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Increasing control reliability of *Orobanche cumana* through integration of a biocontrol agent with a resistance-inducing chemical

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Abstract

Fusarium oxysporum Schlecht. f.sp. *orthoceras* (Appel & Wollenw.) Bilai is a potential biocontrol agent against the root-parasitic weed *Orobanche cumana*. In pot experiments with different sunflower cultivars, the application of *F. oxysporum* was combined with a treatment of BTH (Benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester), a product known to induce resistance against *O. cumana* in sunflower. The combined treatments resulted in highly reliable control and sometimes in increased control level compared to the *Fusarium* treatment alone. In laboratory experiments, no enhancing effect of BTH on virulence and growth of the fungus was observed. Future experiments should further investigate the basic mechanisms of the effect achieved by combining the two control strategies as well as the transfer of this innovative control approach into field experiments.

Introduction

Chlorophyll-lacking root parasitic weeds of the genus *Orobanche* (broomrapes) penetrate their hosts in order to withdraw water, assimilates and inorganic nutrients. A high infestation level can cause total yield loss (Sauerborn, 1991). Sunflower broomrape (*Orobanche cumana*) is a major constraint to sunflower production in the Mediterranean region and southeast Europe (Parker, 1994). Because of the complex relationship between parasite and host, broomrape control is difficult and only partially effective. Therefore farmers often take heavily infested fields out of production with negative consequences for a sound crop rotation and income.

Fusarium oxysporum f. sp. *orthoceras* (FOO) was identified as a host-specific and effective biocontrol agent against *O. cumana* and proved its ability to control the parasite even under field conditions through pre-planting application of the fungal inoculum harvested from organic substrate fermentation (Bedi and Donchev, 1991; Bedi, 1994; Thomas et al., 1998, 1999). Mass production systems and granular formulation techniques have been developed to improve the applicability and storability of the fungus as a bioherbicide (Shabana et al., 2003). Chlamydospores of the fungus can be obtained in a one-step liquid fermentation process using inexpensive natural by-products as a substrate (Müller-Stöver et al., 2002). Different granular formulations were tested under greenhouse conditions (Müller-Stöver et al., 2004). The soil application of wheat-kaolin granules ('Pesta') gave satisfying control of *O. cumana* in the greenhouse, reducing parasite shoot emergence by up to 80% compared to the untreated control. A high proportion of the emerged shoots showed disease symptoms, which leads additionally to a reduced seed production. However, in a field experiment

carried out in Israel, the influence on *Orobanche* emergence and the level of disease was lower compared to the pot experiments and the soil population of the fungus decreased to less than 10% of the initial numbers within two months (Müller-Stöver, unpublished). Thus, the major research aims were to enhance the efficacy of biological control and to make it more reliable under different environmental circumstances. One possibility might be the integration of biological control with other control methods, such as the induction of systemic acquired resistance. BION® (Syngenta, Basel, Switzerland) with its active component benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) is a commercial available resistance inducing product that demonstrated its capability of triggering resistance mechanisms in dicotyledonous and monocotyledonous plants against bacterial, fungal and viral pathogens (Oostendorp et al., 2001). In recent studies Sauerbron et al. (2002) succeeded in enhancing resistance of otherwise susceptible sunflower to infection with *O. cumana* by the use of BTH. The objective of this study was the investigation of the combination of biological control and BTH on the control of *O. cumana*.

Materials and methods

Fungal isolate

An isolate of *F. oxysporum* f.sp. *orthoceras* was received from Dr. J.S. Bedi (Punjab Agricultural University, Ludhiana, India) in 1995 (Thomas et al., 1998). Stock cultures were maintained on special nutrient-poor Agar (SNA, Nirenberg, 1976). For long-term preservation, the isolate was stored on SNA amended with 5% (v/v) glycerol at −80 °C at the Institute for Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Germany.

