

DOES BENZOTHIADIAZOLE-INDUCED RESISTANCE INCREASE FITNESS COST IN BEAN?

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SUMMARY

The fitness cost of benzothiadiazole (BTH)-induced resistance in bean has been investigated both in terms of seed quality and production, and by examining some physiological parameters, such as photosynthesis and ethylene induction and emission. Although BTH treatment induced 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene transcription, no ethylene emission was detected, unless leaves were detached, thus stimulating senescence. Efficiency of photosystem II was not affected by BTH treatment, nor was seed quality, the electrophoretic patterns of seed proteins extracted from BTH-treated and untreated plant being very similar. Only seed production was slightly lower in BTH-treated plants, due to the lower number of pods per plant; however this difference was not significant. All these data, taken together, indicate that BTH-induced resistance in bean does not incur appreciable fitness costs. Moreover, ACC oxidase gene expression, without ethylene emission, suggests a sort of "ethylene priming" induced by BTH. Thus, BTH and ethylene signalling pathways seem to act synergistically in the establishment of systemic acquired resistance.

Key words: BTH, SAR, ACC oxidase, ethylene.

INTRODUCTION

An eucariotic multicellular organism has to increase the level of and possibly reallocate its own metabolic resources in order to defend itself. Resistance, according to Agrios (1988), is the host ability to exclude or overcome, completely or in some degree, the adverse effect of a pathogen. The metabolic changes leading to resistance have an associated fitness cost, which could outweigh benefit. Nevertheless, pathogens can reduce host fitness by reducing its survival and reproduction, that is, decreasing its genetic contribution to the next generation (Purrington, 2000; Heil, 2002).

In resistant plant-pathogen interactions, a cascade of events, from elicitor recognition to signal transduction, activates inducible plant defences at local and systemic level (Dangl and Jones, 2001). These include oxidative burst, hypersensitive cell death, messenger transcription, and protein synthesis, folding, secretion and delivery (Baker and Orlandi, 1995; Hadlington and Denecke, 2000; Heath, 2000). Different molecular signals, such as salicylic acid (SA), nitric oxide (NO), reactive oxygen species (ROS), jasmonic acid (JA) and ethylene cooperate to orchestrate defence responses at cellular level, as well as the programmed cell death (PCD) pathway (Dong, 1998; Raymond and Farmer, 1998; Delledonne *et al.*, 1998; Jabs, 1999; Wang *et al.*, 2002).

Ethylene controls several stages of growth and development in plants: embryogenesis, germination, differentiation and organogenesis, photosynthesis, senescence, leaf abscission and fruit maturation (Johnson and Ecker, 1998; Lund *et al.*, 1998). Ethylene synthesis is strongly stimulated by both biotic and abiotic stresses, such as pathogens, phytophagous, insects, mechanical wounding, water deficiency, extreme temperature and some chemicals (reviewed in Morgan and Drew, 1997). The biosynthesis of ethylene takes place in three steps. In the first, methionine is transformed (adenosylated), by the action of ATP, to S-adenosylmethionine which, under the influence of the key enzyme ACC synthase (second step), leads to 1-aminocyclopropane-1-carboxylic acid (ACC). In a third step, ACC is decomposed to ethylene under the action of a second key enzyme, ACC oxidase. The expression of ACC oxidase genes is then usually investigated to assess the activation of ethylene biosynthesis (Wang *et al.*, 2002).

Recently, plant activators, a novel class of either natural or synthetic compounds, have been employed in crop protection (Hammerschmidt, 1999; Gozzo, 2003). They induce a long-lasting and broad-spectrum systemic acquired resistance (SAR), priming the host against pathogen challenges (Ryals *et al.*, 1996). Resistance inducers, such as benzothiadiazole (BTH) and isonicotinic acid (INA), enhance phenylalanine ammonia lyase (PAL) synthesis, leading to production of phytoalexins and pathogenesis-related (PR) proteins, the molecular markers of SAR (Görlach *et al.*, 1996; Lawton *et al.*, 1996). However, BTH induction of SAR has been negatively correlated to the crop yield, at least in wheat and cauliflower, because of the above mentioned fitness cost (Stadnik and Buchenauer, 1998; Godard *et al.*, 1999; Heil *et al.*, 2000).

