Comparative Efficacy of Systemic Acquired Resistance-Inducing Compounds Against Rust Infection in Sunflower Plants

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ABSTRACT

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Four inducers of systemic acquired resistance (SAR) were examined for their efficacy in controlling rust infection caused by *Puccinia helianthi* in sunflower plants. Of the four compounds, DL-3-amino-n-butanoic acid (DL- β -aminobutyric acid [BABA]) was the most effective and sodium salicylate (NaSA) was the least effective in protecting against rust. In leaf disk assays, full protection was obtained with BABA at 25 μ g/ml, benzothiadiazol-S-methyl ester (BTH) at 100 μ g/ml, 2,6-dichloroisonicotinic acid (INA) at 100 μ g/ml, and NaSA at >200 μ g/ml. L-2-amino-n-butanoic acid (AABA) was partially effective, whereas N-methyl-BABA and 4-aminobutanoic acid (GABA) were ineffective. The R-

enantiomer of BABA, but not the S-enantiomer, was more effective than the racemic mixture. In intact plants, BABA applied as a foliar spray or a root dip, before or after (up to 48 h) inoculation, provided significant protection for 8 days. BTH, INA, and NaSA were less protective and more phytotoxic compared with BABA. BABA did not affect urediospore germination, germ tube growth, appressorial formation, or initial ingress of *P. helianthi*, but strongly suppressed mycelial colonization in the mesophyll and, consequently, pustule and urediospore formation. No accumulation of defense compounds (phenolics, lignin, or callose) was detected in BABA-treated inoculated or noninoculated plants. This is the first report on the activity of BABA against an obligate Basidiomycete pathogen in planta.

Additional keywords: *Helianthus annuus*.

Sunflower rust, caused by the fungus *Puccinia helianthi* Schwein., is a widespread disease in the major sunflower-growing areas of the world (14,30). This pathogen completes its life cycle on sunflower (*Helianthus annuus*) with a high probability of genetic recombination and the formation of new races. Several races of sunflower rust have been identified. Oilseed sunflower hybrids are mostly resistant, whereas most confection hybrids are susceptible to all races, and fungicide application often is required to control the disease (31).

An alternative procedure to protect plants against disease is to activate their own defense mechanisms by specific biotic or abiotic elicitors (39). The classical type of induced resistance is often referred to as systemic acquired resistance (SAR). Sodium salicylate (NaSA), 2,6-dichloroisonicotinic acid (INA), and benzothiadiazole-S-methyl ester (BTH) are well-known elicitors of SAR in various plants against disease (34). The expression of SAR, triggered by either pathogen infection or treatment with NaSA or its functional analogs INA or BTH, is tightly associated with the transcriptional activation of genes encoding pathogenesis-related (PR) proteins (38).

The nonprotein amino acid DL-3-amino-n-butanoic acid (DL- β -aminobutyric acid [BABA]) also activates an induced resistance response. It is capable of inducing systemic resistance against numerous pathogens, including *Tobacco mosaic virus* (TMV) (32), bacteria (2,40), oomycetes (6,33,40), ascomycetes (23,26, 41), and nematodes (20,21). In *Arabidopsis thaliana*, BABA was shown to be effective against both necrotrophic and biotrophic pathogens, as well as abiotic stresses of drought and salinity (17,18,41).

BABA effectively controls diseases in the field (e.g., downy mildew in grapevine [27], moldy core in apple fruit [26], late blight in potato and tomato, and sudden wilt in melon [7]). BABA acts synergistically with BTH or with metalaxyl to protect tobacco against *Peronospora tabacina* (7) and with mancozeb to control downy mildew in cucumbers (1).

The cellular and molecular mechanisms behind this type of induced resistance response are very diverse. In *Arabidopsis*, BABA exerts its function via priming of salicylic acid (SA)-dependent defense mechanisms (40) or through potentiation of abscisic acid (ABA)-dependent signaling pathways (36). Resistance against TMV in tobacco (32) and against *Pseudomonas syringae* cv. tomato DC3000 and *Botrytis cinerea* in *Arabidopsis* resembles SAR in that it requires endogenous accumulation of SA and an intact NPR1/NIM1/SAI1 protein (40,41). In contrast, resistance against *Phytophthora infestans* in tomato (Y. Cohen, unpublished data), *Peronospora tabacina* in tobacco (6), and *P. (Hyaloperonospora) parasitica* in *Arabidopsis*, as well as against the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis*, is unaffected in SA-nonaccumulating plants or in SA-insensitive plants (36,40). Mutants impaired in the production or sensitivity to the stress hormone ABA are blocked in BABA-induced resistance against *Plectosphaerella cucumerina* (36). Resistance, therefore, seems to involve SA-dependent, SA-independent, and ABA-dependent defense mechanisms, and the importance of these defenses varies according to the nature of the challenging pathogen (35).