Inoculum production

Microconidia to be used in formulations in pot experiments were grown in liquid culture using 250-ml Erlenmeyer flasks containing 100 ml autoclaved Potato Dextrose Broth (PDB, Sigma, Taufkirchen, Germany). One agar plug (1 cm diameter) from a fungal culture on SNA was used to inoculate each flask. The flasks were closed with a cotton plug and aluminium foil and incubated on a rotary shaker (150 rpm) at ambient laboratory conditions (20 ± 3 °C) for 5 days. The content of the flasks was homogenised for 5 s in a blender and mycelial fragments were separated by filtration through four layers of cheesecloth.

Preparation of wheat flour-kaolin ('Pesta') granules

Wheat flour-kaolin granules were prepared after the method of Connick et al. (1991). Thirty-two grams of durum wheat flour, 6 g Kaolin and 2 g sucrose were blended and poured into a dish. Microconidial solution (23 ml) was added and the mixture was kneaded with gloved hands and passed through a pasta maker. The resulting dough sheets (1 to 1.5 mm thick) were air-dried on aluminium foil and the dry sheets were milled in a Waring blender. The milled material was sieved to a particle size of 250 μm to 2 mm. The number of cfu g^{-1} were assayed in the formulated material by giving 0.1 g of the preparation with 10 ml sterile H_2O in a test tube. The sample was vortexed from time to time together with 3 glass beads (0.6 cm diameter) until disintegrated. Aliquots (100 μl) of appropriate dilutions were plated on half-strength PDA amended with 100 ppm chloramphenicol (three plates per each of three samples) and cfu g^{-1} formulation were determined after incubation for 3 days at room temperature (20 ± 3 °C). The formulated material was stored in the refrigerator (4 °C) in small plastic bags. Granules were newly produced for every bioassay.

General aspects of plant production

Orobanche cumana (seeds collected in Turkey 1999) and sunflower were grown in a greenhouse at a temperature regime of 25/15 °C (day/night) with supplemental light provided by HQLR-lamps (1000 W) for 13 h. Plastic pots (13 × 13 × 13 cm) were filled with a loamy soil which had been steamed at 80 °C for 4 h up to two-thirds of the pot depth. Approximately 7500 *O. cumana* seeds per pot were sprinkled onto the soil surface and mixed into the top 5 cm. The granular formulation containing the biocontrol agent was incorporated into the soil along with the *O. cumana* seeds. All pots were then filled up with more soil. Three sunflower seeds were sown

in each pot. The experiments were arranged in a completely randomized design with five replicates per treatment. Fourteen days after sowing, each pot was thinned to contain only one sunflower plant and fertilized with 20 ml of a 2% (v/v) Wuxal® solution weekly.

Two pot experiments were performed:

1. *Orobanche cumana* control on sunflower cv. Iregi by FOO, BTH and the combination of both.

The pots were prepared as described above. The experiment comprised a fungal treatment, a BTH treatment and a treatment that combined both control strategies. For the fungal treatments, 1 g of 'Pesta' formulation (approximately 3×10^6 cfu g^{-1}) per pot were incorporated pre-planting into the soil. BTH was first applied as a soil drench at sunflower emergence at a rate of 7.1 μmol per pot. Additional treatments followed every 2 weeks until the end of the experiment. Pots containing *O. cumana* seeds without fungal formulation served as negative control (C−). Pots containing neither fungal formulation nor *O. cumana* seeds were set as a positive control (C+). The experiment was repeated with a fresh 'Pesta' formulation containing approximately 1×10^5 cfu g^{-1}.

2. *Orobanche cumana* control on sunflower cultivar HA89 by FOO, two concentrations of BTH and the combination of both.

The experiment was set with sunflower cultivar HA89 and a 'Pesta' formulation of FOO containing 1×10^6 cfu g^{-1}. Treatments were the same as in the first experiment, except that two concentrations of BTH (2.4 and 1.2 μmol per pot, as HA89 was known to be sensitive to BTH applications from preliminary experiments) were tested.

Parameters measured

Experiments finished 3 weeks after the first emergence of *Orobanche* shoots (which was observed approximately 8 weeks after sowing). Emerged shoots were counted in each pot. Soil was washed from sunflower roots and the total number and dry weight of parasite shoots and the aboveground dry weight of the host plant (in the flowering stage) was determined.

