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Mechanisms of induced resistance in lettuce against *Bremia lactucae* by DL-β-amino-butyric acid (BABA)

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Abstract BABA induced local and systemic resistance in lettuce (*Lactuca sativa*) against the Oomycete *Bremia lactucae*. Structure-activity analysis showed no induced resistance by related amino-butanoic acids or β-alanine. The R-enantiomer of BABA induced resistance whereas the S-enantiomer did not, suggesting binding to a specific receptor. Other compounds known to be involved in SAR signaling, including abscisic acid, methyl-jasmonate, ethylene, sodium-salicylate and Bion® (BTH) did not induce resistance. Systemic translocation of ^{14}C-BABA and systemic protection against downy mildew were tightly correlated. BABA did not affect spore germination, appressorium formation, or penetration of *B. lactucae* into the host. Epifluorescence and confocal microscopy revealed that BABA induced rapid encasement with callose of the primary infection structures of the pathogen, thus preventing it from further developing intercellular hyphae and haustoria. Invaded host cells treated with BABA did not accumulate phenolics, callose or lignin, or express HR. In contrast, cells of genetically-resistant cultivars accumulated phenolics, callose and lignin and exhibited HR within one day after inoculation. The callose synthesis inhibitor DDG did not inhibit callose encasement nor compromised the resistance induced by BABA. PR-proteins accumulated too late to be responsible for the induced resistance. DAB staining indicated that BABA induced a rapid accumulation of H_2O_2 in the penetrated epidermal host cells. Whether H_2O_2 stops the pathogen directly or via another metabolic route is not known.

Keywords Downy mildew · Induced resistance · Butanoic acids

Abbreviations

ABA	Abscisic acid
AABA	DL-α-aminobutyric acid
BABA	DL-β-aminobutyric acid
BTH	Benzothiadiazole-S-methyl ester (Bion®)
CLSM	Confocal laser scanning microscopy
DAB	Diaminobenzidine
DDG	2-deoxy-D-glucose
dpi	Days post inoculation
GABA	γ-aminobutyric acid
hpi	Hours post inoculation
JA	Jasmonic acid
NaSA	Sodium salicylate
ROS	Reactive oxygen species
PV	Primary vesicle
SA	Salicylic acid
SAR	Systemic acquired resistance
SV	Secondary vesicle

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Introduction

Downy mildew caused by the Oomycete *Bremia lactucae* Regel is the most serious disease of lettuce (Lebeda et al. 2008). The pathogen produces moldy, yellow lesions on the leaves which turn necrotic and make the heads unmarketable. The disease can be controlled by chemicals including phenylamide (e.g., metalaxyl/mefenoxam)-based compounds, but isolates insensitive to these compounds are frequent. Disease resistance (*Dm*)-genes are also used to control the disease, but isolates evading recognition conferred by the *Dm*-genes often occur (Lebeda et al. 2008).

An alternative procedure to protect plants against disease is to activate their inherent defense mechanisms by specific biotic or abiotic elicitors. 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, Bion®), are well known elicitors of SAR in various plants against disease. The expression of SAR, triggered by either pathogen infection or treatment with NaSA or its functional analogues INA or BTH, is closely associated with the transcriptional activation of genes encoding pathogenesis related (PR) proteins (Goellner and Conrath 2008).

The non-protein amino acid DL-β-amino-butyric acid (DL-3-amino-*n*-butanoic acid, BABA) also activates induced resistance responses (Cohen 2002; Jakab et al. 2001). β-amino acids occur naturally in diverse forms. They have been found as free forms of metabolites, such as β-alanine and β-aminobutyric acid in mammals and lower organisms (Cohen 2002; Kim et al. 2007). BABA is capable of inducing local and systemic resistance against numerous plant pathogens (see review by Cohen 2002), including tobacco mosaic virus (Siegrist et al. 2000), bacteria (Flor et al. 2008), Oomycetes (Cohen 1994; Silue et al. 2002; Zimmerli et al. 2000), Ascomycetes (Porat et al. 2003; Reuveni et al. 2003; Zimmerli et al. 2001), Basidiomycetes (Amzalek and Cohen 2007), nematodes (Oka and Cohen 2001) and insects (Hodge et al. 2006).

Among the Oomycetes suppressed by BABA in their respective hosts are *Peronospora tabacina* in tobacco (Cohen 1994), *Hyaloperonospora parasitica* in *Arabidopsis* (Zimmerli et al. 2000) and cauliflower (Silue et al. 2002), *Plasmopara viticola* in grape leaves (Cohen et al. 1999; Hamiduzzaman et al. 2005; Reuveni et al. 2001; Slaughter et al. 2008), *Phytophthora infestans* in tomato (Cohen et al. 1994) and potato (Cohen 2002), *Phytophthora capsici* in pepper (Hwang et al. 1997), *Plasmopara halstedii* in sunflower (Tosi et al. 1998), and *Pseudoperonospora cubensis* in cucumber (Walz and Simon 2008). Ascomycetes/Fungi Imperfecti controlled by BABA are *Fusarium oxysporum* f. sp *solani* in tomato (Cohen 2002), *Botrytis cinerea* and *Plectosphaerella cucumerina* in *Arabidopsis* (Ton and Mauch-Mani 2004; Zimmerli et al. 2001), *Monosporascus cannanbolis* in melon (Cohen 2002), *Alternania alternata* in apple fruits (Reuveni et al. 2003), *Alternania brassisicola* in *Arabidopsis* (Ton and Mauch-Mani 2004), *Penicillium digitatum* in grapefruit (Porat et al. 2003), and *Colletotrichum cucumerinum* in cucumber (Walz and Simon 2008). *Puccinia helianthi* in sunflower is a basidiomycete controlled by BABA (Amzalek and Cohen 2007).

In *Arabidopsis thaliana* and cucumber, BABA was shown to be effective against both necrotrophic and biotrophic pathogens, as well as abiotic stresses like drought and salinity (Jakab et al. 2001; Jakab et al. 2005; Walz and Simon 2008; Zimmerli et al. 2001). BABA effectively controlled diseases in the field, e.g. downy mildew in grapevines (Reuveni et al. 2001), moldy core in apple fruits (Reuveni et al. 2003), late blight in potato and tomato, and sudden wilt in melon (Cohen 2002). BABA acts synergistically with Bion® or with metalaxyl to protect tobacco against downy mildew (Cohen 2002).

The cellular mechanisms behind BABA-induced resistance are diverse. In *Arabidopsis* inoculated with *Hyaloperonospora parasitica*, BABA induced callose deposition in sites of penetration, along walls of the penetrated host cells and in the pistil which causes female sterility (Jakab et al. 2001; Zimmerli et al. 2000); in grape leaves inoculated with *Plasmopara viticola*, it induces a massive accumulation of lignin (Cohen et al. 1999), callose (Hamiduzzaman et al. 2005) and pterostilbene (Slaughter et al. 2008); in tobacco inoculated with *Peronospora tabacina* (Cohen 1994) and sunflower inoculated with *Puccinia helianthi* (Amzalek and Cohen 2007), no microscopic cellular responses were seen.

The molecular mechanisms behind BABA-induced resistance are also very diverse. In *Arabidopsis*, BABA exerts its function via priming of SA-

dependent defense mechanisms (Goellner and Conrath 2008; Zimmerli et al. 2000) or through potentiation of ABA-dependent signaling pathways (Ton and Mauch-Mani 2004). Resistance against TMV in tobacco (Siegrist et al. 2000), and against *Pseudomonas syringae* pv *tomato* DC3000 and *Botrytis cinerea* in *Arabidopsis* resembles SAR in that it requires endogenous accumulation of SA and an intact NPR1/NIM1/SAI1 protein (Zimmerli et al. 2000; Zimmerli et al. 2001). In contrast, resistance against *Phytophthora infestans* in tomato (Y. Cohen, unpublished), *Peronospora tabacina* in tobacco (Cohen 1994) and *Hyaloperonospora parasitica* in *Arabidopsis*, as well as against the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis* is unaffected in SA-non accumulating plants and in SA-insensitive plants (Ton and Mauch-Mani 2004; Zimmerli et al. 2000). Mutants impaired in the production or sensitivity to the stress hormone abscisic acid (ABA) are blocked in BABA-induced resistance against *Plectosphaerella cucumerina* (Ton and Mauch-Mani 2004). An *Arabidopsis* mutant impaired in the production of callose (*pmr4-1*) failed to show BABA-induced resistance against *Alternaria brassicicola* (Flors et al. 2008). 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 (Ton et al. 2005).

In a recent study (Cohen et al. 2007), we showed that two sprays of BABA, applied to field-grown lettuce, effectively controlled downy mildew caused by the Oomycete *Bremia lactucae*. The objective of this study was to reveal the mechanisms by which BABA acts against downy mildew in lettuce. Microscopic examinations showed that BABA stopped *Bremia lactucae* in lettuce soon after it penetrated into the host. Unlike in *Arabidopsis*, SA, JA and ABA play no role in BABA-induced resistance against downy mildew in lettuce.

Materials and methods

Plants The susceptible lettuce (*Lactuca sativa* L) cultivar Noga (cup type, Hazera Genetics, Mivhor, Israel) was used unless stated otherwise. Plants were grown from seeds in 175 ml pots containing peat/vermiculite mixture (1/1, v/v), ~20 plants per pot. Plants were grown in the greenhouse (18–26°C) and used one week after seeding, when they had developed both cotyledons. In some experiments, plants were grown in Speedling trays (25 ml per cell), 1 plant/cell and used when had 5–6 true leaves, or in 0.25 L pots, 1 plant/pot and used at the 5–10 true leaf stage. Plants were fertilized with 0.5% N:P:K (20:20:20) once a week.

Pathogen Six isolates of *Bremia lactucae* Regel were used: IL60, BL18, BL21, BL22, BL24, and BL25. Isolate IL60 was a gift from K. Sharaf (University of Haifa, Israel) and the others, a gift from A. Lebeda (University of Olomuc, Czech Republic). The pathogen was maintained by repeated inoculation of detached cotyledons in growth chambers.

Chemicals The following aminobutyric acids and related compounds were tested (Fig. 1): DL-2-amino-*n*-butanoic acid (AABA), 2-amino-isobutanoic acid (iso-AABA), DL-3-amino-*n*-butanoic acid (BABA), DL-3-amino-isobutanoic acid (iso-BABA), 4-amino-butanoic acid (GABA), (R)-3-amino-butanoic acid (R-BABA), (S)-3-amino-butanoic acid (S-BABA), 3-amino-3-methyl-butanoic acid, DL-3-methylamino-butanoic acid, (DL)-3-benzylamino-butanoic acid, and 3-aminopropionic acid (β-alanine). The two SAR compounds sodium salicylate (NaSA) and benzothiadiazole-S-methyl ester (BTH, Bion®) and the SAR modulators abscicic acid, methyl jasmonate and ethephone were also tested. All compounds as well as 2-deoxy-D-glucose (DDG), ethephone (39.8%) and calcofluor were purchased from Sigma, Israel, except Bion® which was a gift from Syngenta, Switzerland. All compounds were dissolved in water before use.

Application of compounds Compounds were each diluted in water to a series of concentrations and applied to lettuce plants by either spraying onto leaf surfaces until run-off or to the root system as a soil drench. The compounds were applied at 0– 2 days before inoculation, depending on the experiment (see below). In other experiments, 10 µl droplets of BABA were applied to the abaxial surface of detached cotyledons placed on moist filter paper in Petri dishes, one droplet per cotyledon.

