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Local and systemic activity of BABA (DL-3-aminobutyric acid) against *Plasmopara viticola* in grapevines

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Abstract

The non-protein amino acid BABA (DL-3-amino-*n*-butanoic acid, β-aminobutyric acid) is reported here to induce local and systemic resistance against downy mildew in grape leaves. Leaf discs of susceptible cultivars placed on BABA solutions and inoculated with *Plasmopara viticola* on the counter surface produced brownish restricted lesions below the inoculation site (hypersensitive-like response, HR) which failed to support fungal sporulation. Histochemical analyses of such HR lesions revealed the accumulation of lignin-like deposits in the host cells. In contrast, water-treated inoculated discs produced expanded chlorotic lesions with profuse sporulation in which no lignin accumulation was observed. Mock-inoculated BABA-treated leaf discs showed no HR or lignin accumulation. Concentrations as low as 25 μg/ml (0.25 mM) of BABA sufficed to prevent tissue colonization with the fungus. Five other isomers of aminobutyric acid, namely L-2 aminobutyric acid, 2-amino isobutyric acid, DL-2-aminobutyric acid (AABA), DL-3-amino isobutyric acid, and 4-aminobutyric acid (GABA) gave no protection against the downy mildew fungus. Of the two (*R* and *S*) enantiomers of BABA only the *R* form was active in producing HR, suggesting a specific stereostructure requirement for activity. BABA could stop fungal colonization even when applied post-infectionally to leaf discs. Resistance of BABA-pulse-loaded leaf discs persisted for more than 14 days. BABA provided systemic protection against the disease when applied via the root system or via the lower leaves of grape plants. Application of ^{14}C-BABA to a single leaf of intact plants showed the accumulation of the ^{14}C label in upper leaves (and root tips), suggesting sink-oriented transport.

Abbreviations: AABA – DL-2-amino-*n*-butanoic (DL-α-aminobutyric) acid; BABA – DL-3-amino-*n*-butanoic (DL-β-aminobutyric) acid; BTH – benzo[1,2,3]thiadiazole-7-carbothionic acid-*S*-methyl ester (Bion®); GABA – 4-aminobutanoic(γ-aminobutyric) acid; INA – 2,6-dichloroisonicotinic acid; PR – pathogenesis-related (proteins); SA – salicylic acid, sodium salt; WP – wettable powder; WG – wettable granular; TLC – thin layer chromatography; MCW – methanol : chloroform : water (12 : 5 : 3).

Introduction

A new technology for plant disease control is based on the activation of the plant's own defense system with the aid of low molecular weight synthetic molecules. Compounds such as salicylic acid (SA), 2,6-dichloroisonicotic acid (INA) and benzo[1,2,3]thiadiazole-7-carbothionic acid-*S*-methyl ester (BTH) are able to induce systemic acquired resistance (SAR) in a variety of plants against a wide range of microbial pathogens without possessing direct antimicrobial activity *in vitro* or in planta. SA, INA and BTH are functionaly related, sharing the activation of similar genes (coding for PR-protein synthesis) in the plant. Both INA and BTH act independently of the presence of SA. They are active in monocot as well as dicot plants and normally require about 2 days for gene activation (Kessmann et al., 1994; Ryals et al., 1996;

Sticher et al., 1997). They failed to induce SAR in *nim* mutants (non-inducible) of Arabidopsis (Delaney, 1997), thus proving again that they work only through the plants. One major hurdle with commercial application of INA-like compounds is their phytotoxic effect to certain crops (Sticher et al., 1997).

A new class of resistance-inducing compounds recently reported by Cohen and coworkers, belongs to aminobutyric acids (Cohen, 1996). A recent review by Sticher et al. (1997) states that 'local treatments with DL-3-aminobutyric acid (BABA) protect tomato, potato, and tobacco, systemically, against *Phytophthora infestans* and *Peronospora tabacina*, respectively. BABA has a curative effect, which is surprising for an SAR inducer. A hypothesis was formulated whereby the action of BABA is based on covalent cell-wall modification, since after [^{14}C]-BABA treatment, covalently bound label is found in the cell wall that can only be released with cell-wall degrading enzymes (P. Schweizer and Y. Cohen, unpublished data).'

SAR was extensively studied with at least 20 plant species (Sticher et al., 1997); none of them was a woody, perennial species. In the present study, we show that BABA provides a durable, local and systemic protection of grape plants against the downy mildew disease incited by the fungus *Plasmopara viticola* when applied pre- or post-infectionally. This widely distributed and highly destructive disease of grapevines (Pearson and Goheen, 1988) is currently controlled by fungicides whose efficacy is often hampered by resistant fungal mutants (LeRoux and Clerjeau, 1985). BABA may offer an important opportunity to develop a new technology to control downy mildew in grapes. The high tolerance of BABA by grape plants adds to its value in agricultural practice.