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In the present study we have further investigated the important aspect of fitness cost associated with BTH-activated SAR in bean plants. We previously demonstrated that BTH induces in this plant species a hydrogen peroxide (H_2O_2) burst and SAR onset in a cell death-independent manner (Iriti and Faoro, 2003). As oxidative stress stimulates ethylene biosynthesis (Wang *et al.*, 2002) and in turn also influences plant fitness, we have examined, besides photosynthetic efficiency and seed production, the activation of the ACC oxidase gene and ethylene emission of BTH-treated plants.

MATERIALS AND METHODS

Plant material and treatments. For all experiments, seeds of *Phaseolus vulgaris*, cv. Borlotto nano lingua di fuoco (BLF), were sown in 12 cm pots in soil improved with 30% of a blend of white and frozen through black sphagnum peat, and grown in a greenhouse at $24\pm 2^\circ C$, RH $60\pm 5\%$, 16 h/8 h light/dark period. Ten to twelve days after sowing, when the primary leaves were almost completely expanded, plants were sprayed with a water suspension of BTH [Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester] (trade name Bion®, Syngenta, CH) at a concentration of 0.3 mM, prepared from a wettable formulation containing 50% (w/w) active ingredient (a.i.). BTH was used at the above concentration, corresponding to 0.14 mg ml^{-1} of a.i., because a single spray of this suspension was found to be sufficient to fully protect bean plants from the rust fungus *Uromyces appendiculatus* for at least a month (Iriti and Faoro, 2003). Control plants were sprayed with water containing the wettable powder alone.

Effect of BTH on seed production. In June 2001 and 2002, 500 BLF seeds were planted singly in 20 cm pots using the same soil mixture as above, in an insect-proof screen house located in a field. When the primary leaves were fully expanded, 400 homogeneous plants were selected, and half sprayed with 0.3 mM BTH while the others were sprayed with the wettable powder alone. Treated and control plants were arranged in a randomised block. Seeds were collected from plants as soon as pods desiccated, starting from the end of July to the middle of August, thus avoiding seeds from the second flowering. Seeds were completely dried in desiccators at $35^\circ C$ for 2 days. The following traits were statistically analysed (MSTAT-C, Michigan State University, USA): grain yield, number of pods/plant, number of seeds/pod and 1000 seed weight.

In all experiments, the effective establishment of SAR, following BTH treatment, was assessed on ten plants by verifying their complete protection against *U. appendiculatus* infection (Iriti and Faoro, 2003).

Seed protein analysis. Twelve seeds randomly collected from the whole seed production of either treated or control plants were frozen in liquid nitrogen, ground with a mortar and pestle and extracted in sodium phosphate buffer (PBS). The supernatant obtained by centrifugation was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 15% acrylamide gel run at 100 mV until the loading dye ran off. After staining with Coomassie Brilliant Blue, the gels were fixed with water/methanol/acetic acid (80/10/10 v/v/v).

Leaf chlorophyll *a* fluorescence analysis. *In vivo* chlorophyll *a* fluorescence of photosystem II (PSII) was excited and detected with a pulse amplitude modulation fluorimeter (PAM-2000, Heinz Walz, Effeltrich, Germany), on the adaxial surface of interveinal regions of the two youngest fully expanded leaves, from 50 plants, 7 days after BTH treatment, and from 50 control plants. Before the beginning of each measurement, leaves were dark-adapted for 40 min (Björkman and Demmig, 1987). The maximum quantum yield (Φ_{PSII}) was assessed as $(F_m - F_0)/F_m = F_v/F_m - F_0$, defined as dark-adapted minimal fluorescence, is the initial level of the chlorophyll *a* fluorescence when all PSII reaction centres are open, and was measured under low ambient background light. F_m is the dark-adapted maximal fluorescence when all PSII reaction centres are closed, and was measured by applying a saturating light pulse (5 s duration). Finally, F_v is the result of $F_m - F_0$ and represents the variable fluorescence in dark-adapted leaves.

ACC oxidase mRNA analysis. Total RNA was extracted from 1 g of leaves of either treated or untreated plants at different times after BTH application, using Trizol Reagent (Gibco, BRL) according to the manufacturer's specifications. RNA (10 μ g per lane) was size-fractionated by electrophoresis on 1% formaldehyde agarose gel and blotted to a nitrocellulose membrane, using the standard technique (Sambrook *et al.*, 1989). The membranes were prehybridised at $68^\circ C$ in a high SDS hybridisation buffer, then hybridised, in the same solution, with the addition of the radioactively-labelled [^{32}P]dCTP ACC oxidase probe. After hybridisation, membranes were washed twice in 2 x SSC and 0.1% SDS at room temperature for 15 min, and twice in 0.5 x SSC and 0.1% SDS at $68^\circ C$ for 15 min.