BABA was also applied to dry seeds or to germinating seeds of lettuce. Dry seeds were agitated in BABA solutions of various concentrations (1,000,

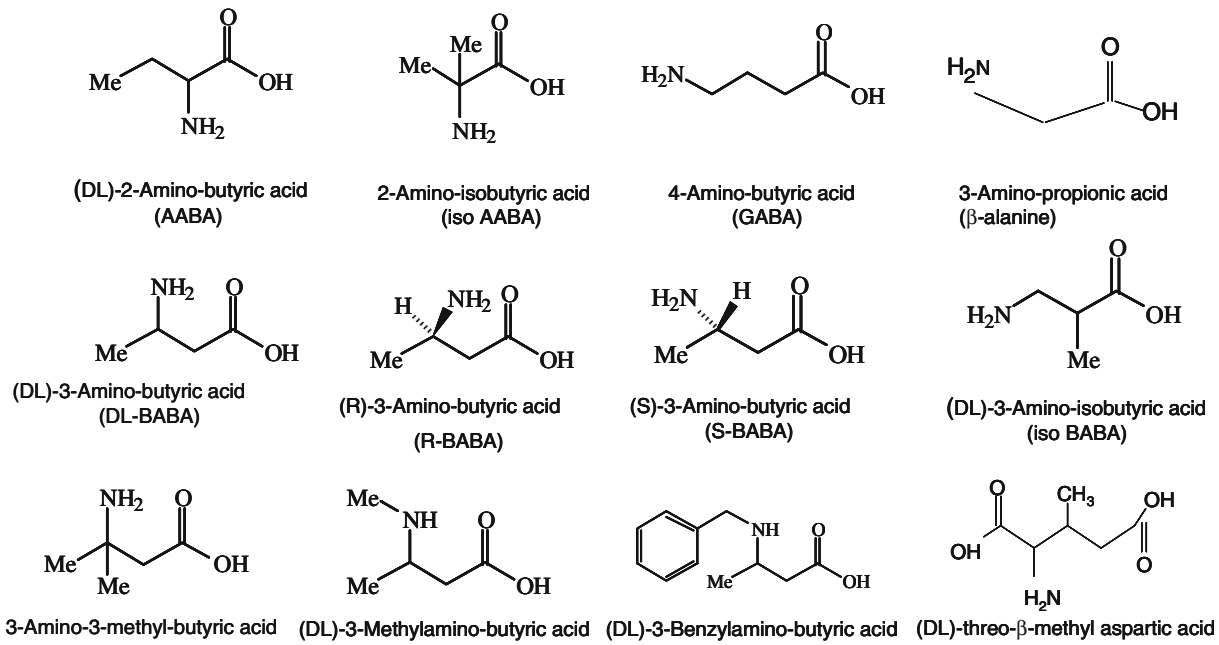


Fig. 1 Chemical structure of aminobutyric acids and related compounds tested for ability to protect lettuce against *Bremia lactucae*

2,000, 5,000 or 10,000 µg ml^{-1}) for 12, 24 or 48 h, sown in soil without wash and inoculated 7 days later when they had both cotyledons expanded. Other seeds were placed on moist filter paper in Petri dishes, incubated at 4°C for 2d to induce germination and then at 20°C (12 h light/day) for 2d to produce rootlets. The germinating seeds were then soaked in BABA for 1d and transplanted into soil in pots. Plants were inoculated a week later when the cotyledons were fully expanded.

Inoculation Spores of *B. lactucae* Regel were collected from freshly sporulating lettuce leaves into ice-cool double distilled water. The spore concentration was adjusted to 1×10^4 spores/ml and sprayed onto the upper leaf surfaces of the test plants until run-off, using a glass atomizer. Subsequently, plants were placed in a dew chamber (100% relative humidity, 18°C, darkness) for 20 h and then transferred to a growth chamber at 18–20°C (12 h light /day, 100 µE.m^{-2}.s^{-1}). At 5–7 days post inoculation (dpi), plants were placed in transparent plastic boxes (100% relative humidity) for one or two days to induce sporulation of the pathogen on the leaves. In some experiments, compounds were mixed with spores and applied as droplets of 20 µl to detached cotyledons or leaf discs placed on a moist filter paper in Petri dishes (see below).

Disease assessment For plants at their cotyledon stage, the number of sporulating plants was determined with the aid of a magnifying lens at ×10. To quantify spore production, sporulating cotyledons were detached, placed each in 2 ml 50% ethanol, shaken at 100 rpm for 10 min and the number of spores was counted using a haemocytometer. For plants having true leaves, the proportion of infected leaf area or the proportion of leaf area showing sporulation in each plant or leaf was visually assessed. Unless stated otherwise, records were made at 7 dpi.

Abscisic acid, methyl-jasmonate and ethephone

Seven day-old plants were treated via the root system (5 ml per 20 plants growing in a 175 ml pot) with abscisic acid (ABA, 1, 10, 100 or 1,000 µM) at concentrations 0.0075, 0.075, 0.75, or 7.5 µg ml^{-1} soil, alone or with BABA at 28.6 µg ml^{-1} soil. Methyl-jasmonate (MJ, 1.25, 2.5, 5, 10 or 20 mM) was added to soil at concentrations of 3.58, 7.15, 14.3, 28.6 or 57.2 µg ml^{-1} soil, alone or with BABA

at 28.6 µg ml^{-1} soil. Ethephone (ethylene releasing compound), was applied to the foliage at 0.015, 0.03, 0.06, 0.125, 0.25 or 0.5% (active ingredient), alone or with 1,000 µg ml^{-1} BABA. Plants were inoculated with *B. lactucae* two days after treatment. Disease records were made at 7 dpi by counting the number of sporulating plants in a pot.

Systemic translocation of ^{14}C BABA Radiolabelled DL-BABA (CH_3-^{14}CH (NH_2)-CH_2-COOH) with a specific activity of 9.68 µCi mg^{-1} (1 mCi $mmol^{-1}$) and a purity of >98% was supplied by the former Sandoz Pharma AG, Basel, Switzerland. The compound was dissolved in water (50×10^6 decompositions per minute (dpm) ml^{-1}) and applied to lettuce plants as follows: (a) 20 µl (1×10^6 dpm) to the root system of 7-day-old plants growing in perlite in 20 ml glass vials, 10 plants per vial; (b) 18 droplets, 5 µl each (total 4.5×10^6 dpm), onto the surface of leaf no. 4 (from stem base) of a 7-leaf plant growing in 175 ml pots. Plants were incubated in a growth chamber at 20°C (12 h light/day). At 2, 18 and 45 h, plants growing in vials were carefully uprooted, washed copiously with water, blotted dry, and pressed between thick papers for a week, and then exposed to X-ray film for 50 h. Other plants were washed as above, extracted with MCW (methanol:chloroform: water, 12:5:3) as described before (Cohen and Gisi 1994) and the radioactivity of the extract was measured using a Packard Tri-Carb Liquid Scintillation Analyzer 1600 CA (Canberra Packard, Victoria). The same procedure was performed with the 7-leaf plants except that sampling was done once, at 48 h after labeling.

Systemic protection with BABA BABA (1,000 and 2,000 µg ml^{-1}) was sprayed onto both surfaces of leaf no. 4 of 7-leaf plants (n=4). Control plants were left untreated. Plants were spray-inoculated with *B. lactucae* 4 h later, and sporulation intensity of the pathogen on each leaf was recorded at 7 dpi as described above.

Spore germination in vitro Spores were mixed with BABA and applied to depression glass slides (20 µl/depression, n=3). Slides were kept on moist filter paper in Petri dishes at 13–15°C for 20 h in the dark. Germination was recorded with the aid of a dissecting microscope at ×160. Records were taken from 100 spores/depression.

Spore germination in planta Tests were performed with cotyledons detached from 7 day-old plants or with leaf discs (12 mm diameter, taken from leaf no. 5 from the stem base of 8-leaf plants). Two types of assays were performed: (a) cotyledons or leaf discs (n=7) were placed on a 7 cm diameter Whatman no. 1 filter paper saturated with 3 ml BABA solution (15.6, 31.25, 62.5, 125, and 250 µg ml^{-1}) in 9 cm Petri dishes and each inoculated with a 20 µl droplet of spore suspension containing 500 spores. Dishes were incubated as above. (b) Spores were mixed with BABA in test tubes and a 20 µl droplet was applied to cotyledons or leaf discs (n=7). Cotyledons or leaf discs were placed on moist filter paper in Petri dishes and incubated as described above. In both assays, 20 µl of 0.02% calcofluor was added to each leaf or leaf disc and germination was assessed with the aid of a UV epi-fluorescence microscope (see below) at ×160. Fungal structures fluoresced blue. Records were made from 100 spores per leaf or leaf disc.

Microscopy of pathogen development and host responses Cotyledons were detached from 7 day-old plants and placed with the abaxial surface up on moist filter paper in Petri dishes. Leaves were each inoculated with a 20 µl water droplet containing 200 spores of *B. lactucae* and 5 µg BABA. Dishes were incubated at 15°C (first 20 h in the dark and then, at cycles of 12 h light, 100 $\mu E.m^{-2}.sec^{-1}$ and darkness). Cotyledons were harvested at different time intervals post inoculation and examined for the presence of phenolics (autofluorescence), callose, lignin and necrotic cells (hypersensitive response, HR). For detecting phenolics, leaves were placed on a glass slide, covered with a glass slip and examined with the aid of an Olympus AX70 epi-fluorescence microscope equipped with excitation filter BP 330–385 nm and emission filter 420 LP. Phenolics emitted blue fluorescence (Cohen et al. 1990). To detect callose, leaves were cleared in boiling ethanol for 10 min, placed in 0.05% aniline blue in 70 mM potassium phosphate buffer (pH 8.9) at 4°C for 24 h, placed on a glass slide, treated with a drop of 0.02% calcofluor and examined with the aid of the epifluorescence microscope described above. Callose fluoresced yellow while spores and germ-tubes fluoresced blue (Cohen et al. 1990). For lignin detection, leaves were cleared as above, placed on a glass slide and treated with 2% phloroglucinol dissolved in ethanol followed

by 25% HCl (Cohen et al. 1989; Cohen et al. 1990). Lignin stained red. HR of host cells was examined with a Carl Ziess microscope under bright light.

Confocal laser scanning microscopy CLSM was used to obtain three-dimensional images of *B.lactucae* in control and BABA-treated lettuce leaves. Cotyledons were detached from 7 day-old plants and placed with the abaxial surface up on moist filter paper in Petri dishes. Leaves were each inoculated with 20 µl water droplet containing 200 spores of *B. lactucae* and 5 µg BABA. Leaves were harvested at 1 and 5 dpi, cleared in boiling ethanol for 5 min, placed in 0.05% aniline blue in 70 mM potassium phosphate buffer (pH 8.9) at 4°C for 24 h, placed on a glass slide, treated with a drop of 0.02% calcofluor and examined with the aid of a Zeiss confocal microscope LSM 510 META (excitation 405 nm, emission with a spectral detector).

