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Post infection application of DL-3-amino-butyric acid (BABA) induces multiple forms of resistance against *Bremia lactucae* in lettuce

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Abstract DL-3-amino-butyric acid (BABA) induces local and systemic resistance against disease in numerous plant species. In a recent study we showed that preventive application of BABA to lettuce (*Lactuca sativa*) plants induced resistance against downy mildew caused by the oomycete *Bremia lactucae* by callose encasement of the primary infection structures of the pathogen. Now we show that post-infection application of BABA to the foliage or the roots, even at progressive stages of disease development, is highly protective against *B. lactucae*. Resistance induced by BABA is manifested in multiple microscopic forms, depending on the time of its application. When applied at 1 day post inoculation (dpi) BABA induced HR in penetrated epidermal cells; at 2 dpi it caused massive encasement with callose of the primary haustoria; and, at 3 or 4 dpi it enhanced the accumulation of H_2O_2 in the developing mycelia runners and altered their colour to red. The pronounced change in the colour of the mycelium was visually apparent to the naked eye. In all cases the pathogen failed to sporulate on the treated plants. This is the first indication that an immunizing compound may be protective at advanced stages of disease development.

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Keywords Curative disease control · Downy mildew · Induced resistance · Butanoic acids

Abbreviations

ABA	Abscisic acid
DAB	Diaminobenzidine
dpi	Days post inoculation
JA	Jasmonic acid
NaSA	Sodium salicylate
ROS	Reactive oxygen species
PR-1	Pathogenesis-related protein 1
SA	Salicylic acid
SAR	Systemic acquired resistance

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. Disease resistance (*Dm*) genes are also used to control the disease but recombinant isolates evading recognition conferred by the *Dm*-genes often occur (Lebeda et al. 2008). The disease can be controlled by fungicides such metalaxyl/mefenoxam (Crute et al. 1994) and carboxylic acid amides (Cohen et al. 2008). However, resistance of *B.lactucae* to metalaxyl/mefenoxam (Crute et al. 1994) avoids its use.

Plants may be immunized against disease by biotic or abiotic agents (Goellner and Conrath 2008; Kuc 2001; Walters et al. 2005). The classical type of immunization 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 (Sticher et al. 1997). The expression of SAR, triggered by either pathogen infection or treatment with NaSA or its functional analogues INA or BTH, is tightly associated with the transcriptional activation of genes encoding pathogenesis related (PR) proteins (van Loon 1997).

DL-3-amino-*n*-butanoic acid (DL-β-aminobutyric acid, BABA) is a non-protein amino acid shown to induce resistance against many plant pathogens in a large number of annual and perennial agricultural crops (Cohen 2002; Jakab et al. 2001). BABA is capable of inducing local and systemic resistance against tobacco mosaic virus (TMV) (Siegrist et al. 2000), bacteria (Baysal et al. 2005; Wu et al. 2009), Oomycetes (Biton et al. 2001; Cohen 1994, 2002; Hamiduzzaman et al. 2005; Hwang et al. 1997; Papavizas and Davey 1963; Reuveni et al. 2001; Shailasree et al. 2001; Silue et al. 2002; Slaughter et al. 2008; Tosi et al. 1998; Walz and Simon 2009; Zimmerli et al. 2000), Ascomycetes and Fungi Imperfecti (Porat et al. 2003; Reuveni et al. 2003; Ton and Mauch-Mani 2004; Zimmerli et al. 2001), Basidiomycetes (Amzalek and Cohen 2007), nematodes (Oka and Cohen 2001; Oka et al. 1999) and aphids and chewing insects (Hodge et al. 2005, 2006). In *Arabidopsis thaliana* BABA is effective against both necrotrophic and biotrophic pathogens, as well as abiotic stresses of drought and salinity (Jakab et al. 2001, 2005; Zimmerli et al. 2001). BABA also enhances acquired thermotolerance in *Arabidopsis* (Zimmerli et al. 2008). BABA effectively controls diseases in the field, e.g. downy mildew in grapevines (Reuveni et al. 2001), downy mildew in lettuce (Cohen et al. 2007), 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), with mancozeb to control downy mildew in cucumbers (Baider and Cohen 2003) and with NaCl to control bacterial speck in tomato (Baysal et al. 2007).