Table 1. Seed production (means \pm SD) of BTH-treated and untreated bean plants. Data refer to two growing seasons (2001 and 2002) and to a total of 200 plants per trial; no significant differences were found after analysis of variance in any of the parameters reported.

Treatment	Grain yield/plant (g)	N. of seeds/pod	N. of pods/plant	1000 seed dry weight (g)
None	4.34 \pm 1.08	2.97 \pm 0.84	4.10 \pm 1.47	433.26
BTH	3.95 \pm 1.78	2.97 \pm 0.88	3.73 \pm 1.53	393.37

Ethylene detection. Fully expanded primary leaves (approximately 1 g in fresh weight), from treated and untreated plants were detached and the cut surface of the petiole sealed with liquid paraffin. Leaves were then gently rolled and singly placed in 50 ml glass tubes tightly air-sealed with a silicone plug. At the indicated intervals, 1 ml gas samples were withdrawn with a syringe and injected into a gas chromatograph (GC) equipped with a flame ionisation detector for ethylene measurements. Two different experiments were carried out. In the first, ethylene was measured on leaves collected at different times (2, 6, 12, 24, 48 and 72 h) from treated plants and controls, to correlate ethylene production with ACC oxidase transcript accumulation. In another set of experiments, leaves from BTH-sprayed and control plants were detached 2 h after treatment when they were completely dry. They were placed in 50 ml tightly air-sealed glass tubes and kept in the greenhouse under the same conditions as bean plants, to evaluate long term gas emission. Ethylene was checked daily up to the tenth day, when leaves underwent rapid dehydration. All data were corrected for recovery by including internal controls.

RESULTS

Effects on plant growth and seed set. BTH-treated and untreated plants did not detectably differ in growth rate. Internode length and leaf expansion, randomly measured at 15, 30, 45 and 60 days after BTH application, showed no significant differences between treated and untreated plants, grown either in the greenhouse or in the field (data not shown). However, in the field experiments, in two different years, some differences in seed set were apparent (Table 1). Grain yield of BTH treated plants was slightly lower compared with controls (reduced number of pods per plant and lower seed dry weight), but analysis of variance showed that these differences were not significant.

Seed protein content. Total protein extracted from seed of treated and untreated bean plants, analysed by SDS-PAGE, showed no difference in their protein electrophoretic profiles (Fig. 1). All major bean seed proteins (in particular legumin, phaseolin, phytohemagglutinin and α -amylase inhibitors) were present in apparently similar amounts. Except for legumin, which is naturally unglycosylated, all the other glycosylated proteins were resolved in more than one band, corresponding to different aggregation forms of their monomers. These forms were present in a similar pattern in all samples (Fig. 1).

Photochemical efficiency of photosystem II (PSII). To assess whether BTH influenced the photochemical efficiency of photosynthesis, leaf chlorophyll fluorescence measurements were carried out when SAR was fully established, that is to say 7 days after treatment (Iriti and Faoro, 2003). The ratio of variable to maximal fluorescence (F_v/F_m), which reflects the maximal photochemical yield of PSII centres, is highly correlated with the quantum yield of net photosynthesis of treated and untreated leaves. All pa-

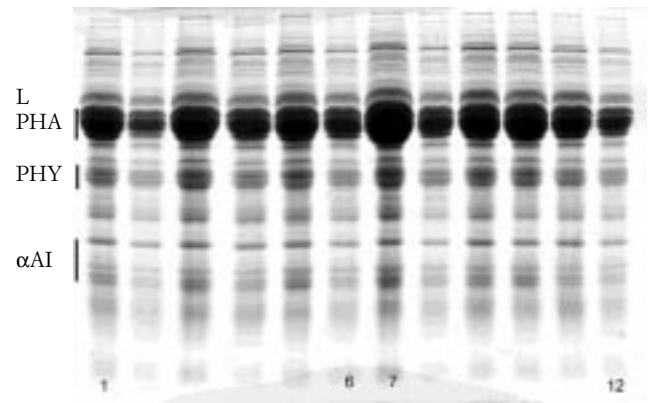


Fig. 1. SDS-PAGE of protein extracts from 12 randomly chosen bean seeds matured on BTH-treated (lane 1-6) and untreated (lane 7-12) plants; L = legumin; PHA = phaseolin; PHY = phytohemagglutinin; α AI = α -amylase inhibitors; different bands for the same protein correspond to different monomeric aggregation states.

rameters calculated from chlorophyll fluorescence measurements are reported in Table 2. The basal (F_0) and maximum (F_m) fluorescence were not affected by BTH treatment. Accordingly, no significant differences were detected in the ratio of F_v to F_m between treated and control plants.