Materials and methods

Chemicals

DL-2-amino-*n*-butanoic acid (AABA), L-2-amino-*n*-butanoic acid, DL-3-amino-*n*-butanoic acid (BABA), 2-amino *iso*butanoic acid, DL-3-amino *iso*butanoic acid and 4-amino-*n*-butanoic acid (GABA) were purchased from Sigma. The *R* and *S* enantiomers of BABA and the ^{14}C-BABA [CH_3-$^{14}CH(NH_2)$-CH_2-COOH] with a specific activity of 9.68 μCi/mg (1 mCi/mmol) and a purity of >98% were kindly supplied by Novartis (Sandoz) Pharma, Basel. INA 25% WP and CGA 245704 (BTH) formulated as 50% WG were a gift of Novartis AgroResearch, Basel.

Plant and fungal materials

Plants were raised from either cuttings or seeds. Unless stated otherwise, the cultivar used was Emerald Riesling. Plants were grown in 1-liter pots containing a mix of (1 : 1 : 1 v/v) sand, vermiculite and peat. Plants were used when they had 4–10 expanded leaves. For leaf disc assays, leaf discs were punched from the second and third leaves from the top of a plant using a 10 or 12 mm diameter cork borer. Leaf discs were floated, lower surface uppermost, on test solutions in 24-well titer plates (10 mm discs) or in 9 cm petri dishes (ten 12 mm diameter discs/dish). For whole plant assays, plants were sprayed first (see Results) and thereafter inoculated on their lower leaf surfaces.

Experiments were carried out using an isolate of *Plasmopara viticola* collected in 1996 in the coastal plain of Israel. The fungus was maintained on detached leaves or whole potted plants by repeated inoculations at 20 °C.

Inoculation and disease development assessment and fungal sporulation

Sporangia of *P. viticola* were harvested from freshly sporulating leaves into ice-cold double distilled water, using a camel hair brush. Concentration of sporangia was adjusted to 1×10^4/ml using a haemocytometer, unless otherwise stated. To inoculate whole plants, the sporangial suspension was sprayed onto the lower leaf surfaces with the aid of a glass sprayer. To inoculate leaf discs, one or two 10 μl inoculum droplet(s) were applied to each disc using an Eppendorf pipette dispenser. Inoculated plants were immediately transferred to a dew chamber (18 °C, darkness) for 20 h and then maintained in a growth chamber at 20 °C (12 h photoperiod, 100 μE/m^2/s light intensity and RH of 60–70%) until symptoms developed or sporulation was induced. Petri dishes and titer plates with inoculated leaf discs were placed in a 20 °C growth chamber. To induce fungal sporulation infected plants were returned to the dew chamber for 24 h.

Disease intensity in leaf discs was visually assessed using a 0–4 scale, in which: 0 = no symptoms visible; 1 = up to 25% of the leaf disc area is chlorotic or necrotic; 2 = 25–50%; 3 = 50–75% and 4 = >75% of the leaf disc area is chlorotic or necrotic. Fungal

sporulation in whole plants was assessed visually using a 0–4 scale in which, 0 = no sporulation visible and 4 = all of the abaxial leaf surface covered with sporangia and sporangiophores. For quantitative determination of sporulation in leaf discs, infected leaf discs were placed in 5 ml per disc of 50% ethanol, shaken for 5 min and the number of sporangia released was counted with the aid of a haemocytometer.

Microscopical examinations

(a) Autofluorescence
Inoculated leaf discs were placed on a glass slide, a few water droplets were added, the preparation was covered with a cover slip and examined with a Zeiss UV epifluorescent microscope equipped with filter combination 390-420/FT 425/LP450. Under such conditions normal host cells fluoresced red (chlorophyll), necrotic host cells looked dark and phenolics fluoresced bluely. Fungal structures were not visible (Cohen et al., 1990).

(b) Fungal structures
Inoculated leaf discs were treated with a few droplets of aqueous Calcofluor (0.01%, Sigma) and similarly examined. Sporangiophores and sporangia fluoresced strongly blue. Fungal structures inside the leaf could not be seen.

(c) Callose accumulation
Inoculated leaf discs were first clarified in boiling ethanol and then treated with basic (pH 9.8) aniline blue as described (Cohen et al., 1990). Callose deposits in the host or the fungus fluoresced strongly yellow.

(d) Lignin deposition
Inoculated leaf discs were clarified with ethanol and then treated with phloroglucinol (Sigma) followed by 25% HCl as described (Cohen et al., 1990). Leaf discs were examined microscopically under bright field illumination. Lignin deposits in host cells stained red.

Systemic translocation of ^{14}C-BABA

Plants were produced from 10 cm cuttings and when they had 7 leaves they were treated with ^{14}C-BABA on a single leaf. Fifty 10 µl droplets of the radioactive compound were placed onto the upper leaf surface of either leaf number (from stem base) 1, 4 or 6 (total of 1 µCi/leaf) and plants were kept in the greenhouse for 5 days until assayed for radioactivity.

The radioactivity extractable from the plants was measured. Leaves were detached, washed with excessive water, blotted dry and extracted in MCW (methanol : chloroform : water, 12 : 5 : 3 v/v) as described (Bieleski and Turner, 1996; Cohen and Gisi, 1994a). An aliquot of 0.1 ml was taken for measuring radioactivity (Cohen and Gisi, 1994a).