PR-proteins BABA or Bion® (each at 500, 1,000 or 2,000 µg ml^{-1}) was applied to the foliage of plants at the second true leaf stage. Untreated plants served as controls. At 3 h after treatment, plants were spray-inoculated with *B. lactucae* (1×10^4 spores ml^{-1}) or with water as a control. Leaves were harvested at various time intervals after inoculation, and PR-1a and PR-5 were detected in extracts by Western blotting as described before (Cohen 1994).

Detection of hydrogen peroxide H_2O_2 was detected macroscopically and microscopically by diaminobenzidine (DAB) (Sigma fast DAB tablets) staining. DAB polymerizes instantly, and develops a localized dark color as soon as it comes into contact with H_2O_2 in the presence of peroxidase (Thordal-Christensen et al. 1997). BABA was applied as a soil drench (28.6 µg ml^{-1}) to two week-old plants growing in 175 ml pots. Plants were inoculated one day later. The first true leaf was detached at 6 h post inoculation (hpi), infiltrated *in vaccuo* with DAB for 10 h at 20°C in light, washed with water, placed on a glass slide, stained with calcofluor and photographed using an epifluorescence microscope under mixed illumination of UV and incandescent lights. In other experiments, cotyledons or first true leaves were excised from two week-old lettuce plants, placed with the abaxial side up on moist filter paper in Petri dishes, and inoculated with two 20 µl droplets of spores of *B. lactucae* suspended in either water or BABA (final concentration 62.5 µg ml^{-1}). At 2 dpi, the cotyledons and leaf discs (10 mm diameter) that were removed from the inoculated true leaves, were each immersed in 1 ml DAB (1 mg ml^{-1}) under vacuum for 5 h at 20°C in the light, washed with water and photographed with the aid of a stereo microscope.

Compromising the resistance induced by BABA The callose synthesis inhibitor 2-deoxy-D-glucose (DDG) was applied to either intact or detached inoculated cotyledons with or without BABA, and its effect on the development of *B. lactucae* was examined. Intact, 7 day-old plants (~20 plants/pot, 10 pots per treatment) were sprayed with either water, 1,000 µg ml^{-1} (10 mM) BABA, 1,640 µg ml^{-1} (10 mM) DDG or 1,000 µg ml^{-1} BABA followed (after 3 h when the spray droplets have dried up) by 1,640 µg ml^{-1} DDG. Plants were inoculated 3 h after the second spray and sporulation was evaluated at 7 dpi. In other experiments, detached cotyledons were treated with DDG, BABA or their mixture thereof and the development of *B. lactucae* in the leaves was examined microscopically, or the number of sporophores produced was counted after calcofluor staining.

Statistical analysis Experiments were repeated at least there times. One representative set of data are presented. Number of replicates per treatment varied according to the experiment: for intact 7 day-old plants, there were 3–10 replicated pots with ~20 plants/pot per treatment; for adult plants, there were 4–10 replicated plants per treatment; for detached cotyledons or leaf discs, there were 7–12 leaves or leaf discs per treatment. The data were subjected to one-way analysis of variance (ANOVA) after using the Fit Y by X model, using the SAS JMP® 7.0 software. The means were separated with student's *t*-test at $\alpha = 0.05$ and different letters were used to indicate significant differences between means.

Results

Protection induced by aminobutanoic acids, not by NaSA and BTH (Bion®)

The protective efficacy of DL-AABA, DL-BABA, R-BABA, S-BABA, GABA, NaSA and Bion (BTH)

against *B. lactucae* is shown in Fig. 2. The compounds were applied to intact plants at their cotyledon stage (7 day-old) as a foliar spray (Fig. 2a) or as a soil drench (Fig. 2b). For both modes of application, only DL-BABA and R-BABA gave significant protection against downy mildew. A foliar spray with 125 µg ml^{-1} DL-BABA reduced the number of diseased plants by 70% and ≥500 µg ml^{-1} gave

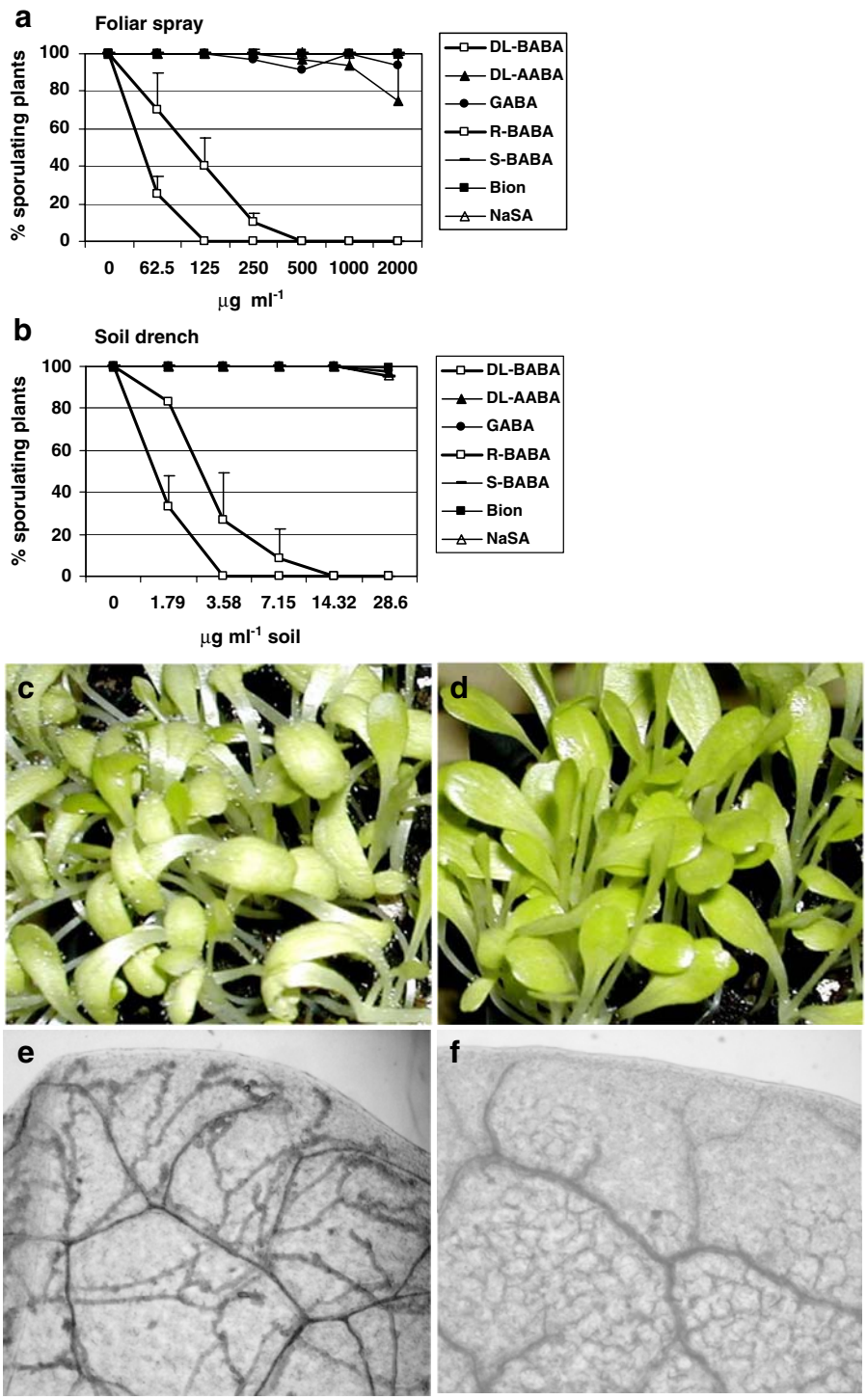


Fig. 2 Protection of lettuce induced against *Bremia lactucae* by aminobutyric acids, Bion and NaSA. **a** foliar spray; **b** soil drench. Plants were sprayed or treated via the roots with different concentrations of the compounds and inoculated with *Bremia lactucae* two days later. Percentage plants showing sporulation of the pathogen at 8 dpi is presented. The error bars represent the standard deviation (±SD) of the means. Note that DL-BABA and R-BABA were the only effective compounds. Disease rating values for the other compounds tested approached 100% and are not distinguishable due to overlapping. Photographs were taken at 8 dpi. **c** and **e** water-treated, control inoculated plants.**d** and **F)** BABA-treated (28.6 µg ml^{-1} soil) inoculated plants. Note sporulation in **c**, but not in **d. e–f** Trypan blue staining of inoculated cotyledons. Note mycelia in **e**, but not in **f**

complete protection (Fig. 2a). Plants treated with 14.3 μg DL-BABA ml^{-1} soil (Fig. 2b) were fully protected from the disease. The R-enantiomer of BABA was significantly (α=0.05) more effective than DL-BABA, whereas the S-enantiomer was totally ineffective. The other aminobutyric acids (Fig. 1) did not give protection, i.e., 2-amino-isobutyric acid, (DL)-3-amino-isobutyric acid, 3-amino-3-methyl-butyric acid, (DL)-3-methylamino-butyric acid and (DL)-3-benzylamino-butyric acid. The related compounds DL-threo-β-methyl-aspartic (2-amino-3-methylsuccinic acid) acid and 3-aminopropanoic acid (β- alanine) did not give protection either. Compounds with a phenyl group (3-phenyl-butyric acid) or bromine (3-bromo-butyric acid) at the β-position were also ineffective. NaSA and its functional analogue BTH (Bion®) applied to the foliage or the root system were both ineffective in protecting against the disease (Fig. 2a, b), suggesting that the protection induced by BABA is mediated by SA-independent pathway(s).

Whereas control plants allowed for profuse sporulation of the pathogen at 7 dpi (Fig. 2c), plants treated with BABA via the root system (28.6 μg ml^{-1} soil) and inoculated with *B. lactucae* one day later did not allow the pathogen to develop any symptoms at 7 dpi nor to sporulate (Fig. 2d). Trypan blue staining of cleared cotyledons showed mycelial colonization in control leaves (Fig. 2e), but not in BABA-treated leaves (Fig. 2f).

BABA protects lettuce after various modes of application

Soaking dry seeds BABA did not give protection when applied to dry seeds of lettuce. Plants developed from seeds that were agitated in BABA solutions (up to 10,000 μg ml^{-1}, for up to 48 h), showed sporulation of the pathogen as did the control untreated plants (data not shown).

Soaking germinating seeds Soaking germinating seeds of lettuce in BABA protected them from downy mildew attack. Percentage inhibition of the disease was 50 and 100 % for germlings treated with 125 and 2,000 μg BABA ml^{-1}, respectively. No signs of toxicity (chlorosis or necrosis) were seen on BABA- treated plants before or after inoculation (data not shown).

Seedlings in perlite culture and speedings BABA applied to the root systems of 7 day-old plants growing in perlite cultures gave ~90% and 100% protection for 12.5 and 50 μg BABA ml^{-1} perlite, respectively (Fig. 3a). BABA administrated to the soil surface of commercial 5-leaf plants of *cvs*. Paris Island and Noga, growing in one-inch cells in Speedling trays, provided significant increased levels of protection as the concentration increased. At a rate of 5 or 10 μg ml^{-1} soil, the number of lesions was reduced by ~80 and 95%, respectively, relative to control plants (Fig. 3b).

Isolates and cultivars Protection induced by BABA was independent of the isolate or the cultivar used for inoculation. Detached cotyledons of *cvs.* Noga and Cobham Green (*Dm0*) were inoculated with each of six isolates of *B. lactucae***.** Spore yield per leaf at 7 dpi was significantly suppressed in BABA-treated leaves relative to the controls in both cultivars and all isolates (Fig. 3c and d).