In spite of the intriguing capacity of BABA to confer protection against an extraordinarily wide range of biotic (7,17) and abiotic (18) stresses, no information is available on the activity of BABA against plant diseases caused by basidiomycetes. Therefore, our major objective was to explore the ability of BABA to protect sunflower against rust infection caused by the Basidiomycete *Puccinia helianthi*. The fact that BABA successfully protects

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sunflowers against downy mildew caused by *Plasmopara halstedii* (37) indicated that BABA also may facilitate defense responses in sunflower against rust. Specific objectives were to (i) compare resistance induced by BABA with that induced by other SAR activators, (ii) measure resistance induced by isomers and enantiomers of BABA, and (iii) identify defense mechanisms induced by BABA in sunflower against rust.

MATERIALS AND METHODS

Plants and pathogen. All experiments were conducted with the sunflower (*Helianthus annuus* L.) cv. HA-567 (Hazera Genetics, Mivhor, Israel). Plants were raised from seed in 100-ml pots containing ≈ 25 g of potting mixture (peat/perlite, 1:1, vol/vol), one plant per pot, in the greenhouse (19 to 34°C). Plants were fertilized once a week with 0.5% of N:P:K (20:20:20) fertilizer. Plants with two or three pairs of leaves were used in all experiments, unless stated otherwise.

Sunflower leaves infected with *Puccinia helianthi* race 3 (30) were collected from nature in summer 2000 (Mivhor region) and the rust was maintained on sunflower plants by repeated inoculations.

Inoculation. Urediospores were collected from leaves by gentle shaking over waxed paper. They were used immediately for inoculation or kept in 1.5-ml Eppendorf tubes at 4°C until used. Whole plants were inoculated by spraying (2.5 to 15 ml/plant, depending on size) with urediospores suspended in distilled water containing 0.05% Tween-20 (30). Spore concentration was adjusted with the aid of a hemacytometer to 2, 4, 6, or 8×10^3 spores/ml, depending on the experiment. Inoculated plants were placed in a dark dew chamber (16 to 18°C) for 20 h and then transferred to a growth chamber (22°C, 14 h of light/day, $100 \mu\text{Em}^{-2} \text{s}^{-1}$) for 10 to 14 days to allow for disease development (30).

Leaf disks (12 mm in diameter) were removed from the third leaf pair. They were placed, lower surface uppermost, in 24-well plates, 1 disk/well, on 1 ml of water or the test solution. Plates were placed, with no lids, in an inoculation tower and inoculated with a mixture (1:10, wt/wt) of urediospores and talcum. Plates then were covered with their lids and incubated at 22°C (as above) for 7 to 10 days, when disease development was recorded.

Disease records and statistical analysis. The number of rust pustules (uredia) developing on whole plants or on leaf disks was recorded at 10 days postinoculation (dpi) unless stated otherwise. Percent protection of treated plants or disks was calculated as $100(1 - x/y)$, where x = mean number of pustules in the treated plants (or disks) and y = mean number of pustules in the control plants (or disks).

Most experiments were repeated twice. Three to five plants (or four leaf disks) were used with each concentration of a tested chemical. Mean values of pustule counts were subjected to Fisher's protected least significant difference analysis ($P \leq 0.05$) to establish significant differences among treatments.

SAR compounds and their application. AABA, BABA, GABA, and NaSA were purchased from Sigma. INA and BTH were a gift from Syngenta, Basel, Switzerland. S-BABA and R-BABA were a gift from the former company Sandoz, Basel, Switzerland. N-methyl-BABA was a gift from Makhteshim, Beer-Sheba, Israel.