Table 2. Chlorophyll fluorescence parameters. Measurements were carried out in fully expanded leaves of *Phaseolus vulgaris* dark-adapted 40 min. Mean values for $n=50$ are shown. Differences are not significant (Student's t -test. NS= $P>0.05$).

Treatment	F_0	F_m	F_v	F_v/F_0
None	0.588	4028	3353	0.850
BTH	0.585	3900	3314	0.849

ACC oxidase mRNA expression. Northern blot analysis showed that BTH-induced accumulation of ACC oxidase transcript occurred as early as 6 h after treatment. The level increased to a maximum at 12 h, then gradually decreased (Fig. 2). At 72 h after treatment, the ACC oxidase expression level was comparable to untreated controls (not shown).

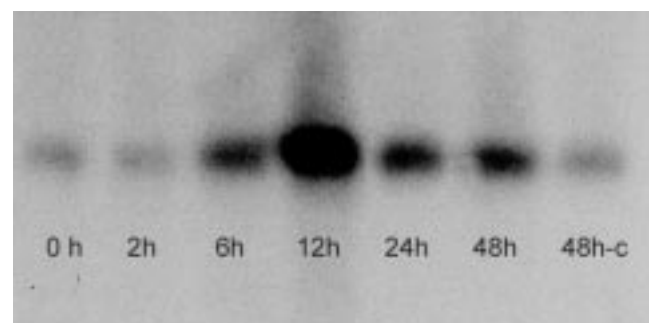


Fig. 2. Northern blot analysis of total RNA extracted from BTH-treated and untreated bean leaves at different times after treatment and probed with ACC oxidase. Time 0 refers to untreated control.

Leaf ethylene emission. No ethylene emission was detected from either BTH-treated or control leaves collected 2, 6, 12, 24, 48 and 72 h after treatment. However, when leaves were detached 2 h after treatment and kept in air-sealed vials, gas emission was detectable only from BTH-sprayed leaves, starting at the seventh day of treatment, with a peak at the ninth day (Fig. 3). At the same time, leaf yellowing occurred while untreated leaves remained green until desiccation. No ethylene emission was detected from control leaves, at any time.

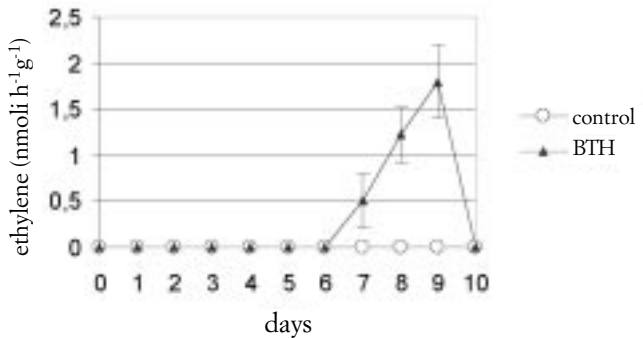


Fig. 3. Time course of ethylene production in BTH-treated and untreated leaves. Values represent the means of three replicates.

DISCUSSION

SAR induction by chemicals opens new and interesting prospects in crop protection (Gozzo, 2003), though it is generally assumed that the activation of resistance might divert energy and carbon resources from vital processes, possibly leading to less productive plants. Such a cost has been reported for BTH-activated resistance in wheat (Stadnik and Buchenauer, 1998; Heil *et al.* 2000) and cauliflower (Godard *et al.*, 1999). However, the present work shows no evidence of a measurable cost associated with bean treatments, at least regarding seed protein quality and content. In fact, the electrophoretic profiles of total seed proteins extracted from BTH-treated and untreated plants appeared the same. The main protein fractions of bean seed were present (Duranti and Gius, 1997), including the globulins legumin and phaseolin and the antinutritional phytohemagglutinin and α -amylase inhibitor. This indicates that BTH treatment does not interfere with seed development.