In other experiments, seeds were allowed to germinate in 9-cm Petri dishes filled with perlite. At 7 days, BABA was added to the perlite (25 μg ml^{-1}) and plants were spray-inoculated with either one of the above isolates of *B. lactucae*. At 10 dpi, 55–95% of the control-inoculated plants (depending on the isolate) died of the disease as compared to 0–6% of the treated plants. Similar results were obtained with isolate CH inoculated on *cv.* Crispa (both obtained from Syngenta, Stein, Switzerland). Complete control of the disease on plants at the first true leaf stage was obtained with a spray with 500 μg ml^{-1} or with a soil drench of 25 μg ml^{-1} soil (data not shown).

Mixing BABA with spores BABA mixed with spores of *B. lactucae* during droplet inoculation was inhibitory to infection (Fig. 3e). The quantity of BABA required to totally suppress subsequent sporulation in *cv.* Noga inoculated with isolate IL60 was 1.25 and 2.5 μg per droplet (site inoculated) for detached cotyledons and leaf discs, respectively.

Floating leaves on BABA solutions BABA reduced the development of the pathogen in leaf discs inoculated and immediately floated on BABA. Thus, at 7 dpi, spore production was totally inhibited in leaf discs floating on a solution with ≥15.6 μg BABA ml^{-1}. BABA also suppressed spore production of *B. lactucae* when applied to already infected cotyledons (Fig. 3f). BABA significantly suppressed sporulation

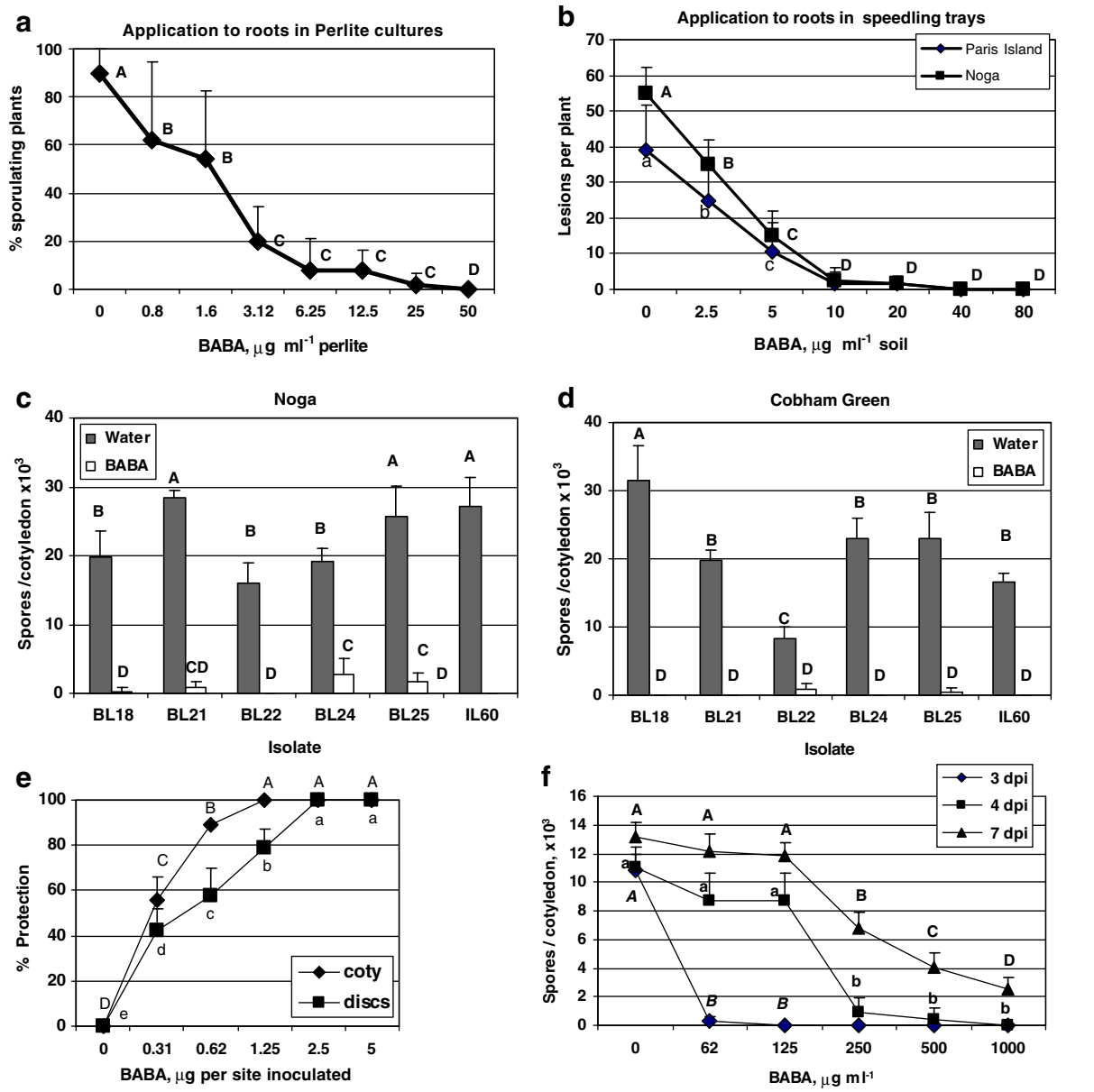


Fig. 3 **a** Protection of lettuce cv. Noga induced against *Bremia lactucae* by BABA applied to the roots of seedlings growing in perlite. Percent sporulating plants was recorded at 7 dpi. Different letters on bars indicate significant (α=0.05) differences between means. **b** Protection induced by BABA applied at various doses to soil in which two lettuce cultivars were grown in Speedlings trays. Different letters on bars indicate significant (α=0.05) differences between means. **c** Protection induced by BABA against 6 isolates of *B. lactucae* in the lettuce cultivar Noga. The error bars represent the standard deviation (±SD) of the mean. Different letters on bars indicate significant (α=0.05) differences between means. **d** As in **C,** but on cultivar Cobham Green (*Dm0*). Assay was performed in detached cotyledons. The error bars represent the standard deviation (±SD) of the mean. Different letters on bars indicate significant (α=0.05) differences between means. **e** Mixing BABA with spores prevented infection in detached cotyledons or leaf discs of lettuce Number of spores of *B. lactucae* produced in controls was $12.7\pm1.6\times10^3$ per cotyledon, and $50.8\pm6.4\times10^3$ per leaf disc (20 mm in diameter). Different letters on bars indicate significant (α=0.05) differences between means. **f** Floating infected cotyledons on BABA suppressed sporulation of *B. lactucae*. Infected cotyledons were detached at 3, 4, or 7 dpi, floated on water or BABA for 4, 3 and 1 day, respectively, and the number of spores per leaf was counted. Different letters on curves indicate significant (α=0.05) differences between means

relative to the controls; the older the infection, the higher the concentration of BABA was required to suppress sporulation. BABA also significantly reduced spore yields in lesions of downy mildew collected from the field. Thus, sporulation in leaf discs, each carrying a single lesion and floated on 250, 500 or 1,000 µg BABA ml^{-1} for 24 h displayed reduced sporulation of 48.3, 70.5 and 96.5%, respectively, compared to control discs floated on water which produced 11.2± 1.5×10^3 spores per lesion per disc.

Adult plants BABA was highly effective in protecting adult plants against downy mildew (Fig. 4). Plants at their 10-leaf stage were either sprayed or soil-drenched with BABA of various concentrations, inoculated with *B. lactucae* 1 day later, and examined for sporulating leaf area at 8 dpi. There was a dose-dependent reduction of sporulating leaf area (Fig. 4a, b) relative to control plants (Fig. 4c) with a complete suppression for spraying with 1,000 µg BABA ml^{-1} (Fig. 4a) or a 28.6 µg ml^{-1} soil drench (Fig. 4b, d). No signs of toxicity (chlorosis and necrosis) were seen on the treated plants (data not shown).

Systemic translocation of ^{14}C-BABA is related to protection with BABA Uptake and acropetal translocation of radioactive BABA from the root system of 7 day-old plants is shown in Fig. 5 (a–c). The compound was taken up by the root within 2 h (Fig. 5a) and reached the cotyledons in 18 h (Fig. 5b). Heavy labeling was seen in all organs by 45 h (Fig. 5c). The amount of ^{14}C-BABA taken up by each plant at 3 days after application ranged between 1.02–3.15% of the totally applied labeled BABA (mean=1.7±0.6%). When applied to leaf number 4 of 7-leaf plants, the compound translocated within 48 h to the top leaf and to the root (Fig. 5d). Thirty percent of the applied ^{14}C-BABA accumulated in the treated leaf (no. 4), 38% was translocated acropetally to leaf no. 7, 26% was translocated basipetally to the roots and only 9% was translocated to leaves no. 1, 2, 3, 5 and 6. The protection provided by BABA applied as a spray to leaf no. 4 is shown in Fig. 5e. Leaf no. 4 and the leaves above, i.e., nos. 5, 6 and 7, were fully protected whereas the leaves below the treated leaf, i.e., nos. 1, 2 and 3, were only partially protected, more so with 1,000 µg ml^{-1} than with 2,000 µg ml^{-1}(Fig. 5e).

The protection from BABA applied as a spray or root treatment, is shown in Fig. 5f. Seven day-old plants with both cotyledons developed and growing in 175 ml pots (~20 plants/pot) were either sprayed (1,000 µg ml^{-1}) or drenched (40 µg ml^{-1} soil) with BABA. Plants were incubated in a growth chamber until inoculated 27 days later, when they had developed 5 new true leaves. Percent protection of these true leaves relative to control plants, as evaluated by sporulation of the pathogen was 82 and 98% for sprayed and drenched plants, respectively. In a similar experiment, 7 day-old plants were drenched with 28.6 µg BABA ml^{-1} soil and inoculated at 14 days after treatment, when 3 new true leaves were developed. The protective efficacy ranged between 90–95% relative to control plants (data not shown).

Histochemistry of the lettuce -*B. lactucae* interaction in control plants

The dynamics of pathogen development in untreated, detached inoculated cotyledons is shown in Fig. 6. Spore germination on the leaf surfaces occurred at about 3 h (Fig. 6a), penetration into host epidermal cells and formation of a primary vesicle (PV) at about 6 h (Fig. 6b), formation of a secondary vesicle (SC) and initial hyphae at about 12 h (Fig. 6c), formation of the first haustoria in the mesophyll at 36 h (Fig. 6d). Mycelial runners were seen at 2 dpi (Fig. 6e) and their extensive spread at 4 dpi (Fig. 6 g). Intercellular mycelium runners, 0.5–1.5 mm long, carried frequent "holes" which emit yellow fluorescence (Fig. 6d, e). These holes represent the callose collars surrounding the haustorial necks (Fig. 6f). Fig. 6h, i show the emergence of sporophores from stomata at 7 dpi. When inoculation was done with a mixture of spores belonging to opposite mating types, mating between an antheridium and an oogonium was seen and resulted in the formation of oospores at 8 dpi (data not shown).