Results

Leaf disc assays

Six isomers of aminobutyric acid were tested for their efficacy to suppress downy mildew development in grape leaf discs. They were: DL-2-amino, L-2-amino, DL-3-amino, 4-amino, 2-amino *iso* and DL-3-amino *iso*butyric acids. Test solutions contained either 6.25, 12.5, 25, 50 or 100 µg/ml of tested compound (∼0.06–1 mM). BTH was also used, for comparison, at 0.3, 1.25, 5, 20 and 40 µg/ml (∼0.001–0.2 mM). Nine days after inoculation, all leaf discs, except those treated with BABA or BTH, showed profused sporulation of *Plasmopara viticola*. Sporangial counts for AABA, BABA, and GABA are given in Figure 1. Sporangial counts for L-2-aminobutyric acid, 2-amino *iso*butyric acid and DL-3-amino *iso*butyric acid were similar to the water control (data not shown). Untreated-inoculated (control) leaf discs yielded a mean of 120×10^3 sporangia/disc (Figure 1). BABA of 6.25, 12.5, 25, 50 and 100 µg/ml reduced this yield by −11, 85, 96, 99 and 99%, respectively (Figure 1). AABA at 100 µg/ml significantly reduced sporulation by 37% whereas GABA at all concentrations enhanced it, although insignificantly. BTH at 0.3, 1.25, 5 and 20 µg/ml reduced sporulation by −4, 27, 75 and 99%, respectively. At 40 µg/ml, it was phytotoxic.

Inoculated leaf discs treated with BABA of ≥12.5 µg/ml showed pale-brownish lesions beneath the inoculum droplets. No such lesions were seen in water-treated inoculated discs (profuse sporulation was seen), or in BABA-treated leaf discs mock-inoculated with water. Bright-field microscopical examination of non-stained leaf discs revealed a hypersensitive-like response (HR) in the BABA-treated inoculated tissue as against normally-looking tissue bearing sporangia in water-treated inoculated tissue. BTH (20 µg/ml) also induced HR. No further studies were devoted to BTH.

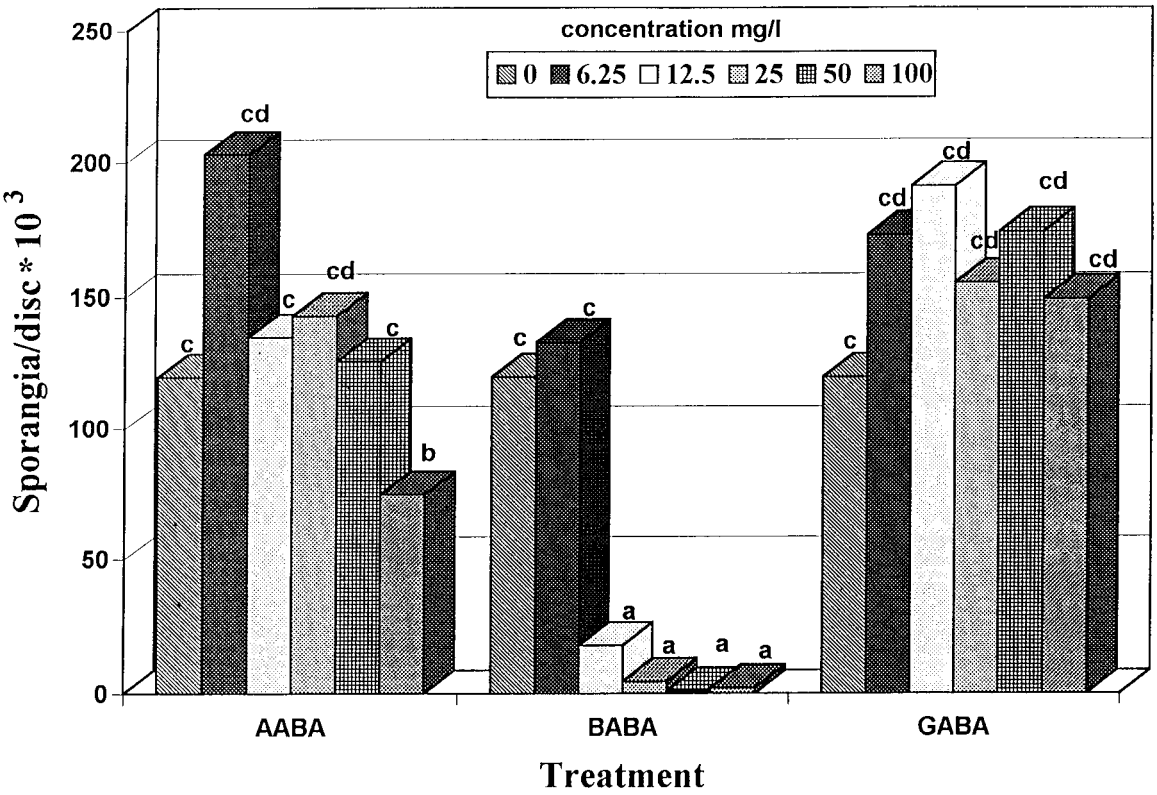

Figure 1. Downy mildew (*Plasmopara viticola*) development on grape leaf discs (2.27 cm^2) floating on aminobutyric acids of various concentrations. AABA = DL-2-aminobutyric; BABA = DL-3-aminobutyric; GABA = 4-aminobutyric acid. Data collected at 10 days post-inoculation.