The compounds, except S-BABA, R-BABA, and N-methyl BABA, were applied by spraying whole plants or by dipping bare root systems. Spraying was done with the aid of a fine atomizer onto the upper leaf surfaces. Sprays of 5 ml were applied to 2- to 4-leaf plants and 25 ml to 12-leaf plants. The root dip was done with two-leaf plants. Plants were removed from pots, and root systems were washed with water to remove the potting mixture and dipped in the test solution at 5 ml/plant for 24 h. Plants then were transplanted into a new potting mixture in pots and inocu-

lated. BABA was applied as a soil drench by pouring 50 ml of the test solution onto the soil surface of 12-leaf potted plants. Leaf disk assays with all SAR compounds were conducted by floating leaf disks (12 mm in diameter) on 1 ml of the test solution in 24-well plates. The potential for BABA to protect plants was tested using seed treatments. Twenty seed were placed in 20 ml of the test solution and agitated for 6 to 12 h at room temperature. Seed then were sown in 0.25-liter pots and inoculated at the cotyledonary or the two-leaf stage. To examine whether untreated leaves can be protected as a result of treating other leaves, we used six-leaf plants in which only one or two leaves were treated. Depending on leaf size, 30 or 50 droplets of BABA at 10 $\mu\text{l}/\text{leaf}$ (2,000 $\mu\text{g}/\text{ml}$) were applied to one or both leaves in leaf pair 1 (from the stem base) or leaf pair 2. Control plants were treated similarly with water. Control and treated plants were kept moist for 24 h in 100% relative humidity (RH) in the light to facilitate uptake of the compound and, thereafter, inoculated with the rust pathogen.

Field tests. Sunflower seed were sown in the field (Bar-Ilan Farm) on June 2002. The field was divided into 10 plots with 20 plants per plot. Half of the plots, randomly chosen, were sprayed to run-off with BABA at 1,000 $\mu\text{g}/\text{ml}$ at the four-leaf stage using a backpack sprayer. One day later, all plants were inoculated with 4,000 urediospores/ml. A second spray with BABA was applied 2 weeks later at the eight-leaf stage. Pustule counts were taken four times at weekly intervals.

Urediospore germination in vitro. Urediospores were suspended in BABA solutions (0 to 500 $\mu\text{g}/\text{ml}$) and 10- μl droplets (containing ≈ 200 urediospores) were placed in depressions on glass slides. Slides were placed in 100% RH at 22°C (12 h light/day) and percent germinated spores and germ tube length were measured microscopically at 48 h.

Urediospore germination, appressorial formation, and mycelium growth in vivo. Two-leaf plants were sprayed with water or BABA at 2,000 $\mu\text{g}/\text{ml}$ and inoculated 24 h later with 4,000 urediospores/ml. Leaf disks (1 cm in diameter) were removed at 24 and 48 h postinoculation for microscopic examinations. Microscopic examinations also were conducted with leaf disks floating on water or BABA at 200 $\mu\text{g}/\text{ml}$ in multiwell plates. Such disks were inoculated with dry urediospores in an inoculation tower and removed for examination at 2, 5, and 7 dpi. Leaf disks were clarified and stained with trypan blue (24).

Histochemistry. Plants at the two-leaf stage were sprayed with water (containing 0.01% Tween-20) or BABA at 2,000 $\mu\text{g}/\text{ml}$ and inoculated 2 days later. Leaf disks (1 cm in diameter) were removed at different intervals postinoculation and examined for autofluorescence (the presence of phenolics), callose, lignin, peroxidase, and catalase activity and for reactive oxygen species (ROS) production.

For detecting phenolics, leaf disks were placed on a glass slide, covered with water, and examined with the aid of an epifluorescent microscope equipped with an excitation filter of 390 to 420 nm and an emission filter of 425 to 450 nm (5,8,11). To detect callose, leaf disks were clarified by boiling in 90% ethanol for 30 min and then placed in 70 mM potassium phosphate buffer (pH 8.9) at 4°C for 24 h. Thereafter, disks were placed in 0.005% aniline blue in the same buffer for another 24 h at 4°C and then examined with the aid of epifluorescent microscope as above (8,9). For lignin detection, leaf disks were clarified as above, placed on a glass slide, and treated with 2% phloroglucinol dissolved in ethanol followed by 25% HCl (8,9).

Microscopic assays for ROS production were performed with leaf disks floating on water or on BABA at 200 $\mu\text{g}/\text{ml}$. Leaf disks were inoculated with dry urediospores and removed for examination at 6 h to 6 dpi. H_2O_2 accumulation was visualized by using 3,3'-diaminobenzidine-4 HCl (DAB). Leaf disks were infiltrated in vacuo with 0.2% DAB for 5 min. Disks then were exposed to light for 30 min and, thereafter, placed in 0.15% trichloroacetic

acid (wt/vol) in chloroform/ethanol (1:4, vol/vol) for 24 h in the dark (28,32). For O₂⁻ detection, leaf disks were placed in a solution containing 0.1% nitroblue tetrazolium (NBT) in water (wt/vol), 10 mM sodium azide, and 50 mM potassium phosphate (pH 6.4) (28,32).