Histochemistry of lettuce-*B. lactucae* interaction in BABA-treated plants

BABA does not affect spore germination, appressorium formation and penetration BABA mixed with spores of *B. lactucae* had no inhibitory effect on germination in vitro. In water, mean germination was 70% and mean germ-tube length was 50 µm. In the presence of 5–20 µg BABA per 10 µl droplet (500–2,000 µg ml^{-1}),

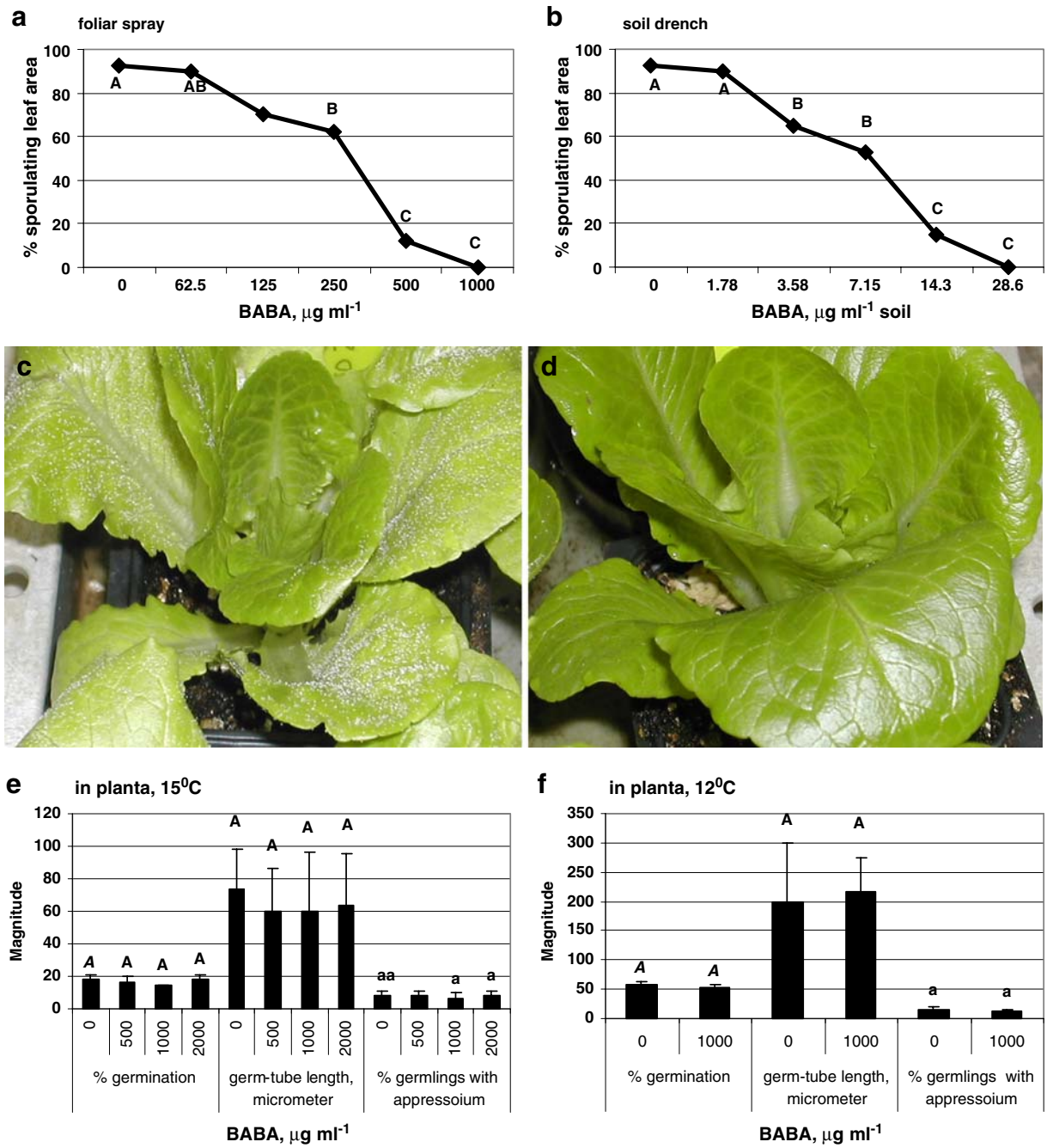


Fig. 4 Protection induced by BABA against *Bremia lactucae* in 10-leaf stage lettuce plants. **a** foliar spray; **b** soil drench. The percentage of sporulating leaf area was visually estimated at 7 dpi. Different letters on curves indicate on significant (α= 0.05) differences between means. **c** Control inoculated plant, note the white sporophores and spores on leaves surface. **d** BABA-treated (28.6 μg ml^{-1} soil) inoculated plants; note that no spores are seen. **e–f** Conidial germination, germ-tube extension and appressorium formation of *B. lactucae* on detached cotyledons as affected by BABA. **e** at 15°C and **f** at 12°C; Records were made at 24 hpi. The error bars represent the standard deviation (±SD) of the mean. Different letters on bars indicate significant (α=0.05) differences between means

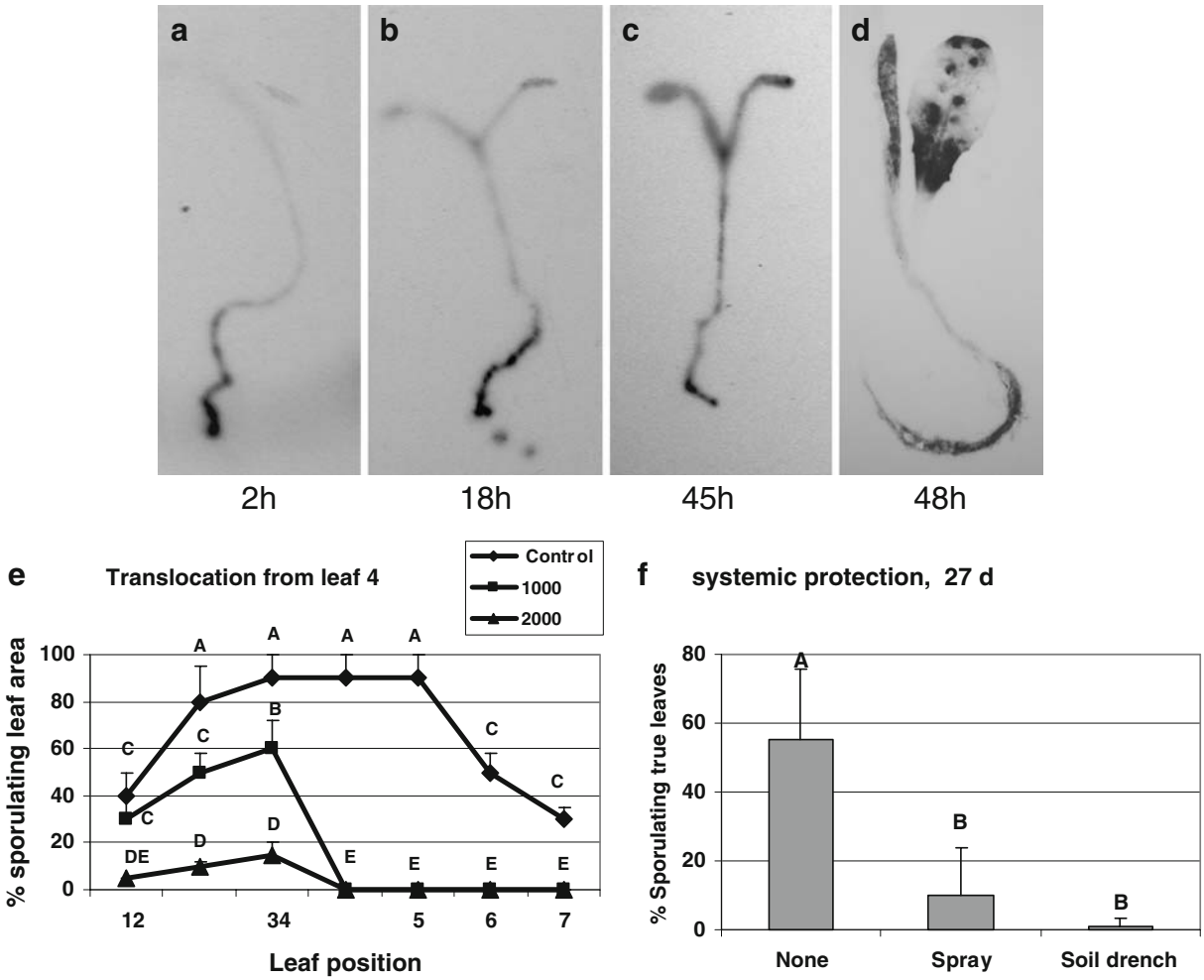


Fig. 5 Uptake of ^{14}C-BABA by the roots of 7 day-old lettuce plants at: **a** 2 h; **b** 18 h; **c** 45 h, after administration. **d** Uptake of ^{14}C-BABA by leaf no 4. of 7-leaf stage plants at 48 h. Note heavy label in the proximal part of leaf 4, leaf 7, stem and root. Leaves no. 1, 2, 3, 5, and 6 show no labeling. **e** Systemic protection against *Bremia lactucae* by BABA translocation from leaf 4. Plants were sprayed with 2 ml BABA onto leaf 4 and, at 7 dpi, % sporulating leaf area was recorded on each leaf. Different letters on bars indicate significant (α=0.05) differences between means. **f** Durability of systemic protection of lettuce plants against *Bremia lactucae*. Seven day-old plants with two cotyledons were either left untreated (CK), sprayed (1,000 µg ml^{-1}), or drenched (40 µg ml^{-1} soil) with BABA, and inoculated 27 days after treatment when they had five true leaves. Disease development was assessed at 7 dpi. Different letters on bars indicate significant (α=0.05) differences between means

the corresponding values were 60–73% and 50–65 µm, respectively. Germination in planta was tested at 15 and 12°C with detached cotyledons inoculated with spores mixed with BABA. Data in Fig. 4e, f show that BABA had no effect on spore germination, germ-tube length or appressorium formation relative to the water controls at either 15°C (Fig. 4e) or 12°C (Fig. 4f). BABA did not inhibit penetration (see below) when mixed with spores and inoculated onto detached cotyledons, but strongly inhibited colonization as could be judged from the percentage of leaves showing sporulation at 7 dpi. Thus, the number of sporulating cotyledons was reduced by 50 and 100% when spores were mixed with 0.32 and 1.25 µg BABA per site inoculated (32 and 125 µg ml^{-1}), respectively.