UV micrographs of calcofluor-stained water-treated and BABA-treated inoculated leaf discs showed that, compared to water, BABA strongly suppressed both steps of sporulation: the emergence of sporangiophores from stomata and thus the sporangial production. In water-treated tissue, sporangiophores emerged in groups of 3–5/stoma, they were branched and bear abundant sporangia whereas in BABA-treated tissue, only a few non-branched sporangiophores, 1/stoma, with only a few sporangia, were developed. The numerical data collected from this experiment are given in Table 1.

Other microscopical examinations were aimed at ascertaining whether the HR response of inoculated BABA treated leaf discs was associated with autofluorescence of the tissue, callose formation or lignification. We found, at 10 days after inoculation, no autofluorescence in either water control-inoculated or BABA-treated inoculated leaf discs, when mounted in water and examined under UV light using an epifluorescence microscope. Similarly, when first clarified with ethanol and then placed for 48 h in basic (pH 9.8) aniline-blue solution and examined under UV light no yellow fluorescence was visible in the affected plant tissue, indicating that resistance acquired after BABA treatment was not associated with callose accumulation. With basic aniline-blue staining, however, we observed a strong yellow emission of fluorescence from the sporangiophores indicating the presence of β-1,3-glucans in walls and cytoplasm of the sporangiophore.

Phloroglucinol–HCl staining for lignin was negative for control-infected leaf discs but positive for BABA-treated inoculated discs. While no red-stained cells were observed in the control-infected tissue, many such cells were seen in the mesophyll (not in the epidermis – probably because fungal penetration occurs via stomata) of BABA-treated infected tissue. The red staining, characteristic for lignin accumulation, was mostly confined to the cells surrounding the inoculated

Table 1. Sporulation of *Plasmopara viticola* on grape leaf discs as affected by BABA (DL-3-aminobutyric acid). A UV-microscopical analysis with calcofluor

BABA, mg/L	Sporangia/mm^2	% Protection	Sporangiophores/mm^2	% Protection
0	508 ± 102 a	—	72 ± 7 a	—
12.5	28 ± 11 b	94	11 ± 6 bc	85
25	19 ± 5 b	96	10 ± 2 bc	86
50	14 ± 14 b	97	10 ± 1 bc	86
100	7 ± 7 b	99	4 ± 4 c	94

Ten mm diameter leaf discs were floated on 1 ml of the test solution, lower side uppermost, in 24-well titer plates. Two 10 µl droplets of 2×10^4 sporangia/ml of *P. viticola* were applied to each disc one day after floating. Fourteen days post-inoculation discs were placed on glass slides and 0.2 ml calcofluor (0.01% in water) was pipetted on each disc and covered with a cover slip. Sporulation was determined with the aid of a UV epifluorescence microscope. Figures followed by different letters are significantly different at 5% level ($n = 4$, Duncan's multiple range test).

site. The walls and cytoplasm of such cells were intensively stained.

The effect of BABA on mildew development was examined in leaf discs of 3 other cultivars. The cultivars used in addition to Emerald Reisling were Sultanina and Chardonnay. No significant differences were found between cultivars in their response to BABA. Visible sporulation was totally suppressed at $\geq$25 µg/ml. At 12.5 µg/ml, Chardonnay, Sultanina and Emerald Reisling showed sporulation in about 25% of the inoculated leaf discs.

To evaluate how long BABA activity can persist in grape leaf tissue, the following experiment was conducted: Leaf discs in petri dishes were placed on BABA solution of 200 µg/ml, or water, for one day, removed, blotted dry between paper towels and placed on water. Inoculation took place 1–14 days after such pulse-loading with BABA or water. Twelve days after inoculation sporangial counts were taken from the leaf discs to determine the magnitude of protection. Results (Figure 2) show that between 1 and 14 days after treatment, sporangial production in BABA-treated leaf discs were significantly reduced by approximately 80%. The lower counts at 14 days seemed to result from tissue senescence. It should be noted that leaf discs, which were inoculated 0 days after floating, were continuously maintained on BABA (Figure 2).

The post-infectional activity against grape downy mildew was examined in leaf discs inoculated with a 50 µl droplet/disc containing 1500 sporangia of *P. viticola*. At 0, 24, 48, 72 and 96 h after inoculation discs were transferred to petri dishes containing 200 µg/ml BABA solution on filter paper. Nine days after inoculation disease intensity was visually estimated and sporangial yield in discs was determined using a haemocytometer. The data presented in Figure 3 show that BABA induced a strong post-infectional resistance against *P. viticola*. When applied at time 0, sporulation was completely inhibited but the tissue beneath the inoculated site showed an HR. When BABA was applied 24 h after inoculation the HR symptoms remained but a few sporangia were produced. At 48 h, the lesions expanded slightly, but sporulation was still almost completely (98.7%) suppressed. Inhibition was only partially removed at 72 h. At 96 h, symptoms appeared almost normal but sporulation was still inhibited by 66% (Figure 3). Other experiments revealed that BABA had no effect on sporulation when applied to leaf discs bearing chlorotic lesions (8 days after inoculation).