Influence of BTH on growth and sporulation of FOO in growth medium

Growth of FOO was determined on PDA (Potato Dextrose Agar, Sigma, Germany) plates containing BTH at 0, 3.6 and 5.9 μmol l^{-1} and in a second experiment 71.3 and 214.0 μmol l^{-1} with 5 replications per medium type in a fully randomised experimental design. BTH was added to the agar prior to autoclaving. The thermal stability of BTH had been proved by quantitative HPLC before and after autoclaving (unpublished data). Inoculum plugs (1 cm diameter) were aseptically transferred from fungal cultures on SNA to the centre of each test plate. The inoculated plates were sealed with Parafilm and arranged randomly in a growth chamber with 12 h/12 h light/dark and constant 25 °C. Every 24 h, two colony diameters per plate were measured.

Sporulation of FOO was measured under ambient laboratory conditions (20 ± 3 °C) in 100 ml Malt extract medium (20 ml Biomalt, Kirn, Germany, in 1 l deionized H_2O). Treatments with BTH at 0, 5.9 and 142.7 μmol l^{-1} in the growth medium were prepared with three replicates per treatment and incubated on a rotary shaker at 150 rpm. Forty-eight, 72 and 96 h after inoculation, the content of one Erlenmeyer flask (three per treatment) was blended in a Waring blender and mycelium was separated from conidia by filtration through four layers of cheesecloth. Conidial densities in the resulting solution were determined using a hemacytometer. The experiments were conducted twice.

Influence of the application of BTH on fungal growth in soil

The same soil that had been used for the pot experiments was sieved to pass a 5 cm mesh screen. Petri dishes with 42.5 g dry soil each were prepared. Treatments were: control with no additives, a fungal treatment (FOO) with 20 mg 'Pesta' granules per Petri dish, a BTH treatment (0.001 μmol g^{-1} of dry soil at the indicated application dates) and a combined treatment (FOO + BTH) with 20 mg 'Pesta' granules per dish and 0.001 μg of BTH per g of dry soil at the indicated application dates. 'Pesta' granules with a concentration of approximately 1.6×10^6 cfu g^{-1} (containing microconidia produced in liquid Malt extract medium) were carefully mixed with the soil

at the beginning of the experiment and the water content was set to 50% of the maximum water capacity of the soil. To prevent evaporation, the Petri dishes were sealed with parafilm and incubated at constant 25 °C in the dark. After 1 week of incubation, the content of each dish was thoroughly mixed and two samples of 0.5 g were taken out of each Petri dish. Each sample was put in a test tube and 10 ml of water were added. The content of the tube was mixed for 30 s using a vortex and was let sit approximately 30 min. Afterwards, the content was mixed again for another 30 s and proper dilutions were prepared of which two samples of 0.2 ml were taken and spread over plates with PDA–PCNB agar with chloramphenicol (Fauzi and Paulitz, 1994). The agar plates were incubated at room temperature and cfu counts were taken after 4 days. Moisture content of the petri dishes containing soil was checked and water was added when necessary. An aqueous solution of BTH was added to the treatments BTH and FOO + BTH to give a final concentration of 0.001 μmol g^{-1} of dry soil. The samples were incubated under the same conditions as before and population counts were repeated 2 weeks after the BTH application. One more BTH application was performed followed by another 2 weeks incubation period until the experiment was finished. The experiment was set with four replicates per treatment and repeated with a fresh 'Pesta' formulation containing approximately 1.4×10^6 cfu g^{-1}. Data were corrected for percent soil moisture and for background soil population densities of *Fusarium* spp. recorded in the untreated control and in the BTH treatment before analysis.