To detect peroxidase activity, treated and untreated leaf disks (0.2 g) were homogenized in 2 ml of 15 mM phosphate buffer (pH 6) at 4°C and centrifuged at 8,000 × g for 10 min. The reaction mixture contained 0.2 ml of leaf extract, 0.1 ml of 0.05 M guaiacol, 0.1 ml of 0.5 M H₂O₂, and 5 ml of phosphate buffer. The change in optical density at 470 nm was followed for 3 min. A reaction mixture containing no plant extract served as a blank. Protein content was determined according to Bradford (4). For determining catalase activity, 10 treated and untreated leaf disks (5 mm in diameter) were agitated in 9 ml of 5 mM phosphate buffer (pH 7.1) and 1 ml of 0.1 M H₂O₂. The change in optical density at 410 nm was determined at 10-min intervals. Protein content was determined as above.

Staining fungal structures. Four-leaf plants were sprayed with BABA at 2,000 µg/ml and inoculated 1 day later with 4,000 urediospores/ml. Leaf disks were removed from the second leaf pair at 1 dpi, stained with 0.01% calcofluor (Sigma), and examined with an epifluorescent microscope. Fungal colonization inside the mesophyll was observed with bright-field microscopy in leaf disks at 3 to 7 dpi. Disks were clarified in acidic ethanol for 24 h and stained in 0.01% trypan blue in lactophenol-ethanol for 10 min (24). Chloral hydrate was used to remove the excessive stain.

RESULTS

Foliar spray. BABA protected plants (inoculated with 4 × 10³ urediospores/ml) from rust infection in a dose-dependent manner (Table 1). At 250 µg/ml, BABA was ineffective and, at 2,000 µg/ml, it provided 100% protection in younger plants and 97% protection in older plants. In other experiments, six-leaf plants were inoculated with 2 or 8 × 10³ urediospores/ml. In plants inoculated with 2 × 10³ urediospores/ml, BABA applied at 500, 1,000 and 2,000 µg/ml provided protection of 79, 94, and 100%, respectively; whereas, in plants inoculated with 8 × 10³ urediospores/ml, it provided protection of 0, 87, and 100%, respectively, relative to control-inoculated plants.

Soil drench. The number of rust pustules that developed in 12-leaf plants is shown in Table 2. The efficacy of BABA in protecting the plants from the rust was dose dependent: 12.5 and 100 mg of BABA provided 40 and 81% protection, respectively.

Root dip. At 10 dpi, the mean number of pustules developed per cotyledon was 30 and 0 for plants treated with water and BABA, respectively. In two-leaf, plants 50 and 0 pustules per leaf developed in water and BABA-treated plants, respectively. Other experiments revealed that BABA, applied as a root dip to two-leaf plants, was effective for at least 10 days, until plants

reached the four- to six-leaf stage. Such plants exhibited 95 to 97% protection against rust compared with water-treated control-inoculated plants.

Persistence of a foliar spray. BABA significantly reduced the number of rust pustules at all inoculation times (Table 3). However, the magnitude of protection was significantly lower at 8 days (74%) and 10 days (40%) after treatment compared with 2 to 6 days (86 to 98%). Such a decrease in efficacy with time may result from the translocation of BABA to the new developing leaves (10,13).

Postinfection activity of BABA. Four-leaf plants first were inoculated (4 × 10³ urediospores/ml) and then, at 1, 2, 3, 4, and 6 dpi, were sprayed with 5 ml of BABA at 1,000 µg/ml. Untreated control-inoculated plants developed 130 ± 20 pustules per plant at 14 dpi. Percent protection observed in BABA-treated plants was 83, 70, 19, 0, and 0 for plants treated at 1, 2, 3, 4, and 6 dpi, respectively. These data suggest that BABA can stop disease development in 1- and 2-day-old infection sites but not in sites with more advanced infections.

Seed treatment. Sunflower seed were agitated in BABA of various concentrations (up to 1% = 100 mM) for up to 12 h and planted in soil, and the developing plants were inoculated with *P. helianthi* at either the cotyledonary or the two-leaf stage. No protection against rust was observed compared with water-treated control-inoculated plants. Also, no adverse effects of BABA were observed on seed germination or plant development.

