$2.96 \times 10^5 \pm 4.29 \times 10^4$ (11) a	$2.02 \times 10^5 \pm 1.51 \times 10^4$ (9) a

Figures captions

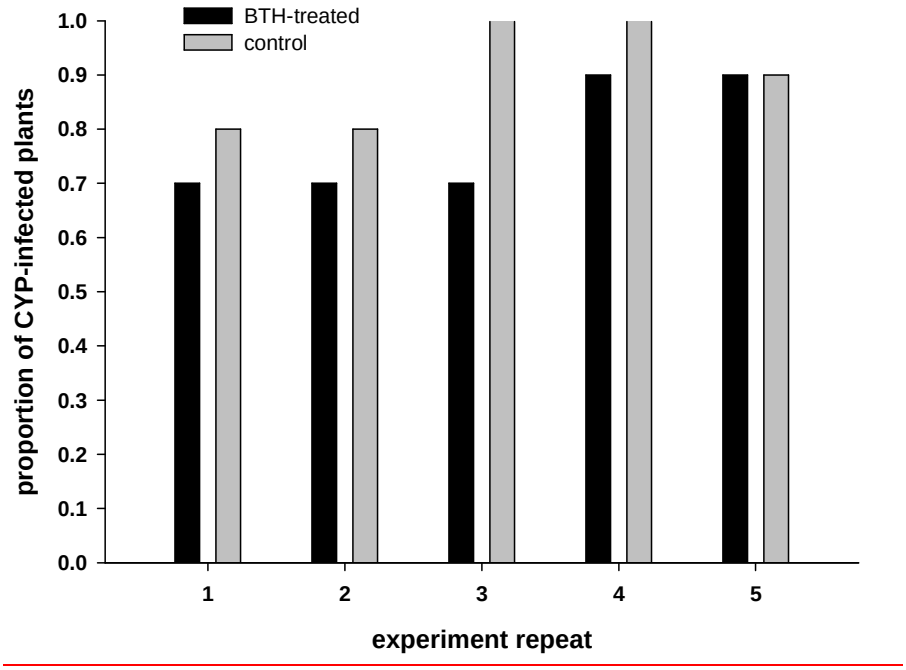
Figure 1. CYP symptom severity observed in the preliminary experiments in BTH-treated and control plants. Symptoms were observed from 11 until 32 dpi on 20 plants at each observation date.

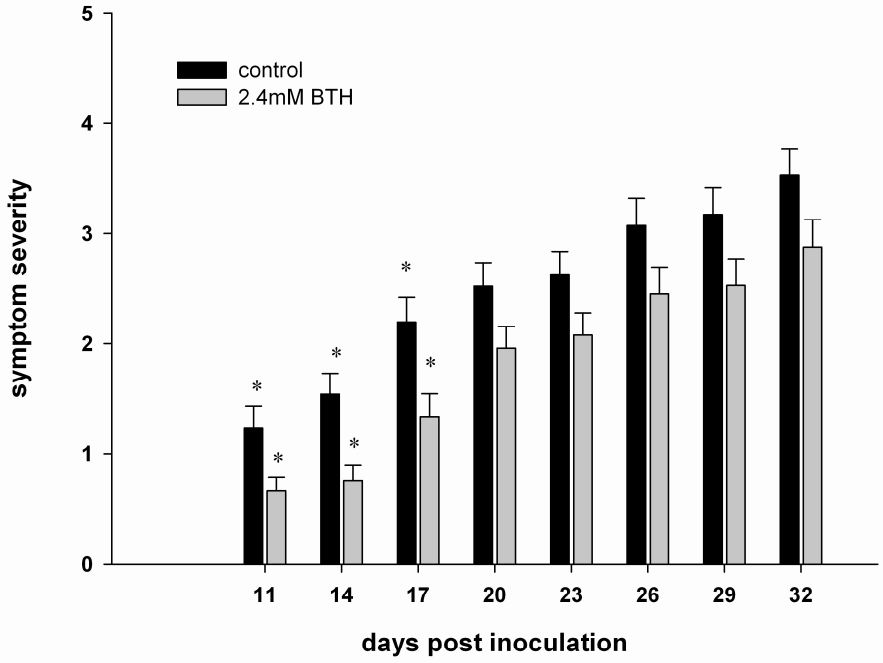
Figure 2. Proportion of CYP-infected daisy plants observed in the five experiment repeats on 2.4 mM BTH-treated and control plants.

Figure 3. CYP symptom severity recorded on 2.4 mM BTH-treated and control plants observed from 11 until 32 dpi. Asterisks indicate significant differences between the two plant treatments according to the *t* test. Fifty plants were observed at each dpi**.**


1.2 mM BTH
4.8 mM BTH
control
2.4 mM BTH
symptom severity
0
1
2
3
4
5
11
14
17
20
23
26
29
32
days post inoculation

460
461


BTH-treated
control
proportion of CYP-infected plants
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
experiment repeat


control
2.4mM BTH
symptom severity
days post inoculation
0
1
2
3
4
5
11
14
17
20
23
26
29
32
*

464