Influence of BTH on the virulence of FOO

The influence of BTH in the growth medium on the subsequent virulence of the fungus was tested in root chambers (Linke et al., 2001). Surface-sterilized *O. cumana* seeds were sprinkled evenly onto a glass fibre paper strip moistened with H_2O that was fixed between the clear Plexiglas front of a root chamber and the hollow space filled with autoclaved soil. The root chambers were wrapped into black plastic and left for pre-conditioning for 7 days at ambient laboratory conditions. A pre-germinated sunflower seed was placed in each chamber between the filter paper and the lid and the chambers were placed in the growth chamber at an angle of 30° to force the host roots to grow along the surface of the lid. When *Orobanche* tubercles had developed, the Plexiglas cover was removed from the chambers and 10 ml of a conidia suspension diluted with deionized H_2O to contain 1×10^5 conidia ml^{-1} were pipetted onto the host roots on the filter paper strip. Conidia had been produced in 100 ml of a Malt extract medium with 0 or 142.7 μmol BTH l^{-1}, respectively, as described above, and had been harvested after 5 days of incubation. The control chambers were treated with 10 ml of Malt extract medium only. Afterwards, the chambers were closed and wrapped in black plastic again. Fourteen days after inoculation, the percentage of dead (brownish) tubercles was determined under the binocular. The experiment was set with four replicates per treatment and repeated.

Statistical analysis

Statistical analysis was conducted to the combined data of repeated experiments when they had homogenous variances. Comparison of two means was performed using Fisher's *t*-test. Multiple mean comparisons consisted of analysis of variance (ANOVA) and Tukey's studentized range test (HSD). All tests of significance were conducted at $P \leq 0.05$. When data were not normally distributed or showed heterogeneity of variances they were square-root or log-transformed before analysis. Percentage data were arcsine-transformed before analysis (Gomez and Gomez, 1984).

Results

Pot experiments

Due to heterogeneity of variances, the repeated experiments were analysed separately. In the pot experiments carried out with sunflower cv. Iregi, the *Fusarium* treatments and the combined treatments with the fungal biocontrol agent and BTH performed best regarding the control of *O. cumana* (Figure 1). However, the combined treatments differed from the control and/or BTH treatment at a higher level of significance ($P \leq 0.01$) than the *Fusarium* treatment. In the first experiment, the