BABA stops pathogen colonization soon after infection The dynamics of pathogen development in detached, BABA-treated, inoculated cotyledons is

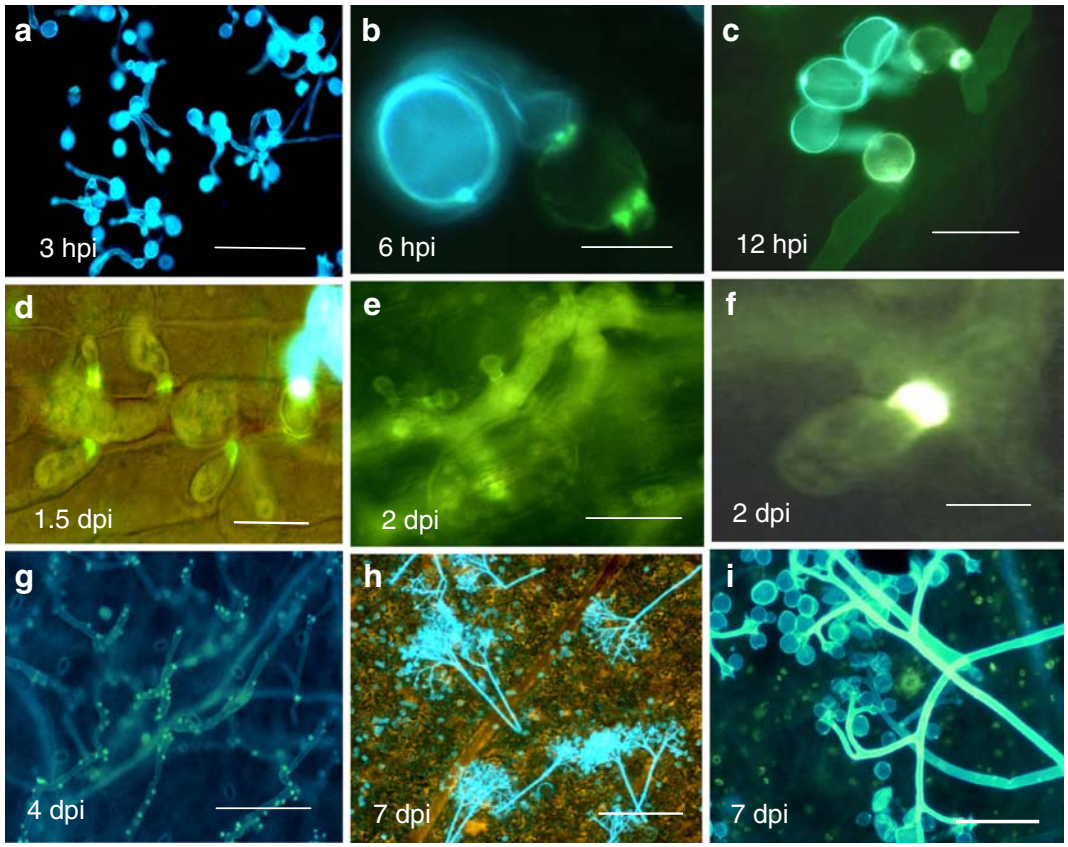


Fig. 6 Microscopy of *Bremia lactucae* developing in detached cotyledons of lettuce. Cotyledons were stained with alkaline aniline blue and calcofluor and examined with the aid of an epifluorescence microscope under UV light (except **d)** and **h)** with mixed UV and incandescent light). **a** Spore germination on leaf surface at 3 h. Bar=100 µm. **b** Penetration and formation of primary infection structures in an epidermal cell at 6 h. Bar=20 µm. **c** Formation of initial hypha at 12 h. Bar=50 µm. **d** Formation of haustoria in the mesophyll at 36 hpi. Bar=20 µm. **e** Mycelium runners with haustoria in the mesophyll at 2 dpi. Bar=50 µm. **f** A single haustorium in a mesophyll cell at 2 dpi. Note the shining yellow collar neck. Bar=20 µm. **g** Extensive mycelium colonization in the mesophyll at 4 dpi. Bar=100 µm. **h** Sporophores carrying spores emerging from stomata at 7 dpi. Bar=100 µm. **i** Higher magnification of sporophores carrying spores at 7 dpi. Bar=80 µm

shown in Fig. 7. Spore germination on BABA-treated cotyledons occurred at about 3 h (Fig. 7a) similar to BABA-free leaves (compare with Fig. 6). Penetration and formation of primary vesicles (PV) occurred at about 6 h (Fig. 7b) and similarly to the BABA-free leaves. At 1 dpi, secondary vesicles (SV) were seen (Fig. 7c). At 2 dpi, no mycelium was detected between the mesophyll cells. Rather, the primary and secondary vesicles that developed in the epidermal cells emitted strong yellow fluorescence, indicating on a massive accumulation of callose in these structures (Fig. 7d). Mixed bright-field and UV-epifluorescence microscopy revealed a minor hypha 'attempting' to emerge from a callose-encased secondary vesicles (Fig. 7e, f). The pathogen failed to sustain mycelium growth or formation of haustoria. Callose and lignin were deposited in the infected host cells as late as 5 and 8 dpi, respectively. At 5 dpi, penetrated epidermal cells accumulated callose in their walls (Fig. 7g). At 8 dpi, penetrated host cells became lignified including the secondary vesicle of the pathogen (Fig. 8h). Lignification, which was a late response of the host to fungal invasion in BABA-treated leaves, was always associated with penetration by the pathogen (Fig. 8i). Similar results were obtained with plants treated with BABA as a foliar spray. Comparative experiments done with genetically resistant cultivars revealed massive callose, lignin and HR production at 1 dpi (data not shown).

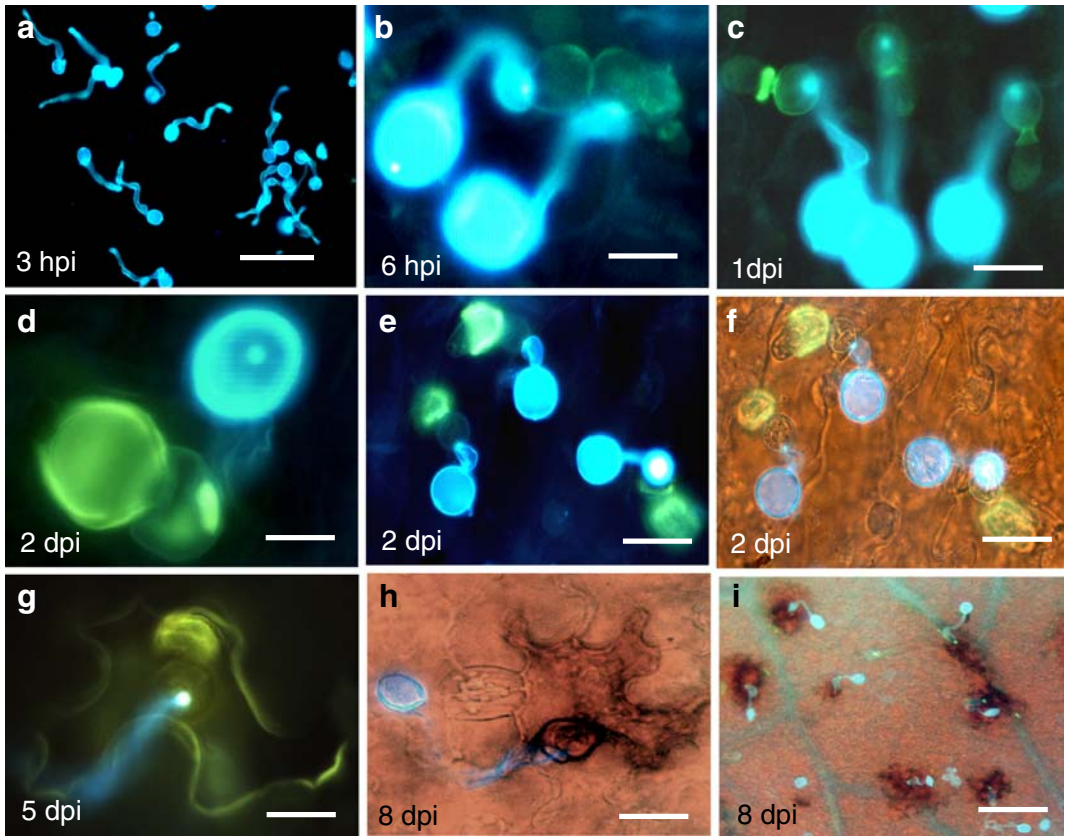


Fig. 7 Microscopy of *Bremia lactucae* developing in detached cotyledons of lettuce treated with 5 µg BABA. Cotyledons were stained with alkaline aniline blue and calcofluor (except **h)** and **i)**, which were stained with phloroglucinol-HCl) and examined with the aid of an epifluorescence microscope under UV light (except **f), h)** and **i)** under mixed UV and incandescent light). **a** Spore germination on leaf surface at 3 h. Bar=100 µm. **b** Formation of primary vesicles in epidermal cells at 6 h. Bar=10 µm. **c)** Formation of secondary vesicles in epidermal cells at 1 dpi. Bar=20 µm. **d** Encasement of the primary and the secondary vesicles with callose at 2 dpi. Bar=10 µm. **e** Emergence of a primary hypha from the secondary vesicle. UV light at 2 dpi. Bar=50 µm. **f** Emergence of a primary hypha from the secondary vesicle (mixed UV and incandescent light) at 2 dpi. Bar=50 µm. **g** Accumulation of callose in the cell walls of a penetrated epidermal cell at 5 dpi. Bar=50 µm. **h** Accumulation of lignin in a penetrated epidermal cell at 5 dpi. Bar=50 µm. **e** Accumulation of lignin in epidermal cells was always associated with penetration of the pathogen at 8 dpi. Bar=100 µm

Confocal laser scanning microscopy Figure 8 provides four images of *B. lactucae* in lettuce cotyledons in the presence or absence of BABA. In control leaves at 1 dpi, spore and germ tubes of the pathogen (fluoresce blue) are seen on the leaf surface, and mycelial runners of about 180 µm (fluoresce yellow) developing inside the mesophyll. The point of penetration looks white. At the same time, in BABA-treated leaves, the primary and secondary vesicles invading epidermal cells are heavily encased with callose and no mycelium appears to be developing. At 5 dpi, control leaves showed sporulation, whereas the pathogen was still inhibited as at 1 dpi in BABA-treated leaves (Fig. 8).

Abscisic acid, methyl-jasmonate and ethylene are not involved in protection of lettuce

ABA, MJ and ethephone failed to protect lettuce seedlings against downy mildew when applied alone (Fig. 9). When applied with BABA to the roots (ABA, MJ) or foliage (ethephone), neither ABA, nor MJ (Fig. 9) or ethephone (data not shown) had any effect on the resistance induced by BABA.