Systemic protection. BABA effectively protected sunflower plants against rust when applied to the root system (Table 2). When BABA was applied to leaves, complete control of the disease was observed in the treated leaves, but only 50% control in the leaf opposite to the treated leaf. No control was seen in leaves above or below the treated leaves (data not shown).

Field observations. BABA-treated plants developed significantly fewer rust pustules compared with the untreated control-inoculated plants. The mean number of pustules per plant in control plots was 50, 250, 280, and 390 at 1, 2, 3, and 4 weeks after inoculation, respectively, compared with 20, 90, 130, and

TABLE 2. Effect of soil drench with DL-3-amino-n-butanoic acid (BABA) on sunflower plants against rust infection^z

BABA mg/kg of soil	Pustules per plant	Protection (%)
0	510 c	...
12.5	306 b	40
25	265 b	48
50	130 a	75
100	99 a	81

^z Figures followed by the same letter are not significantly different (Fisher's protected least significant difference test, *P* > 0.05). Inoculation was done with 6 × 10³ urediospores/ml. Pustules were counted at 10 days post-inoculation.

TABLE 3. Persistence of DL-3-amino-n-butanoic acid (BABA) efficacy against rust infection in sunflower plants^y

Days ^z	Pustules/plant		Protection (%)
	H ₂ O	BABA	
2	145*	20 a	86
4	150*	6 a	96
6	245*	4 a	98
8	172*	45 b	74
10	225*	135 c	40

^y Asterisks indicate a significant difference at α = 0.05 between H₂O and BABA treatment at each inoculation time (*t* test). Different letters in a column indicate significant differences between inoculation times (Fisher's protected least significant difference test, *P* < 0.05). Plants were inoculated with 4 × 10³ urediospores/ml. Pustules were counted at 10 days post-inoculation.

^z Inoculation, days after treatment.

TABLE 1. Effect of foliar spray with DL-3-amino-n-butanoic acid (BABA) on sunflower plants against rust caused by *Puccinia helianthi*^z

BABA (µg/ml)	Pustules/plant	
	6-leaf plants	12-leaf plants
0	270 (0) c	600 (0) c
250	240 (10) c	610 (0) c
500	180 (33) b	240 (60) b
1,000	30 (89) a	120 (80) ab
2,000	0 (100) a	18 (97) a

^z Number in parenthesis represents percent protection. Figures followed by the same letter are not significantly different (*P* < 0.05) according to Fisher's protected least significant difference test. Plants were inoculated with 4 × 10³ urediospores/ml and pustules counted at 10 days postinoculation.

190 pustules/plant in BABA-treated plots. Therefore, percent protection reached 60, 64, 54, and 67% at 1, 2, 3, and 4 weeks after inoculation. Area under disease progress curve was 5,460 in control plots compared with 2,660 in BABA-treated plots. Seed yield per plant was 31 and 38 g in control and BABA-treated plants, respectively (not significantly different, $\alpha = 0.05$), weight of 100 seed was 3.9 and 4.3 g (not significantly different, $\alpha = 0.05$), and percent germination of seed was 55 and 51% (not significantly different, $\alpha = 0.05$) in control and BABA-treated plants, respectively. The data suggest that BABA, although providing $\approx 60\%$ protection against rust, did not increase seed production or reduce seed germination.

Efficacy of isomers and enantiomers. The efficacy of AABA and GABA was examined by foliar spray and root dip treatments. Although a foliar spray with BABA resulted in protection of up to 95%, a foliar spray with AABA was partially effective (37%) and that with GABA was ineffective. AABA was phytotoxic at high concentrations (Table 4).

Somewhat different results were obtained with two-leaf plants treated by a root dip (Table 5). BABA provided 50% protection with a 31.3 $\mu\text{g/ml}$ solution. Protection increased to 100% in a dose-dependent manner. No clear dose response was obtained with either AABA or GABA. At 250 $\mu\text{g/ml}$, they each provided 45% protection compared with 95% protection obtained with BABA. Both were phytotoxic at 500 $\mu\text{g/ml}$.