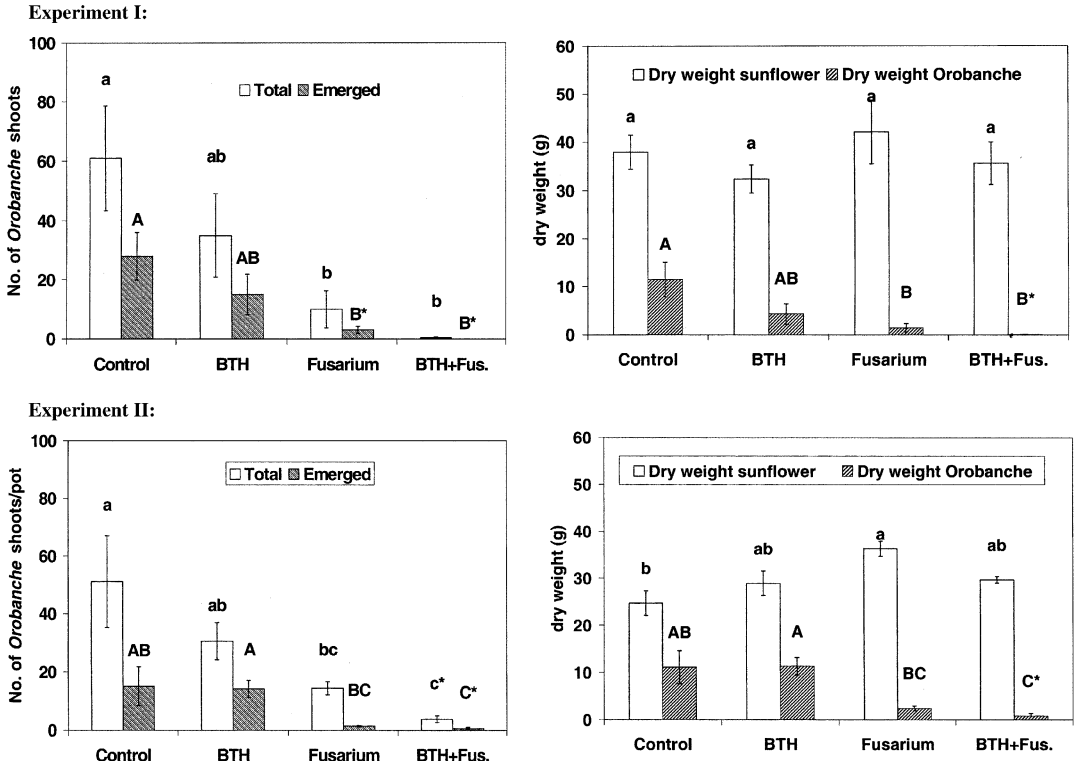

Figure 1. Influence of BTH, *Fusarium oxysporum* f.sp. *orthoceras* and a combined treatment on the number of total and emerged *Orobanche cumana* shoots and the *Orobanche* and sunflower (cv. Iregi) dry weight. Multiple mean comparisons were performed using Tukey's HSD test at $\alpha = 0.05$ level of significance. Bars with the same letter are not significantly different. Letters marked with '*' indicate a statistical difference from the treatment labelled with 'a/A' at the $\alpha = 0.01$ level of significance. Vertical lines indicate SEs.

combined treatment resulted in a complete suppression of emergence, whereas in the second experiment reduction of emergence was 96%. Also the total number of *O. cumana* shoots (99% and 93%, respectively) and the *Orobanche* dry weight (99% and 93%, respectively) were markedly reduced in the combined treatment in both experiments. The BTH treatment alone did not result in control of *O. cumana*. Sunflower dry weights were not significantly affected by the BTH treatments, even compared to the control (C+) (sunflower alone, data not shown).

In the experiment with sunflower cv. HA 89, the combined treatments generally gave the best results regarding *O. cumana* control. *Orobanche* emergence was significantly reduced by the *Fusarium* treatment and the combined treatments compared to the control (Figure 2). As in the first experiments, the reduction resulting from the combined treatments was statistically significant at $P \leq 0.01$. The total number of *Orobanche* shoots was significantly reduced compared to the control only in the combined treatments. A significantly lower *Orobanche* dry matter was recorded in the combined treatment with the higher dosage of BTH. No statistically significant differences regarding sunflower dry weight occurred.

Laboratory experiments

Influence of BTH on growth and sporulation of FOO. Due to heterogeneity of variances, the experiments with 3.5 and 5.9 µmol BTH per l growth medium were analysed separately. After adding the lower dosages of BTH, in the first experiment a reduction of growth could be observed after 48 h with 5.9 µmol BTH (Table 1). In the second experiment, the fungus showed a slightly reduced growth in both BTH treatments after 48 and 72 h. All other sampling dates did not show any differences in fungal growth between the