The efficacy of the *S*- and the *R*-enantiomers of BABA on grape downy mildew development was compared on leaf discs (cultivar Superior). The *S*-enantiomer was totally ineffective, as sporulation occurred on all leaf discs at all concentrations. The racemate behaved as previously described, namely HR was observed at 16 and 32 µg/ml. The R-enantiomer was highly effective in suppressing fungal development. At $\geq$4 µg/ml it induced an HR and prevented visible sporulation (Data not shown).

Activity of aminobutyric acid against P. viticola in vitro

Due to the host-obligatory nature of the fungus, germination was the only phase in the life cycle that sensitivity of *P. viticola* to aminobutyric acids could be tested.

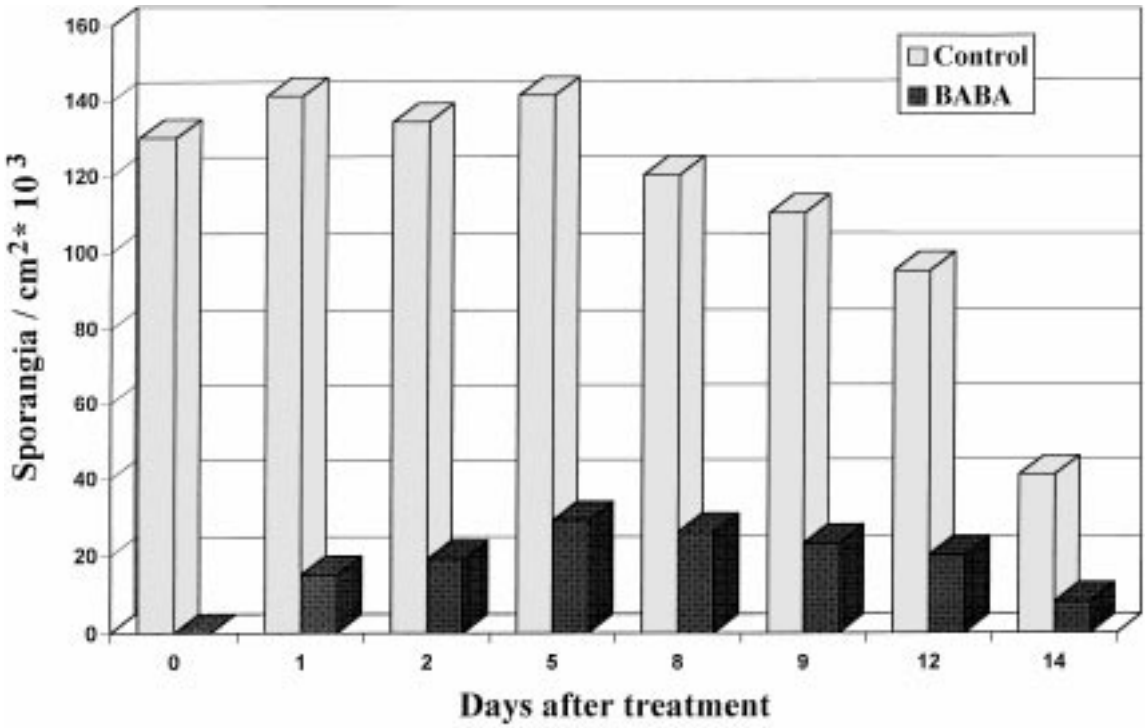

Figure 2. Persistence of BABA activity against *Plasmopara viticola* in grape leaf discs. Leaf discs were pulse-loaded with 200 μg/ml BABA for one day and then transferred to water and inoculated at 1–14 day intervals. Leaf discs in the 0 treatment were continuously kept on BABA. All figures in BABA treatments are significantly different at 5% level from the controls.

To assess such an activity, sporangial suspensions were kept on ice in 1.5 ml tubes and mixed with aqueous solutions of aminobutyric acids to various final concentrations, so that sporangial concentration (5000/ml) remained constant. Twenty μl of the mixtures were pipetted onto depressions of microscope slides and incubated at 14 °C for 20 h in the dark. Microscopical examination revealed that in water controls most of the sporangia were empty, many zoospores were motile and only a few cystospores germinated. A similar picture was observed with DL-2-aminobutyric acid, DL-3-aminobutyric acid and 4-aminobutyric acid at concentrations ranging from 250–2000 μg/ml. Based on our experience on the enhancement of cystospore germination with sucrose, 10 μl of 50 mM sucrose were added to each depression. After an additional 20 h we noticed in the water–sucrose controls germination of approximately 100 cystospores/depression with a germ tube of 200–600 μm in length. With BABA and GABA (+sucrose) of up to 2000 μg/ml the picture was similar to the control. However, in AABA of 1000–2000 μg/ml, germ tubes were much longer, reaching a length of 1000–2000 μm, and ramificated once or twice. Lowering the concentration of this compound to 250 μg/ml diminished this phenomenon.

Whole plant assays

Uptake and systemic translocation of ^{14}C-BABA

Assays with ^{14}C-BABA were conducted as foliar applications. Five days after such applications, most of the ^{14}C-label was recovered from the leaf surface with water. TLC analysis (Cohen and Gisi, 1994a) revealed that this label on the leaf surface is made of ^{14}C-BABA only.