Even seed production was not significantly different, but in this case there were slightly fewer pods per plants in both the 2001 and 2002 trials. This aspect deserves further investigation with large-scale experiments to reduce the effect of variability among plants, though such experiments would require growing numerous plants in "enemy-free" conditions, which is not an easy task to achieve. A reduced number of pods is unlikely to be due to reduction in photosynthetic capacity of BTH-treated plants, since leaf expansion and chlorophyll fluorescence data, as indicators of photochemical efficiency of PSII, were similar in both treated and untreated plants. The ratio F_v/F_m reached values considered normal for healthy plants, indicating that electron transport efficiency around PSII was similar in

both treated and untreated beans, with normal production of NADPH and ATP (Björkman and Demmig, 1987).

The chlorophyll fluorescence assay is an effective and non-invasive tool for investigating the photosynthetic apparatus in intact leaves (Krause and Weis, 1991) and is based on the fact that not all of the absorbed energy can be used for photochemical reactions, and part of it is emitted as heat and fluorescence. Primary damage caused by stress, affecting photosynthetic functions, is expected to influence the extent of fluorescence emission (Genty *et al.*, 1989; Demmig-Adams *et al.*, 1995).

The different behaviour of bean in respect to wheat, in terms of fitness cost associated with BTH treatment, could be due to higher nutritional needs of the latter to maintain primary metabolism during SAR activation. In fact, it has been shown for this crop that nitrogen supplements counterbalance adverse effects of BTH (Stadnik and Buchenauer, 1998). Instead, the fitness cost of BTH-activated resistance in cauliflower could be related to plant age, as seedlings were much more influenced than 30 day-old plants (Godard *et al.*, 1999). In this regard, we have observed a delay of a few days in the complete expansion of primary leaves after BTH treatment of bean seeds (unpublished data).

Another aspect investigated in this study was the induction and emission of ethylene, since increased levels of this compound cause senescence and yield reduction (Morgan and Drew, 1997). Induction of ethylene biosynthesis by stress is tightly regulated and involves induction of two enzymes, ACC synthase and ACC oxidase. In many tissues, ACC synthase is believed to be the rate-limiting step of the process, while ACC oxidase is responsible for the final stage in biosynthesis (Barry *et al.*, 1996). ACC oxidase is a dioxygenase non-heme ferrous enzyme, which requires ascorbate as cofactor (John, 1997). Furthermore, two transcript groups for this enzyme are expressed in vegetative tissue, either stress-induced or constitutive (Pogson *et al.*, 1995; Kim *et al.*, 1998).

As shown by Northern analysis, BTH treatment resulted in activation of ACC oxidase gene, with highest expression 12 h after spraying. However, the rapid induction of ACC oxidase transcripts, upon BTH application, did not rapidly result in ethylene synthesis, since the related emission peak was first detected only on the 7th day after treatment, and only in detached leaves. One possible explanation for this delay is that BTH elicits ACC oxidase but not ACC synthase, so the ACC level remains too low for detectable ethylene emission. However, in detached leaves undergoing senescence, when ACC level is naturally raised by ACC synthase, the presence of BTH-induced ACC oxidase accelerates and strongly enhances ethylene production. This would explain the ethylene peak found on the 9th day and would also account for the rapid leaf yellowing observed at the same time. Untreated leaves remained green until they desiccated and no ethylene emission was detected at any time. Therefore, it seems that in bean plants BTH induces a sort of "ethylene priming", resulting in a positive regulatory interaction with ethylene.

The role of ethylene in plant defence is somewhat controversial, promoting disease establishment in some plant-microbe interactions, while in many systems it interacts

with other molecular signals of SAR in a complex cross talk, in which different pathways influence each other, either positively or negatively (Feys and Parker, 2000). We have previously shown that in bean BTH induces complete and long-lasting resistance against *U. appendiculatus* (Iriti and Faoro, 2003); it seems that in this plant ethylene acts synergistically with other SAR signals, particularly H₂O₂, whose accumulation in BTH-treated tissues is particularly abundant (Iriti and Faoro, 2003). This hypothesis is also supported by the fact that ethylene is intimately involved in the amplification of ROS production under oxidative stress induced by ozone (Moeder *et al.*, 2002), and further explains the aggravation of ozone damage in BTH-treated plants (Iriti *et al.*, 2003).

In conclusion, we found that BTH-induced resistance in bean may occur without appreciable fitness costs, though this result requires support from more extensive field experiments. We show here, for the first time to our knowledge, that BTH-induced signaling pathways cross talk with the ethylene-forming step in SAR induction. This could lead to an enhanced SAR level and, in turn, would explain the well-known efficacy of BTH.

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