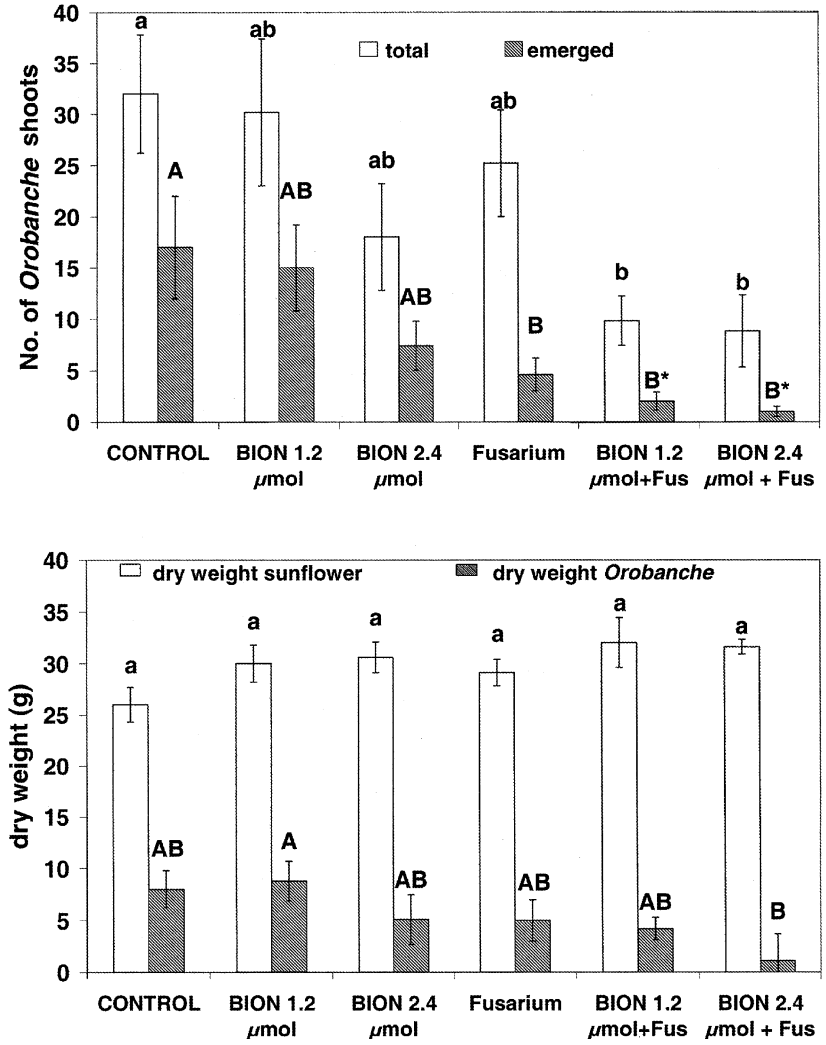

Figure 2. Influence of BTH in two concentrations, *Fusarium oxysporum* f.sp. *orthoceras* and combined treatments on the number of total and emerged *Orobanche cumana* shoots and the *Orobanche* and sunflower (cv. HA89) dry weight. Multiple mean comparisons were performed using Tukey's HSD test at $\alpha = 0.05$ level of significance. Bars with the same letter are not significantly different. Letters marked with '*' indicate a statistical difference from the treatment labelled with 'a/A' at the $\alpha = 0.01$ level of significance. Vertical lines indicate SEs.

tested media. Adding 71.3 or 214 µmol BTH to the growth medium did not cause a reduction of fungal growth at the first sampling date, but resulted in reduced colony diameters up to 5 days after inoculation with only insignificant differences between the two dosages (Table 2).

Sporulation in Malt extract medium was significantly reduced after the addition of both 5.9 and 142.7 µmol BTH l^{-1} at the first sampling date (48 h after inoculation) by 29% and 85%, respectively. After 72 and 96 h, the differences were not statistically significant anymore.

Influence of the application of BTH on fungal growth in soil

Results of one experiment are presented because the repetition had similar results. The fungal population in soil was not significantly affected by the application of BTH at any sampling date (Figure 3).

Influence of BTH on the virulence of FOO

The addition of BTH to the fungal growth medium had no obvious influence on the control effi-

Table 1. Influence of 3.6 and 5.9 μmol BTH l^{-1} in the growth medium on the colony diameter of *Fusarium oxysporum* f.sp. *orthoceras* on Potato Dextrose Agar (PDA) plates

Experiments	24 h	48 h	72 h	96 h	120 h
Experiment 1					
PDA	1.4 (0.03) a	3.0 (0.02) a	4.1 (0.02) a	5.6 (0.04) a	6.8 (0.07) a
BTH 3.6 μmol l^{-1}	1.4 (0) a	3.0 (0.02) a	4.1 (0.04) a	5.7 (0.02) a	6.8 (0.03) a
BTH 5.9 μmol l^{-1}	1.4 (0) a	2.8 (0.05) b	4.1 (0.02) a	5.7 (0.04) a	6.9 (0.15) a
Experiment 2					
PDA	1.7 (0.03) a	3.2 (0.02) a	4.5 (0.02) a	5.9 (0.06) a	7.2 (0.14) a
BTH 3.6 μmol l^{-1}	1.6 (0.04) a	3.1 (0) b*	4.4 (0.03) b	5.7 (0.05) a	7.1 (0.11) a
BTH 5.9 μmol l^{-1}	1.6 (0.04) a	3.1 (0.02) b*	4.3 (0.04) c*	5.7 (0.07) a	7.1 (0.09) a

Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Letters marked with '*' indicate a statistical difference compared to the PDA control at the $\alpha = 0.01$ level of significance. Values in parentheses are SEs.

cacy of FOO on *O. cumana* tubercles in root chambers (Figure 4).