2-deoxy-D-glucose does not reverse protection

DDG of 10 mM did not compromise the resistance induced by 10 mM BABA (Fig. 10a, b). At

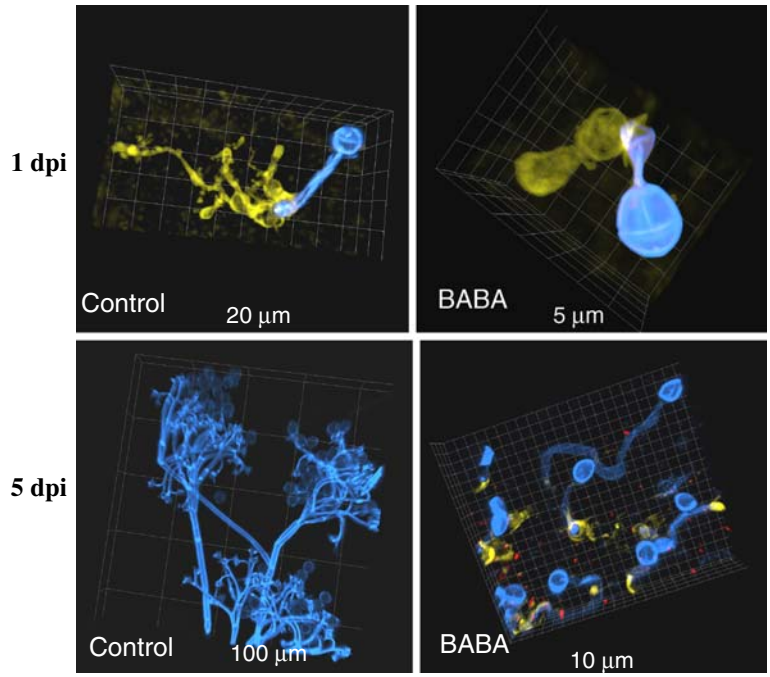


Fig. 8 Confocal microscopy of *Bremia lactucae* developing in detached cotyledons of lettuce treated with water or BABA (10 µg per leaf). Spores, germ-tubes, and sporophores on leaf surface were stained with calcofluor and fluoresce blue. Primary vesicles, secondary vesicles and intercellular hyphae, all inside the leaf, contain β-1, 3-glucans, were stained with alkaline aniline blue and fluoresce yellow. Chloroplasts fluoresce red. The scale indicates the grid size in µm

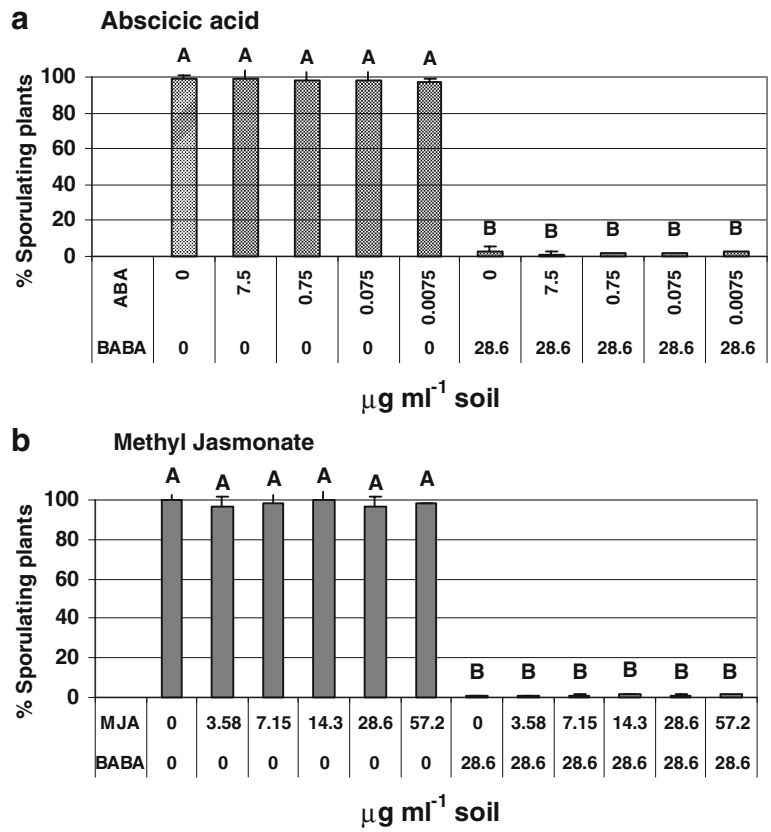


Fig. 9 The effect of abscisic acid or methyl jasmonate, applied alone or in combination with BABA to the root system of lettuce seedlings, on protection against downy mildew. **a** The effect of abscisic acid, alone or with BABA at 28.6 µg ml^{-1} soil. **b** The effect of methyl-jasmonate, alone or with BABA at 28.6 µg ml^{-1} soil. Disease records were made at 7 dpi by counting the number of sporulating plants in each pot ($n=3\times20$). The error bars represent the standard deviation (±SD) of the mean. Different letters on bars indicate significant ($\alpha=0.05$) differences between means

10 dpi, the pathogen sporulated on water-treated control plants and on DDG-treated leaves, but not on leaves treated with BABA or BABA+DDG (Fig. 10c).

A series of BABA concentrations was also used, alone or with 10 mM DDG. As shown in Fig. 10d, the dose-dependent resistance induced by BABA, was not altered by DDG.

The images presented in Fig. 10e show that whereas hyphal development occurred in water-treated control and DDG-treated leaves (at all concentrations) no such growth took place in BABA treated leaves (at either concentration tested). Whenever BABA was present, regardless of DDG, primary and secondary vesicles were encased with callose and growth of the pathogen was halted (Fig. 10e).

A series of DDG concentrations was also used, alone or with 3.3 mM BABA. As shown in Fig. 10f, DDG alone did not affect the development of *B. lactucae* (as measured by sporophore counts) at concentrations of 0.5–8.32 mM. At 16.65 mM, more so at 33.3 mM, DDG alone significantly inhibited sporophore formation. BABA alone did not allow any formation of sporophores, and when combined with DDG of any concentration, almost no sporophores were produced (Fig. 10f).

Fig. 10 The effect of DDG (2-deoxy-D-glucose), BABA, and their combinations on protection of lettuce against *Bremia lactucae*. The compounds were applied to intact lettuce seedlings as a spray or to detached cotyledons in droplets. **a** Plants treated with water, 10 mM BABA, 10 mM DDG or 10 mM BABA followed (after 3 h) by 10 mM DDG and inoculated 3 h after the second spray. Bar represent % sporulating plants at 7 dpi. **b** The appearance of the plants at 7 dpi. Different letters on bars indicate significant (α=0.05) differences between means. **c** Microphotographs of leaves, sampled at 7 dpi, under mixed UV and incandescent light. Bar=100 µm; **d** Percentage protection of seedlings treated with BABA (0.3–20 mM), or with BABA (0.3–20 mM) and 10 mM DDG. The error bars represent the standard deviation (±SD) of the mean. Different letters on bars indicate on a significant (α=0.05) difference between means. **e** Micrographs of inoculated detached cotyledons treated with BABA (3.3 mM), DDG (3.3 mM) and their mixture at 2 dpi. Spores and germ-tubes fluoresce blue, hypha, haustorial necks, primary vesicles, and secondary vesicles fluoresce yellow due to the presence of β-1, 3 glucans. Bar= 20 µm. **f** Sporophore formation at 10 dpi in detached cotyledons treated with DDG (0.5–33.3 mM), BABA (3.3 mM), and their mixtures thereof. Different letters on bars indicate on a significant difference between means

Accumulation of pathogenesis-related proteins is not altered by BABA

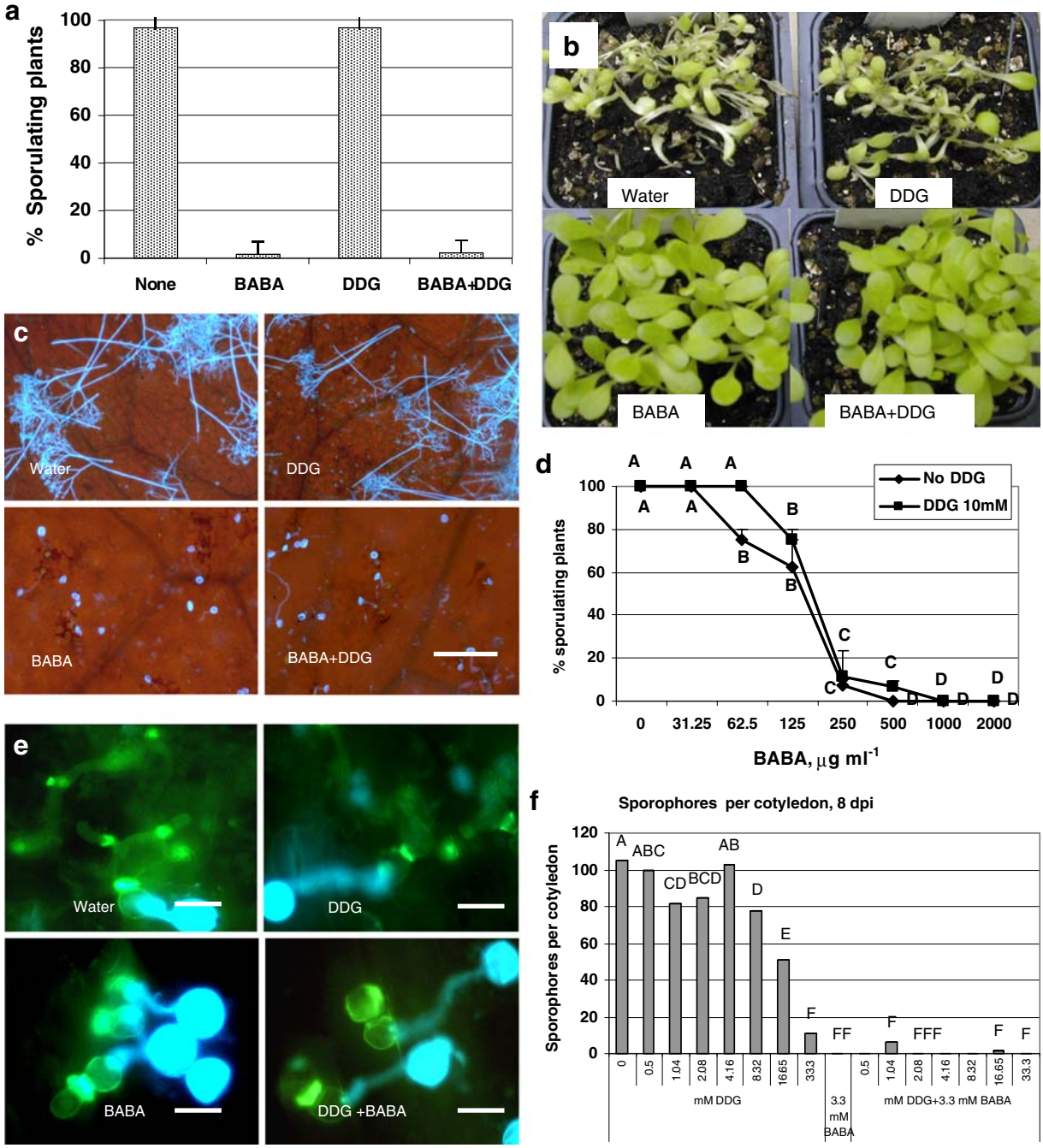
Plants with 2 true leaves were sprayed with various concentrations of BABA or Bion (500–2,000 µg ml^{-1}). Three hours later, half of the plants in each treatment were inoculated with *B. lactucae* and half were left un-inoculated. Protein extraction and Western blotting were performed at 1–4 dpi with one plant per treatment. The results are presented in Fig. 11. Massive accumulations of PR-1a occurred in Bion-treated plants, inoculated or un-inoculated. Only minor accumulation occurred in BABA-treated plants. PR-1a accumulation was seen in mock-inoculated plants at 4 dpi (Fig. 11). Similar results were obtained with PR-5 (data not shown).

BABA induces H_2O_2 production

DAB applied to BABA-treated, inoculated leaf tissue at 6 hpi produced extensive brown color in the epidermal cells penetrated by *B. lactucae* (Fig. 12a–c). No such staining was seen in mock inoculated leaf tissue, nor in non-inoculated leaf tissue (either treated or not treated with BABA), suggesting that penetrated host cells of BABA-treated plants rapidly accumulate H_2O_2. DAB-staining of inoculated leaf discs at 2 dpi produced brown lesions in the inoculated sites of BABA-treated leaf discs, but not in inoculated sites of control inoculated discs (Fig. 12d, e). No staining was seen in non-inoculated leaf discs (treated or not treated with BABA). Similar brown colour developed in BABA-treated inoculated cotyledons (Fig. 12f), but not in control inoculated cotyledons.