The data on the uptake into the treated leaves and translocation to other organs are presented in Table 2. They show that out of the 1 μCi of ^{14}C-BABA applied to leaf 1 or 4, a total of only 5.02% and 3.49% of the label applied could be re-extracted with MCW from the various organs of the plant (leaves, green stem and roots). In contrast, a total of 29.99% could be recovered with MCW when the ^{14}C-BABA was applied to leaf 6 (youngest) indicating that uptake through the upper

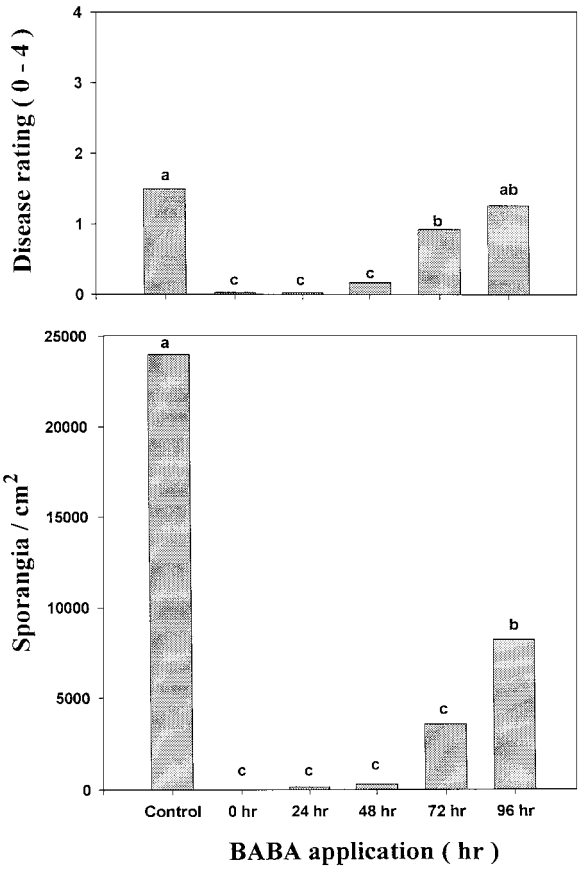

Figure 3. The post-infectional activity of BABA (200 μg/ml) against *Plasmopara viticola* in grape leaf discs. The fungus was inoculated to all leaf discs at time 0. At various time intervals, discs were transferred from water to BABA. Upper panel – disease rating. Lower panel – number of sporangia produced.

surface of young leaves is more efficient compared to old leaves. Most of the penetrating label was recovered from the treated leaf itself. The recovery of ^{14}C-BABA from the remote younger leaves indicates on acropetal transport. Label was recovered also from the root system, especially when leaf 1 was treated, indicating also on basipetal translocation (Table 2). Other experiments, conducted with detached leaves in petri dishes proved that uptake after 1 week ranged between 56.3–92.7% of the applied ^{14}C-BABA, suggesting that the low uptake in intact plants resulted from the hydrophilic nature of BABA and the quick drying of the label droplets. It should be mentioned that MCW extracts of ^{14}C-BABA-treated grape leaves contained ^{14}C-BABA, as proved by thin layer chromatography. This solvent mixture extracted 96% of the label present inside the tissue. The

Table 2. Uptake and distribution of ^{14}C-3-aminobutyric acid in grape plants after foliar application[a]

Analyzed leaf/organ	Percentage of recovered radioactivity treated leaf		
	1	4	6
leaf 1	26.0[b]	1.1	0.2
leaf 2	2.4	1.1	0.2
leaf 3	2.2	1.1	0.3
leaf 4	2.8	31.2[b]	0.3
leaf 5	5.4	3.7	0.4
leaf 6	18.6	12.0	92.6[b]
leaf 7	18.8	21.8	3.2
leaf 8	—[c]	20.3	—[c]
Stem	18.1	6.0	1.9
Root	5.7	1.7	0.9
Total	100.0	100.0	100.0
% of applied[d]	5.02	3.49	29.99

[a]Fifty 10 μl droplets of ^{14}C-BABA, containing a total of 1 μCi label were applied to the upper surface of either leaf1, 4 or 6 of 7-leaf grape plants. Five days later, plants were washed with water and organs extracted with MCW to determine radioactivity inside the tissue. (For details, see text.)
[b]Treated leaf.
[c]Leaf8 does not exist.
[d]The rest of the ^{14}C-BABA applied (1 μCi) was recovered from the leaf surface by water.

remaining 4% were bound to the cell walls, in a similar manner described in tomato (Cohen and Gisi, 1994a).

Spray application to intact plants

'Superior' plants, made from cuttings, carrying 4 expanded leaves were sprayed on both leaf surfaces with aqueous BABA solutions of various concentrations, placed in humid plastic tents in growth chambers at 20 °C for 1 day to facilitate BABA uptake, and inoculated two days afterwards by spraying the lower leaf surfaces with sporangial suspension (1×10^4/ml). Seven days after inoculation plants were placed in a dew chamber (18 °C darkness) for 24 h to induce fungal sporulation. Percentage leaf area (abaxial surfaces) covered with sporangiophores and sporangia was found to significantly decrease as concentration of BABA applied increased. Thus, when plants were sprayed with 0, 250, 500, 1000 and 2000 μg/ml solution of BABA *P. viticola* sporulated on 90, 70, 35, 5 and 0% (mean values, $n = 3$) of the leaf total area, respectively.