In a recent paper (Cohen et al. 2010) we showed that application of BABA to lettuce plants before inoculation prevents further development of the oomycete *Bremia lactucae* soon after it penetrated into the epidermal cell. We showed that PR-1, SA, JA and ABA play no role in BABA-induced resistance in lettuce against *B. lactucae*.

In the present study we were interested to learn if BABA provides protection to lettuce against downy mildew when applied curatively, after *B. lactucae* has already penetrated and established itself in the leaf tissue; and, if it does, to reveal the mechanisms by which such protection operates. The data indeed suggest, that post-infection activity of BABA is robust and the nature of the resistance induced depends on how advanced the pathogen was at the time of immunization.

Materials and methods

Plants The susceptible lettuce (*Lactuca sativa* L) cultivar Noga (cup type, Hazera Genetics, Mivhor, Israel) was used in most experiments. Some were done with cut-salad-type cultivar Strubelpeter (Syngenta, Stein, Switzerland). Plants were grown from seeds in 175 ml pots containing 100 ml (40 g) peat/vermiculite mixture (1/1, v/v), ~20 plants per pot. Plants were grown in the greenhouse (18–26°C) and used 1 week after seeding, when developed two cotyledon leaves. In some experiments, plants were grown in 0.5-l pots, 1 plant/pot and used at the 10th leaf stage. Plants were fertilized with 0.5% N:P:K (20:20:20) once a week.

Pathogen Most experiments were done with isolate IL60 of *Bremia lactucae* Regel. A few were done with isolate CH provided by Syngenta, Stein, Switzerland. The pathogen was maintained by repeated inoculation of detached cotyledon leaves in growth chambers at 13–15°C, 12 h light per day.

BABA DL-3-amino-*n*-butanoic acid (β-aminobutyric acid, BABA) was purchased from Sigma. It was dissolved in water before used.

Application of BABA The compound was diluted in water to a series of concentrations and applied once to the test lettuce plants by either spraying onto the upper leaf surfaces to initial run-off or to the root system as a single soil drench, 5 ml solution per 100 ml soil. The compound was applied curatively, at various time

intervals after inoculation. In some experiments lettuce cotyledons were detached from 7 days old plants, placed lower side upward, on moistened filter paper in 5 cm Petri dishes and each inoculated with 10 μl droplet of water containing 200–500 spores of *B.lactucae*. At 0–4 dpi a 10 μl droplet of BABA was inserted into the inoculum droplet.

Inoculation Spores of *B. lactucae* Regel were collected from freshly sporulating lettuce leaves into ice-cool double distilled water, their concentration was adjusted to 2×10^4 spores ml^{-1} and sprayed onto the upper leaf surfaces of the test plants to initial run-off with the aid of a glass atomizer. Plants were then placed in a dew chamber (~100% relative humidity RH, 18°C, darkness) for 20 h and then transferred to a growth chamber at 18–20°C (12 h light/day, 100 $\mu E.m^{-2}.s^{-1}$). At 5 dpi plants were placed in Perspex boxes (~100% RH, 12 h light/day, 100 $\mu E.m^{-2}.s^{-1}$) for 1 or 2 days to induce sporulation of the pathogen on the leaves.

Disease assessment With plants at their cotyledon stage, the number of sporulating plants was determined with the aid of a magnifying lens at ×10. To estimate spore production, cotyledons were detached, placed each in 1 ml 50% ethanol solution, shaken at 100 rpm for 10 min and the number of spores was counted with the aid of a cytometer. Sporophore formation was followed microscopically, in clarified cotyledon leaves (see below). With plants having true leaves, the proportion of infected leaf area or the proportion of leaf area showing sporulation in each plant was visually assessed. Records were taken at 7 dpi, unless stated otherwise.