Discussion

The greenhouse experiments showed that combining the biocontrol agent with the use of BTH resulted in a more reliable *Orobanche* control than the use of a single control agent. This is especially important considering the variable control levels achieved by the biocontrol agent and the application of BTH alone. Drenched BTH alone did generally not result in control of *O. cumana* in the performed pot experiments. However, effective dosages of BTH are different for each sunflower cultivar (Buschmann, unpublished). The excellent control level in the combined treatments resulted in a lower number and dry weight of *Orobanche* shoots, indicating that the combination of control strategies takes effect already in the early developmental stages of *Orobanche*. This could be either due to an enhanced activity of the fungus against the early underground stages of the parasite (seeds, germination, attachments and tubercles) or to an enhanced induced resistance within the host plant sunflower. In laboratory experiments, we investigated possible direct effects of the resistance inducing chemical on growth and virulence of FOO. While the growth in soil seems to stay generally unaffected by the tested dosage of BTH compared to the treatments containing the fungus only, growth on synthetic media was slightly adversely influenced by the incorporation of BTH. Ishii et al. (1999) got similar results testing the influence of different concentrations of BTH in PDA and Czapek agar plates on various fungi including *F. oxysporum* f.sp. *cucumerinum*. For all tested isolates (except *Didymella bryoniae*), the authors calculated an EC 50 higher than 100 ppm. Further, they did not observe any activity on conidial germination and germ tube growth of some investigated isolates. Only *Cladosporium cucumerinum* showed an increased germ tube growth in the presence of BTH which was not investigated in our own experiments, but might be

Table 2. Influence of 71.3 and 214 μmol BTH l^{-1} in the growth medium on the colony diameter of *Fusarium oxysporum* f.sp. *orthoceras* on Potato Dextrose Agar (PDA) plates

	24 h	48 h	72 h	96 h	120 h	144 h
PDA	1.2 (0.02) a	2.8 (0.04) a	4.1 (0.3) a	5.4 (0.05) a	6.4 (0.08) a	7.4 (0.08) a
BTH 71.3 μmol l^{-1}	1.2 (0.02) a	2.6 (0.03) b*	3.8 (0.7) b*	5.0 (0.05) b*	6.0 (0.05) b*	7.0 (0.07) b*
BTH 214 μmol l^{-1}	1.2 (0.01) a	2.5 (0.02) b*	3.7 (0.3) b*	4.9 (0.07) b*	5.9 (0.07) b*	6.9 (0.1) b*

Values are the means of two experiments. Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Letters marked with '*' indicate a statistical difference compared to the PDA control at the $\alpha = 0.01$ level of significance. Values in parentheses are SEs.

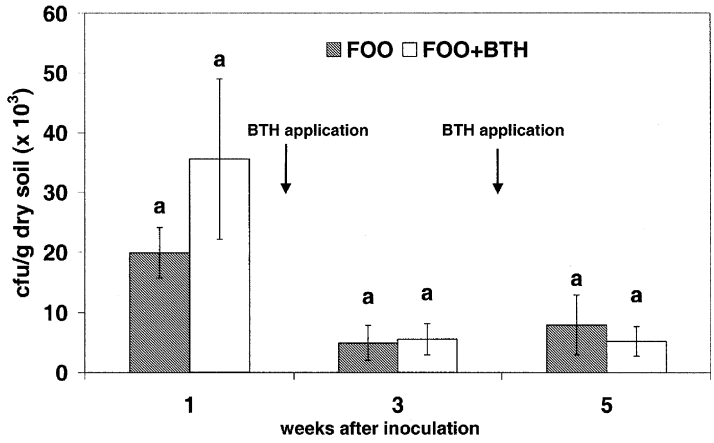

Figure 3. Influence of BTH on *Fusarium oxysporum* f.sp. *orthoceras* populations in soil. Background soil population densities of *Fusarium* spp. recorded in the untreated control and in the BTH treatment have been subtracted from the respective treatment before analysis. Comparison of means was performed using Fisher's *t*-test at $\alpha = 0.05$ level of significance. Bars with the same letter are not significantly different. Vertical lines indicate SEs.

an incidence for a possible enhancing effect of BTH on fungal growth. In our experiments, sporulation in liquid medium was significantly reduced 2 days after inoculation when FOO was grown in Malt extract medium supplemented with BTH. After this sampling date, no significant differences in the number of conidia produced were observed any more. Thus, an increase of fungal population through the addition of BTH seems unlikely. Furthermore, experiments on the possible enhancement of fungal virulence through BTH have been carried out. However, in root chamber experiments, conidia that had been produced in BTH-amended media showed no enhanced virulence against *O. cumana* tubercles.