To evaluate the protection associated with systemic translocation of BABA, the compound was formulated as 25% wettable powder (Sandoz) and applied

Table 3. Systemic activity of BABA against *Plasmopara viticola* in grape plants[1]

No. of leaf from stem base	Percentage leaf area showing sporulation (Treated leaves)			
	Water control (1–8)	Blind formulation (1–8)	25% WP BABA (1–8)	25% WP BABA (1–3)
1	100 a	50 b	5 c	0 c
2	100 a	60 a	7.5 b	15 b
3	100 a	50 b	0 c	0 c
4	100 a	50 b	0 c	95 a
5	100 a	60 b	2.5 c	100 a
6	100 a	80 a	2.5 b	80 a
7	100 a	80 a	5 b	0 b
8	100 a	30 b	0 c	0 c
Mean	100.0	57.5	2.8	36.3

[1]Eight-leaf plants grown from seed (cv. Emerald Reisling), were sprayed with either 0.8% 25 WP BABA or with 0.6% WP blind formulation (containing no BABA) on all leaves or the lower 3 leaves only. Spray was applied to both leaf surfaces. Two days after spraying plants were inoculated with *P. viticola.* Seven days after inoculation the plants were kept for 2 days in moist plastic tents and leaf lower surface areas showing sporulation of the fungus was visually estimated. Figures in rows followed by the same letter are not significantly different ($n = 3$. Duncan's multiple range test).

(2000 μg/ml a.i.) to either the whole plant or to the 3 lower leaves of 8-leaf plants grown from seeds. Two days after spraying plants were inoculated with *P. viticola* and 7 days later were placed in moist plastic tents in a growth chamber for 2 days to induce fungal sporulation. Results shown in Table 3 indicate that a total spray with 25% WP BABA was highly effective in suppressing fungal sporulation. Controls without the active ingredient resulted in a limited inhibition of fungal sporulation as compared to water-treated inoculated plants. When the formulated BABA was applied to the 3 lower leaves, protection was observed in the treated leaves as well as in the youngest two untreated leaves, but not in the middle untreated leaves. The later result stands in accordance with the preferable acropetal translocation of ^{14}C-BABA.

Protection by applying BABA to the root system

An experiment was conducted to evaluate the protection of grape plants from downy mildew attack by root applications of BABA. 'Superior' 4-leaf plants produced from cuttings (having adventive root system) were used. Plants were carefully uprooted, their root system washed with water, and placed in vials containing 50 ml of aqueous BABA solution of various concentrations. Plants were left in the solutions for 3 days and the volume of the solution taken up was measured. Plants were then transferred to new vials filled with water, and inoculated with sporangia of *P. viticola*. A week after inoculation sporulation was induced and the extent of sporulation on lower leaf surfaces was visually estimated. The results showed that exposure of the root system to BABA protected the foliage from the mildew indicating the uptake and translocation of the compound in the vascular system of the plant. The extent of protection was directly related to the quantity of BABA taken up, 7.5 mg/plant sufficed for 50% protection and 15 mg/plant totally abolished fungal sporulation.

Discussion

The data presented in this paper demonstrate that BABA protects grape plants from infection by the obligate fungal parasite *Plasmopara viticola*. A similar capacity was performed by BTH. Five other isomers of aminobutyric acid, namely L-2-amino, DL-2-amino, DL-2-*iso*amino, DL-3-*iso*amino and 4-aminobutyric acids were totally ineffective, suggesting that the amino group has to be in a 3-position to the terminal carboxylic group for activity. The 3-amino acids are not incorporated into proteins in nature (Rosenthal, 1982) except one reported case in scytonemin in blue green alga (Helms et al., 1988). A single report on the occurrence of BABA in nature was as a secretion from

tomato roots following soil solarization (Gamliel and Katan, 1992). The DL racemate of BABA is composed of the *S*- and *R*-enantiomers. When tested separately the *R*-enantiomer was highly active whereas the *S*-enantiomer was totally inactive, indicating a specific stereo structure affinity of the molecule to a putative ligand in the host. Similar differential activity of the enantiomers were reported in tobacco (Cohen, 1994a). BABA (as well as *R*-BABA) had no adverse effect on spore germination of *P. vitocola* as well as on hyphal growth *in vitro* of various fungal pathogens (e.g. *Phytophthora infestans*, *Alternaria solani*, *Fusarium lycopersici*, Y. Cohen, unpublished). We postulate that it acts as an inducer of systemic resistance although no positive evidence is presented. The compound also did not affect fungal penetration into the host as could be judged from the hypersensitive response associated with the inoculum droplets placed on treated leaf discs. Lignification of BABA-treated leaf discs occurred only upon inoculation, thus making the case of induced resistance possible. Unlike INA and BTH, even at higher concentrations BABA caused no phytotoxic symptoms when administrated to either leaf discs (up to 500 μg/ml), whole plants (up to 4000 μg/ml) or the root system (up to 4000 μg/ml) of grape plants (data not shown).