Discussion

We show here that BABA efficiently protected lettuce against downy mildew caused by the Oomycete *Bremia lactucae* Regel. It was effective when applied locally to the foliage, and systemically when applied to the roots. Two isomers, AABA and GABA, were ineffective, suggesting that a β position of the amino group is crucial for activity. The *iso* (branched) isomer of BABA was also ineffective, suggesting that only a straight molecule is recognized by a putative plant receptor. The S-enantiomer of BABA was ineffective whereas the R-enantiomer was more effective than the



racemic DL-BABA, suggesting that a specific stereoscopic arrangement of this 4-carbon molecule is critical for binding and activity. β-alanine (3-aminopropanoic acid) was inactive, showing that a terminal methyl group is required for binding to the target molecule. It could be speculated that plants have a receptor for BABA. The above structure-activity relationships suggest that such a receptor does occur in plants. Recently, Kim et al. (2007) cloned a novel β-transaminase from *Mesorhizobium* sp. which uses β-aminocarboxylic acids (preferably BABA) as donors to transaminate pyruvate or 2-ketoglutarate/oxaloacetate. They suggested that the enantio-selective recognition mode of β-aminocarboxylic acids in the active site is

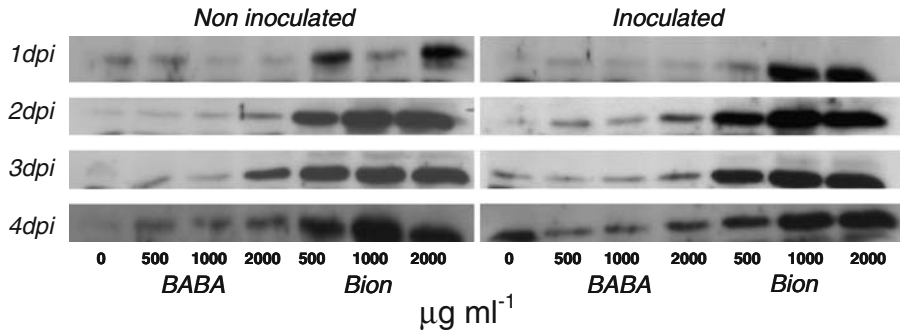


Fig. 11 Western-blot analysis of PR-1a in lettuce plants. Plants with two true leaves were sprayed with the indicated concentrations of BABA or Bion®. At 3 h after spraying, plants were either inoculated with *Bremia lactucae* or left un-treated for comparative purposes

reversed relative to α-amino acids. If this enzyme occurs in plants, it might serve as a receptor for BABA.

NaSA and its functional analogue BTH (Bion®) which operate via the SA-pathway of inducing PR-proteins accumulation did not induce resistance in lettuce against *B. lactucae*. Similar results were reported by Si-Ammour et al. (2003) who showed that BABA, but not BTH protects potato and *Arabidopsis* from *Phytophthora infestans* and *Phytophthora brassicae,* respectively.

^{14}C-BABA was shown to translocate in lettuce plants acropetally and basipetally, corroborating our previous data in tomato and grape (Cohen and Gisi 1994; Cohen et al. 1999). Systemic translocation of BABA from the root system provided the leaves with protection against *B. lactucae* implying that BABA may be used as a soil drench in lettuce production in the field for the control of downy mildew. Spray application of BABA to lettuce in the field was highly effective against *B. lactucae* (Cohen et al. 2007)

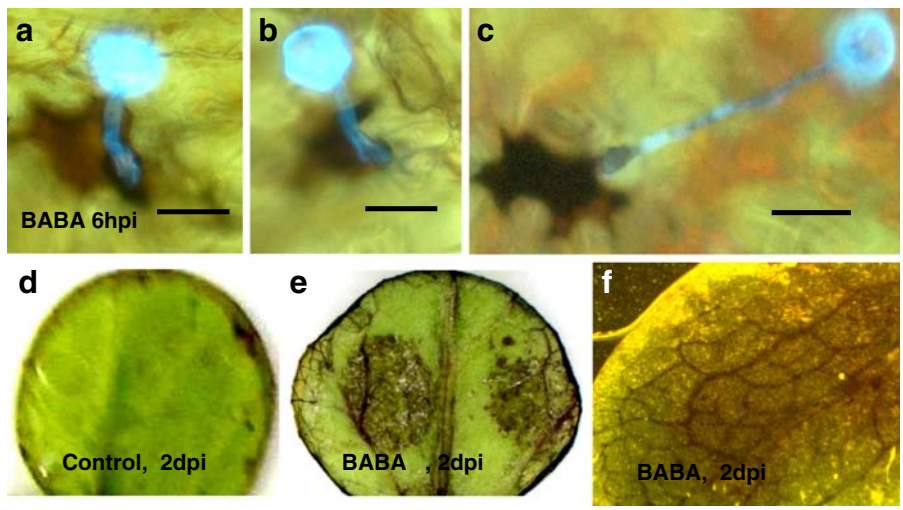


Fig. 12 H_2O_2 accumulation in lettuce leaves inoculated with *Bremia lactucae*. **a–c** BABA was applied as a soil drench (28.6µg ml^{-1}) to 14 day-old plants at 1 day before inoculation. Leaf number 1 was detached at 6 hpi, treated with DAB, stained with calcofluor and photographed. Spores and germ-tubes fluoresce blue. Invaded epidermal cells accumulating H_2O_2 stain dark-brown. No dark-brown cells were seen in BABA-free, control-inoculated leaves (data not shown). Bars in **a** and **b** =20µm and in **c**=30µm. **d** and **e** leaf number 1 was detached and inoculated with two 10µl droplets of spores of *B. lactucae* suspended in water **d)** or in BABA **e)**. At 2 dpi, leaf discs were removed and immersed in DAB and photographed. Note the brown lesions in **e)** (BABA), but not in **d)** (water)**.** Un-inoculated leaf discs**,** treated with water or BABA, produced no dark lesions when treated with DAB. **f)** as in **e)**, but in a cotyledon

probably due to also the systemic translocation of BABA from treated to untreated leaves.

In other pathosystems, BABA was shown to enhance callose and/or lignin deposition in host cells (Zimmerli et al. 2000), ROS accumulation (Siegrist et al. 2000), synthesis of PR-proteins or their transcripts, transcript levels of jasmonic acid and abscisic acid related transcripts (Jakab et al. 2005; Ton and Mauch-Mani 2004). In grape leaves, resistance was associated with lignification of the mesophyll cells (Cohen et al. 1999). PR-proteins were involved in resistance induced by BABA in tomato (Cohen et al. 1994) and *Arabidopsis* (Zimmerli et al. 2000), but not in tobacco (Cohen et al. 1994) or sunflower (Amzalek and Cohen 2007). Here, BABA applied to the foliage (or to the roots, data not shown) of lettuce plants, induced only minor accumulation of PR-1a (and taumatine-like PR-5, data not shown) at ≥3 days after application (see also Pajot et al. 2001). We assume that this minor, late accumulation is not responsible for suppressing hyphal growth from the secondary vesicles, as this suppression occurs as early as 1 dpi. In contrast, Bion enhanced major accumulation of PR-1a in lettuce, thus confirming its priming effect (Goellner and Conrath 2008), but failed to protect against *B. lactucae*. This suggests that PR-proteins, in-spite of their rapid synthesis, do not contribute to resistance of lettuce against downy mildew. Because BABA did not induce PR-proteins in lettuce we suggest that it operates via a SA-independent pathway.

In *Arabidopsis*, BABA at a high concentration completely suppressed hyphal penetration into the host due to callose deposits (papillae) at the site of attempted penetration (Zimmerli et al. 2000). At a lower concentration, cells underwent a phenocopy HR at the site of attack including callose deposition in walls of the invaded host cells and trailing necroses (host cells undergoing necrosis along the growing hyphae) (Zimmerli et al. 2000). Our microscopic observations show that BABA did not affect spore germination, appressorium formation or penetration of *B. lactucae* into the host. It allowed also the formation of primary and secondary vesicles in the epidermal cells, but inhibited the emergence of the infective hyphae from these primary invading structures of the pathogen, thus localizing the pathogen to only the penetrated epidermal cells. BABA induced a massive accumulation of callose (β-1, 3-glucans) around the primary and secondary vesicles (PV/SV) invading the epidermal cells. This heavy encasement with callose enabled these structures to emit strong yellow fluorescence following staining with alkaline aniline blue. We assume that this callose is synthesized by the host, and deposited in the interface zone (extra-haustorial membrane) between the pathogen cell wall and the invaginated host plasma membrane. It might well be that some putative effector proteins, which might be secreted by PV/SV and required for *B.lactucae* colonization, fail to cross this callose encasement. The mechanism by which BABA induced this encasement is not known.

BABA did not provoke any visible microscopic responses of the host cell invaded by *B. lactucae* besides callose encasement of the PV/SV. This lack of response lasted until 5 dpi. At 5 and 8 dpi, respectively, invaded host cells responded by accumulating callose in their walls and lignin in walls and cytoplasm. Interestingly, lignin was also deposited in the infection structures (PV/SV) of the pathogen. In *Arabidopsis*, DDG reversed the resistance induced by BABA due to inhibition of callose synthesis (Jakab et al. 2001). In our experiments, DDG did not prevent callose accumulation around the PV/SV in BABA-treated leaves and therefore failed to compromise resistance. Our data show that DDG applied alone at high concentrations prevented sporophore formation of *B. lactucae*. This proves that DDG is taken up by lettuce leaf tissue. The reasons for the failure of DDG to prevent callose formation in infected BABA-treated lettuce tissue are not known.

We do not know how exactly BABA provokes callose accumulation around the invading vesicles of *B. lactucae*. BABA or a signal molecule induced by BABA in the host (H_2O_2?) may enhance callose synthesis by the host during invasion. Spores of *B. lactucae* did not produce callose in the presence of BABA during germination in vitro or in planta, suggesting that the callose is of a host-origin.

The structural and biochemical features associated with BABA-induced resistance were very different from those associated with genetic resistance. Whereas BABA induced no hypersensitive response (HR) in the host, a rapid HR occurred in lettuce cultivars carrying *Dm* genes for resistance as well as in the non-host *L. saligna* (Lebeda et al. 2008). This HR was manifested as cell death soon after inoculation and was accompanied with a rapid lignin and callose production (Cohen and Rubin, unpublished data).

We here provide evidence that BABA enhanced the production of H_2O_2 in lettuce infected with downy mildew. DAB-staining showed rapid accumulation of H_2O_2 in invaded epidermal cells within 16 h after treatment with BABA, suggesting that H_2O_2 might be responsible for halting the growth of the pathogen. Alternatively, H_2O_2 may halt pathogen development by inducing callose encasement of the PV/SV. Hydrogen peroxide, but not superoxide, was shown to accumulate in *Lactuca* spp challenged with *B. lactucae* and dramatic changes of H_2O_2 were correlated with race-specific resistance where it is associated with early HR onset (Lebeda et al. 2008). H_2O_2 generation is known to play a role in restricting fungal penetration, inhibiting fungal invasion, and activating defense-related genes (Apel and Hirt 2004).

Taken together, BABA is shown here to induce a unique mechanism of resistance against downy mildew in lettuce. Unlike innate resistance which was accompanied with rapid accumulations of lignin and callose and followed by HR, BABA blocked disease development by a rapid encasement with callose of the primary invading structures of the pathogen, with no HR

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