The R and S enantiomers of BABA were compared for their efficacy in leaf disk bioassays with that of DL-BABA or its isomers AABA, GABA, and N-methyl-DL-BABA. The minimal inhibitory concentration for DL-BABA, R-BABA, and S-BABA was 25, 12.5, and 50 $\mu\text{g/ml}$, respectively (Fig. 1). GABA was ineffective at 200 $\mu\text{g/ml}$, whereas AABA provided 62% protection at this concentration. N-methyl-DL-BABA was ineffective (up to 400 $\mu\text{g/ml}$, data not shown). The efficacy of BABA and GABA in controlling rust in sunflower leaf disks is shown in Figure 2A and B.

Comparative activity of BABA and other SAR-inducing compounds. Two-leaf plants were sprayed with 5 ml of BABA (250 to 2,000 $\mu\text{g/ml}$), NaSA (1,000 to 8,000 $\mu\text{g/ml}$), INA (100 to 800 $\mu\text{g/ml}$), or BTH (200 to 1,000 $\mu\text{g/ml}$). These concentrations were chosen after preliminary examinations of the phytotoxic effects of the compounds. At 2 days after treatment, plants were inoculated with the rust pathogen (4×10^3 urediospores/ml) and disease was recorded at 14 dpi. Phytotoxic effects of NaSA, INA, and BTH were seen at concentrations of 2,000, 400, and 800 $\mu\text{g/ml}$, respectively. Disease assessments were not taken for these treatments. BABA provided 43, 59, 92, and 97% protection at 250, 500, 1,000, and 2,000 $\mu\text{g/ml}$, respectively. Treatment with NaSA at 1,000 $\mu\text{g/ml}$ provided no protection. Treatments with INA at 100 and 200 $\mu\text{g/ml}$ provided 0 and 35% protection, respectively, and treatments with BTH at 100 and 200 $\mu\text{g/ml}$ provided 3 and 33% protection, respectively.

TABLE 4. Comparative efficacy of L-2-amino-n-butyric acid (AABA), DL-3-amino-n-butyric acid (BABA), and GABA, applied as a foliar spray, in protecting sunflower plants against rust infection^y

Conc. ($\mu\text{g/ml}$) ^z	Pustules per plant (percent protection)		
	AABA	BABA	GABA
0	235 d	...	...
500	180 cd (23)	138 c (41)	190 cd (19)
1,000	147 c (37)	70 b (70)	235 d (0)
2,000	200* d (15)	12 a (95)	235 d (0)

^y Different letters following figures in rows and columns indicate significant differences (Fisher's protected least significant difference test, $P < 0.05$). The asterisk indicates phytotoxicity. The experiment was conducted with four-leaf plants. Plants were inoculated with 4×10^3 urediospores/ml at 1 day after treatment. Pustules were counted at 10 days postinoculation.

^z Concentration.

Leaf disk bioassays were performed with BABA, INA, BTH, or NaSA each at 12.5, 25, 50, 100, and 200 $\mu\text{g/ml}$. A treatment with BABA at 12.5 $\mu\text{g/ml}$ provided 78% protection and 25 $\mu\text{g/ml}$ or above provided full protection (Fig. 3). NaSA provided no protection at all concentrations while being toxic at 100 and 200 $\mu\text{g/ml}$. INA and BTH at 50 $\mu\text{g/ml}$ provided 75 and 58% protection and full protection at 100 and 200 $\mu\text{g/ml}$, respectively (Fig. 3). At the later concentrations, INA and BTH were phytotoxic.

Urediospore germination in vitro. Percent germination in water droplets as well as in BABA (all concentrations) solutions ranged between 95 and 98%. Germ tube length ranged between 130 and 145 μm in both water and BABA solutions.

Urediospore germination, appressorial formation, and hyphal growth in vivo. UV-epifluorescent microscopy of calcofluor-stained leaf disks revealed profuse urediospore germination on disks derived from either water-treated or BABA-treated plants. Appressoria also were seen on both treated and untreated leaf disks. At 2 dpi, percent germinating urediospores in water and BABA-treated leaf disks was 85 and 87%, respectively, and percent urediospores forming appressoria was 21 and 26%. At 5 dpi, extensive hyphal growth was observed in control disks (Fig. 2C) but only minimal growth in BABA-treated disks (Fig. 2D). At 7 dpi, fungal colonies were seen in control disks whereas no colonies were observed in BABA-treated disks (data not shown).

Host responses. UV-epifluorescence microscopy of fresh leaf disks revealed no fluorescence indicative of phenolic compounds. Similarly, basic aniline blue-staining of leaf disks failed to detect any callose production. Bright-field microscopy of phloroglucinol-HCl-stained leaf disks showed no lignin production, except in the xylem vessels.