One-day feeding of leaf discs with BABA was enough to protect them from disease for at least 14 days. Enhanced senescence of such discs prevented further pursuing the persistence of activity.

We assume that persistence in intact growing plants will decline with time due to dilution and translocation.

SAR normally requires a lapse period between treatment and inoculation (Kessmann et al., 1994; Ryals et al., 1996; Sticher et al., 1997). This allows for signal transduction and gene activation to take place. This is the case with SAR induction by either biological agents (e.g. TMV in N-tobacco) or chemical activators (e.g. SA, INA and BTH). Interestingly, BABA protected grape leaves from the mildew when applied post-infectionally. Even when applied at 48 h after inoculation protection achieved was 98.7%, based on the number of sporangia produced relative to the control. This means that BABA may activate host defense in such a quick and strong manner that it can suppress further development of already-developed fungus. It may be speculated that BABA deteriorates the fungus-penetrated host cells so that translocation of nutrients into the haustoria is blocked, thus prohibiting further mycelial growth and sporangial production (Steiner and Schonbeck, 1997; Stenzel et al., 1985). More histological data are required to prove such a hypothesis. Another hypothesis is that BABA quickly binds to host cell walls in a covalent manner, so modifying them that further fungal ingress is blocked (Schwizer and Cohen, unpublished data).

BABA, as other amino acids, translocates from source to sinks in the plant via the phloem (Peoples and Gifford, 1990). When applied to leaves of intact plants it reaches the youngest leaves, as could be judged from either the acropetal translocation of ^{14}C-BABA or from the protection observed in top leaves following a treatment with BABA given to the basal leaves. Exposing the root system of bare-root intact plants to BABA also protected the leaves from the mildew indicating xylem transportation from the roots. Xylem transportation of ^{14}C-BABA from roots was also demonstrated in tomato (Cohen and Gisi, 1994a). Xylem transport and phloem reallocation of amino acids is well-known in plants (Peoples and Gifford, 1990).

BABA was reported to protect tomato against *Phytophthora infestans* (Cohen, 1994b), tobacco against *Peronospora tabacina* (Cohen, 1994a), peppers against *Phytophthora capsici* (Sunwoo et al., 1996) and peanuts against leaf spot caused by *Cercosporidium personatum* (Zhang et al., 1998). Other reports demonstrated activity of BABA against soil-borne pathogens such as *Aphanomyces euticheS* in peas (Papavizas, 1964), *Fusarium lycopersici* f.sp. *solani* in tomato (Kalix et al., 1996; Li et al., 1996), *Verticilium dahliae* in cotton (Kalix et al., 1996; Li et al., 1996), *Fusarium oxysporium* f.sp. *melonis* in melons (Cohen, 1996) and *Fusarium oxysporium* f.sp. *niveum* in watermelon (Y. Cohen, unpublished). Activity was also observed against *Plasmopara hatstedii* in sunflower attacking via either the roots or the foliage (Y. Cohen, unpublished), and *Alternaria brassicicola* in broccoli (Cohen, 1996). It thus appears that BABA protects several crops against fungal pathogens belonging to various classes.

How exactly BABA confers its activity against fungal disease is partially understood. In tomato root application or foliage spray with BABA induced the accumulation of the pathogenesis-related (PR) proteins PR-1, chitinase and β-1, 3-glucanase (Cohen, 1994a; Cohen et al., 1994b). In tobacco, foliar spray similarly induced PR-proteins accumulation but, surprisingly, no PR-proteins were detected in plants either stem-injected with BABA (Cohen, 1994a) or treated via the roots (Ovadia and Cohen, unpublished), although plants were highly protected. This differential activity

indicates that PR-protein accumulation is not the sole mechanism of resistance. In melons, BABA induced peroxidase and lignification in roots upon inoculation with *Fusarium* (R. Bitton and Y. Cohen, unpublished). Induction of PR proteins by SA or *Botrytis cinerea* in grape leaves was demonstrated but no data on systemic resistance against foliar pathogens are given (Renault et al., 1996). Busam et al. showed that SA induced in grape leaves basic chitinase I and III whereas INA or BTH induces chitinase III only (Busam et al., 1997). The induction by BABA of lignin accumulation in HR-responding mesophyll cells hints at the possible involvement of enzymes like phenylalanine-ammonia lyase (PAL) and peroxidase. In fact, BABA was reported (Newton et al., 1997) to increase PAL activity and induce resistance against late blight in potato leaf discs.

The role of salicylic acid in BABA-treated plants was investigated (A. Ovadia and Y. Cohen, unpublished) in the transgenic NahG tobacco plants which constitutively expresses the salicylic acid hydroxylase gene (Delaney, 1997). BABA was found to be equally effective in NahG as well as the wild type Xanthi nc against blue mold suggesting that BABA may trigger the resistance-mediated signal transduction pathway down-stream of SA.

The data presented in this paper, together with our findings (unpublished) on the efficacy of BABA in vineyards against natural attack by downy mildew, makes BABA an attractive compound for practical agronomic use against *P. viticola* in grapes.

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