Microscopy The effects of BABA on the host and the pathogen were examined as described before (Cohen et al. 1989, 1990, 2010). Briefly, plants (at their cotyledon leaf stage or the 10th leaf stage) were inoculated with *B.lactucae* at time zero and treated with BABA (by spray or soil-drench) at 0–6 dpi. Plants left untreated or treated with water served as controls. Cotyledons or leaf discs were removed 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 or leaf discs were placed on a glass slide, covered with a glass slip and examined with the aid of an Olympus AX70 epi-fluorescent microscope equipped with an excitation filter of 390–420 nm and an emission filter of 425–450 nm. Phenolics emitted blue fluorescence (Cohen and Ibrahim 1975). To observe the discolouration of the hyphae of *B.lactucae*, cotyledons or leaf discs were clarified in boiling ethanol for 5 min, mounted on glass slides in 20% glycerol and examined with the aid of an Olympus AX70 microscope with incandescent illumination. Hyphae looked red in treated leaves. To detect callose, cotyledons or leaf discs were clarified 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, drop treated with 0.02% calcofluor and examined with the aid of the epi-fluorescent microscope described above. Callose fluoresced yellow while spores and germ-tubes fluoresced blue. For lignin detection, leaves were clarified as above, placed on a glass slide and treated with 2% phloroglucinol dissolved in methanol followed by 25% HCl. Lignin stained red. HR in host cell was examined with a bright-field dissecting microscope.

Detection of hydrogen peroxide H_2O_2 was detected by diaminobenzidine (DAB) (Sigma fast DAB tablets) staining. DAB polymerized instantly, and develops a localized dark colour as soon as it comes into contact with H_2O_2 in the presence of peroxidase (Thordal-Christensen et al. 1997). Ten-leaf plants were inoculated with *B.lactucae* and treated with BABA (1,000 μg ml^{-1}) at various time intervals after inoculation. Leaf discs (15 mm diameter) were excised from leaf 5 (from stem base), immersed each in 1 ml DAB (1 mg/ml) and incubated at 20°C in the light for 5 h until examined for dark colour production.

Statistics Experiments were repeated twice or more, with a typical set of results presented. With intact 7 days old plants, 3–4 replicate pots/treatment, 20–25 plants/pot, were used. With 10-leaf plants 3 replicate plants/treatment were used. With detached cotyledons, 10 replicate leaves/treatment were used. Data were analyzed with the aid of JMP7 software. Means were separated using the Tukey-Kramer *t*-test at $\alpha=0.05$.

Results

Protection by spray application BABA applied 1 day post inoculation induced a significant protection against

downy mildew as can be judged from the reduction in the number of plants showing sporulation of *B. lactucae* (Fig. 1). A single spray with 125, 250, 500 μg ml^{-1} BABA caused at 7 dpi 82%, 95% and 100% inhibition, respectively (Fig. 1a). Protection was pronounced even after a prolonged period of 3 weeks (Fig. 1b). BABA applied at 2 dpi was as highly and significantly protective as BABA applied at 1 dpi (Fig. 1c). However, when applied at 3, 4, and 5 dpi it gradually became less protective, as higher doses were required to achieve significant protection (Fig. 1c). Thus, percent protection, relative to control plants, obtained with 250 μg ml^{-1} BABA applied at 1, 2, 3, 4, and 5 dpi was 95, 90, 45, 30 and 20%, respectively; with 500 μg ml^{-1} BABA- 100, 100, 90, 70, and 40%, respectively; and with 1,000 μg ml^{-1} BABA- 100, 100, 100, 95 and 60%, respectively (Fig. 1c).

Post-infection spray application of BABA to 7-days old seedlings not only protected against downy mildew but also induced the development of red color in the inoculated cotyledons (Fig. 2). As shown in Fig. 2a, treatment with 1,000 μg ml^{-1} BABA at 1, 2, 3 or 4 dpi induced strong and significant protection. About 25% of the plants