H_2O_2 -positive blue staining was observed in water control-inoculated disks at 4 days dpi. At 5 dpi, heavy blue staining was seen in the infected sites, accumulating mainly in hyphal tips. No staining was seen in BABA-treated inoculated disks. O_2^- -positive brown staining was observed in water control-inoculated disks at 4 dpi onwards. No staining was seen in BABA-treated inoculated leaf disks.

Peroxidase (POX) was measured in leaf tissue derived from two-leaf plants treated with water or BABA (250 to 2,000 $\mu\text{g/ml}$). At 3 dpi, POX-specific activity in water control and BABA-treated leaf tissues were not significantly different ($P = 0.05$) and ranged between 0.08 and 0.16 optical density (OD) per milligram of protein per minute and 0.05 and 0.13 OD per milligram of protein per minute, respectively. Similarly, insignificant differences in catalase activity were observed (data not shown).

TABLE 5. Comparative efficacy of L-2-amino-n-butyric acid (AABA), DL-3-amino-n-butyric acid (BABA), and GABA, applied by root dipping, in protecting sunflower plants against rust infection^y

Conc. ($\mu\text{g/ml}$) ^z	Pustules per plant (percent protection)		
	AABA	BABA	GABA
0	110 c	...	...
31.3	115 c	55 b (50)	82 bc (25)
62.5	115 c	30 ab (73)	70 b (36)
125	122 c	15 a (86)	60 b (45)
250	60 b (45)	5 a (95)	60 b (45)
500	55* b (50)	0 a (100)	62* b (44)

^y Different letters following figures in rows and columns indicate significant differences (Fisher's protected least significant difference test, $P < 0.05$). Asterisks indicate phytotoxicity. Experiment was conducted with two-leaf plants. Plants with bare roots were placed in test solutions for 24 h, transplanted into soil in pots, and inoculated with 4×10^3 urediospores/ml. Disease was recorded at 10 days postinoculation.

^z Concentration.

DISCUSSION

BABA is a nonprotein amino acid shown to induce resistance against ≈ 50 plant pathogens in a large number of annual and perennial agricultural crops (7,17). Oomycetes suppressed by BABA in their respective host tissues include *Aphanomyces eutiches* in pea (22), *Peronospora tabacina* in tobacco (6), *P. parasitica* in *Arabidopsis* (40) and cauliflower (33), *Phytophthora infestans* in tomato (12) and potato (7), *P. capsici* in pepper (16), *Plasmopara viticola* in grape (13,15), *P. halstedii* in sunflower (37), *Sclerospora graminicola* in sorghum (29),

and *Pseudoperonospora cubensis* in cucumber (3). Ascomycetes/Fungi Imperfecti controlled by BABA are *Fusarium oxysporum* f. sp. *solani* in tomato (7), *B. cinerea* and *Plectosphaerella cucumerina* in *Arabidopsis* (36,41), *Monosporascus cannonbolis* in melon (7), *Alternaria alternata* in apple fruit (26), *A. brassicicola* in *Arabidopsis* (36), and *Penicillium digitatum* in grapefruit (23).

The literature supplies no evidence on the activity of BABA against basidiomycetes in general or against rusts in particular. Rust in sunflower is a worldwide-distributed disease and genetic resistance in confection hybrids is lacking (14). There-

Fig. 1. Efficacy of **A**, DL-3-amino-n-butanoic acid (BABA), its enantiomers **B**, S-BABA and **C**, R-BABA, and its isomers **D**, L-2-amino-n-butanoic acid (AABA), **E**, GABA and **F**, N-methyl-BABA, in controlling rust development in sunflower leaf disks. Different letters on bars indicate significant differences among means according to Fisher's protected least significant difference test ($P < 0.05$).