If there are no direct effects of BTH on the performance of the fungus, another possible interaction between the two control agents might be the increase of some pre-induced resistance mechanisms against *O. cumana* parasitism in sunflower. The mechanisms by which BTH induces resistance to *O. cumana* in sunflower are not yet well known, however, Sauerborn et al. (2002) reported the synthesis of scopoletin and hydrogen peroxide in BTH-treated sunflower roots. They demonstrated also an accumulation of chitinase, a

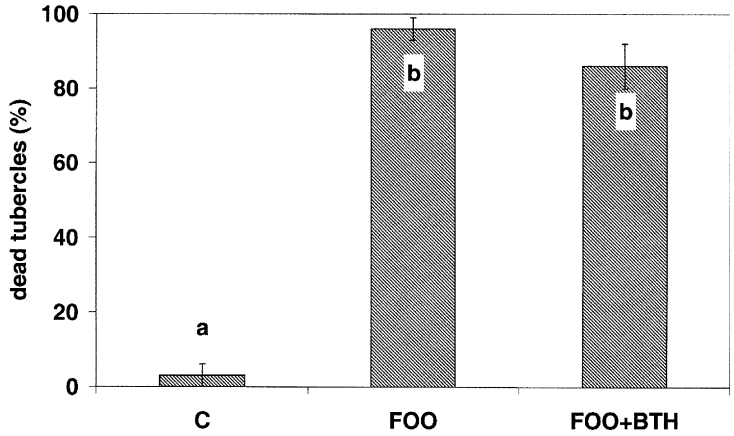

Figure 4. Influence of 142.7 μmol BTH l^{-1} in the fungal growth medium (=FOO+BTH) on the virulence *of Fusarium oxysporum* f.sp. *orthoceras*. Conidial suspensions were applied on *Orobanche* tubercles at a rate of 1×10^5 ml^{-1}. C = control treated with Malt extract medium only. Values are the means of two experiments. Multiple mean comparisons were performed using Tukey's HSD test at $\alpha = 0.05$ level of significance. Bars with the same letter are not significantly different. Vertical lines indicate SEs.

widely used SAR marker in dicotyledonous plants, in roots and stems of induced resistant sunflowers. Several authors observed the ability of non-pathogenic *Fusarium* strains to induce an increased accumulation of enzymes such as chitinase, beta 1-3 glucanase, beta 1-4 glucosidase or peroxidase (Fuchs et al., 1997; Duijff et al., 1998; Cachinero et al., 2002). Cachinero et al. (2002) observed an increase of the phytoalexins maackiain and medicarpin in chickpea (*Cicer arietinum*.) seedlings inoculated with nonhost isolates of *F. oxysporum* that did not accumulate in plant tissues but were released into the inoculum suspension. Benhamou and Garand (2001) demonstrated that pea (*Pisum sativum*.) root inoculation with the non-pathogenic *Fusarium* strain Fo47 triggered a set of plant defence reactions that resulted in the elaboration of permeability barriers. Since there are similarities between fungi and parasitic plants at the very early stage of the infection process, the same array of defence mechanisms might play a role in preventing parasitism (Perez-de-Luque et al., 2004). However, Yu and Muehlbauer (2001) observed the induction of different pathways of defence by BTH and *Fusarium* in wheat. If this could be proven also for sunflower plants exposed to BTH and FOO, it might be an explanation for the effect of the combined treatment against the root parasite. This could be an objective for future investigations, together with the optimisation of the dosage and application mode and time of both control methods to get a maximum control efficacy for practical use.

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