Fig. 2. Protection of leaf disks by DL-3-amino-n-butanoic acid (BABA) and GABA. Multiwell plate assay with **A**, BABA and **B**, GABA of various concentrations. Bright-field micrographs of trypan blue-stained mycelia of *Puccinia helianthi* growing in the mesophyll of sunflower leaves at 5 days postinoculation. **C**, Untreated and **D**, treated with BABA at 2,000 µg/ml. Magnification $\times 400$. Arrow shows hyphal tips.

fore, alternative control methods are needed. We show here that sunflower plants treated with BABA were protected against rust infection. This was true in growth chambers and in the field. Protection was achieved by applying BABA as a foliar spray, soil drench, root-dip, or in floating leaf disks. No protection, however, was seen after soaking seed in the test compounds. Percent protection varied according to the methodology used. In the field, plants treated twice with BABA at 1,000 µg/ml had 60% fewer pustules than control plants. In leaf disks, 100% protection was observed with BABA at 25 µg/ml. Similar to our previous observations with *Phytophthora infestans* in tomato (12), *Plasmopara viticola* in grape (13), and *Peronospora tabacina* in tobacco (6), postinfection applications of BABA were effective in reducing rust in sunflower. This postinfection inhibitory effect implies that BABA operates quite rapidly to stop growth of mycelia in the mesophyll. With BTH, four or more days were required to elicit protection against rust (24).

Compared with BABA, the isomers AABA, N-methyl-BABA, and GABA were far less active, suggesting that activity requires a free amino group residing in a β position. The more effective activity of the R-enantiomer suggests that a specific stereostructure of the molecule is required for activity, probably for binding to its ligand. The R-enantiomer was the most active in other pathosystems (7).

Unlike BTH, which induced resistance in sunflower against rust by suppressing urediospore germination and appressorial formation on the leaf surface (24), BABA exerted no inhibitory effects on urediospore germination, appressoria formation, or fungal penetration. This suggests that the resistance induced by these two compounds operates via different mechanisms. Prats et al. (24) demonstrated that suppression of disease by BTH was accompanied by the production of the coumarins scopolin, scopoletin, and ayapin by the host. Accumulation of scopolin and scopoletin was reported in susceptible, downy mildew-infected sunflower cultivars (5,11).

BABA effectively translocated from the root system to protect the foliage against rust. However, almost no translocation occurred from treated leaves to untreated leaves, indicating poor loading or translocation in the phloem. In contrast, ^{14}C -BABA was translocated from the middle leaves to the upper leaves (but not to lower leaves) and to the root tips in tomato (10) and grape plants (13).

BABA was reported to elicit the formation of diverse defense compounds in plants (7). In *Arabidopsis*, callose accumulated in infection sites (40) as well as in the ovaries (17), and transcripts of PR proteins were detected in the leaves (17). In cauliflower, callose (7) and PR proteins (33) were reported after BABA treatment. In tomato, PR proteins (12), callose and lignin (19,25),

Fig. 3. Control of rust development in sunflower leaf disks by **A**, DL-3-amino-n-butanoic acid (BABA), **B**, 2,6-dichloroisonicotinic acid (INA), **C**, benzothiazol-S-methyl ester (BTH), and **D**, sodium salicylate (NaSA). Different letters on bars indicate significant differences among means according to Fisher's protected least significant difference test ($P < 0.05$); + indicates phytotoxicity.

as well as ROS and enhanced activity of phenylalanine ammonia lyase and peroxidase, were observed (2). In grape, lignin (13) and callose (15) were formed. PR proteins were detected in tobacco plants protected against downy mildew by a foliar spray treatment, but no PR proteins, callose, or lignin were detected in tobacco plants protected by soil application (6). In tobacco inoculated with TMV, ROS production, callose, and peroxides were shown (32). In pepper, phytoalexins as well as PR proteins were reported (16).

Strikingly, BABA did not elicit production of phenolics, lignin, callose, peroxidase, or catalase in protected sunflower plants. This resembles the results obtained with tobacco treated via the soil, in which we failed to detect these defense-related compounds. We also failed to detect PR proteins in BABA-treated sunflower plants (*unpublished data*). ROS (H_2O_2 and O_2^-) were associated with fungal structures in control-inoculated leaves but not in BABA-inoculated leaves. This indicates that other, unknown mechanisms are responsible for halting rust growth in sunflower plants. Further studies are needed to reveal this mechanism or mechanisms.

It is commonly accepted that BABA is a SAR-inducing compound. This notion is based on (i) lack of antimicrobial activity in vitro, (ii) elicitation of a defense compound in the host, (iii) a broad spectrum of activity, and (iv) systemic protecting activity against pests. In the sunflower-rust pathosystem, BABA exerted no antifungal activity in vitro or in vivo toward urediospore germination, germ tube elongation, appressorial formation, and initial ingress of the pathogen into the host. However, because common defense-related compounds were not detected in the host, the mechanism of protection is unclear. We

cannot eliminate the possibility that BABA stops hyphal growth in planta by direct fungicidal action.

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