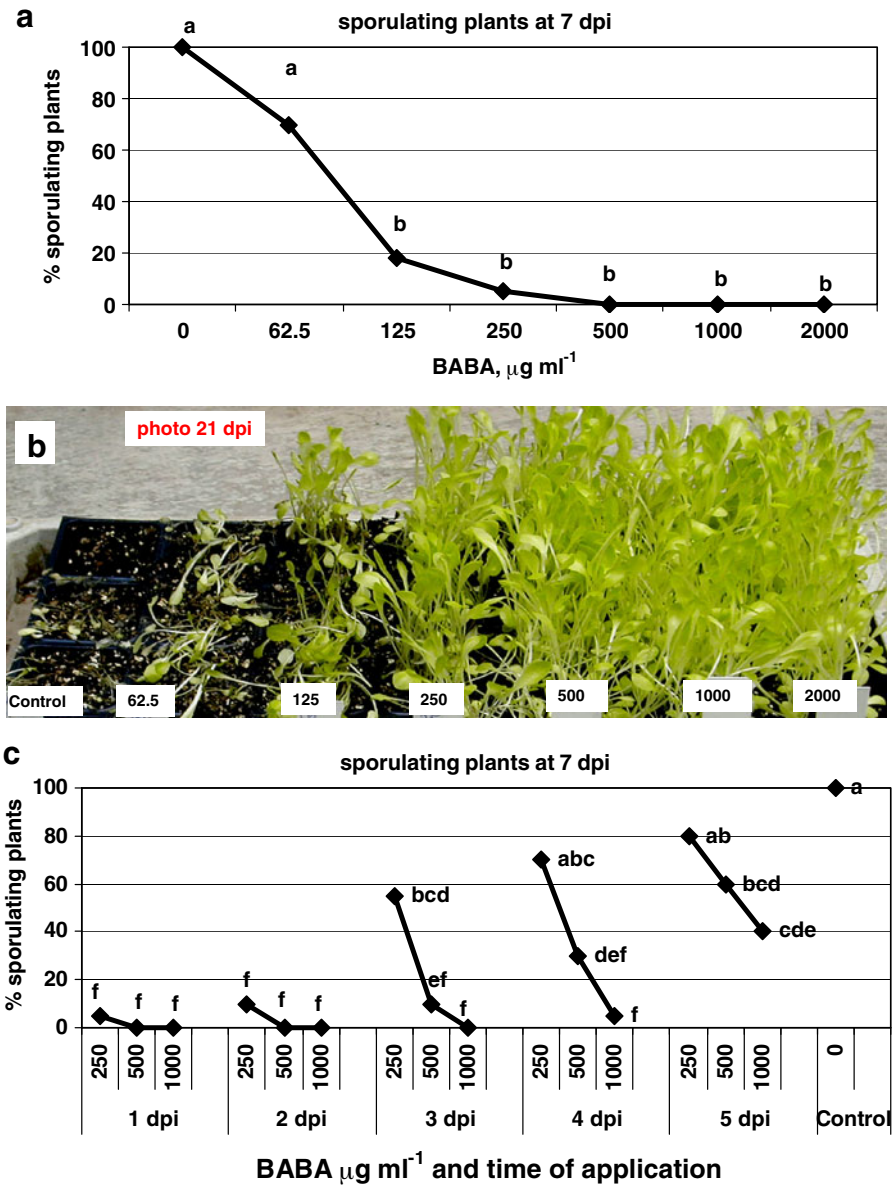

Fig. 1 BABA applied as a post-infection spray induced resistance against *Bremia lactucae* in lettuce seedlings. Seven days old plants (n=4 pots with ~20 plants/pot) were inoculated at time zero and sprayed with various doses of BABA at different time interval after inoculation. Control plants were left untreated. On the 6th day after inoculation the plants were placed in a moist chamber and % sporualting plants was recorded a day later with the aid of a magnifying lens ×10. **a** Plants treated at 1 dpi with 62.5–1,000 μg ml^{-1} BABA. Different letters on figures indicate on significant differences between means (Tukey-Kramaer analysis, α=0.05). **b** Appearance of the plants treated at 1 dpi with 62.5–1,000 μg ml^{-1} BABA. Photograph was taken at 21 dpi. **c** Plants treated at 0–5 dpi with 250–1,000 μg ml^{-1} BABA. Different letters on figures indicate on significant differences between means (Tukey-Kramaer test, α=0.05)

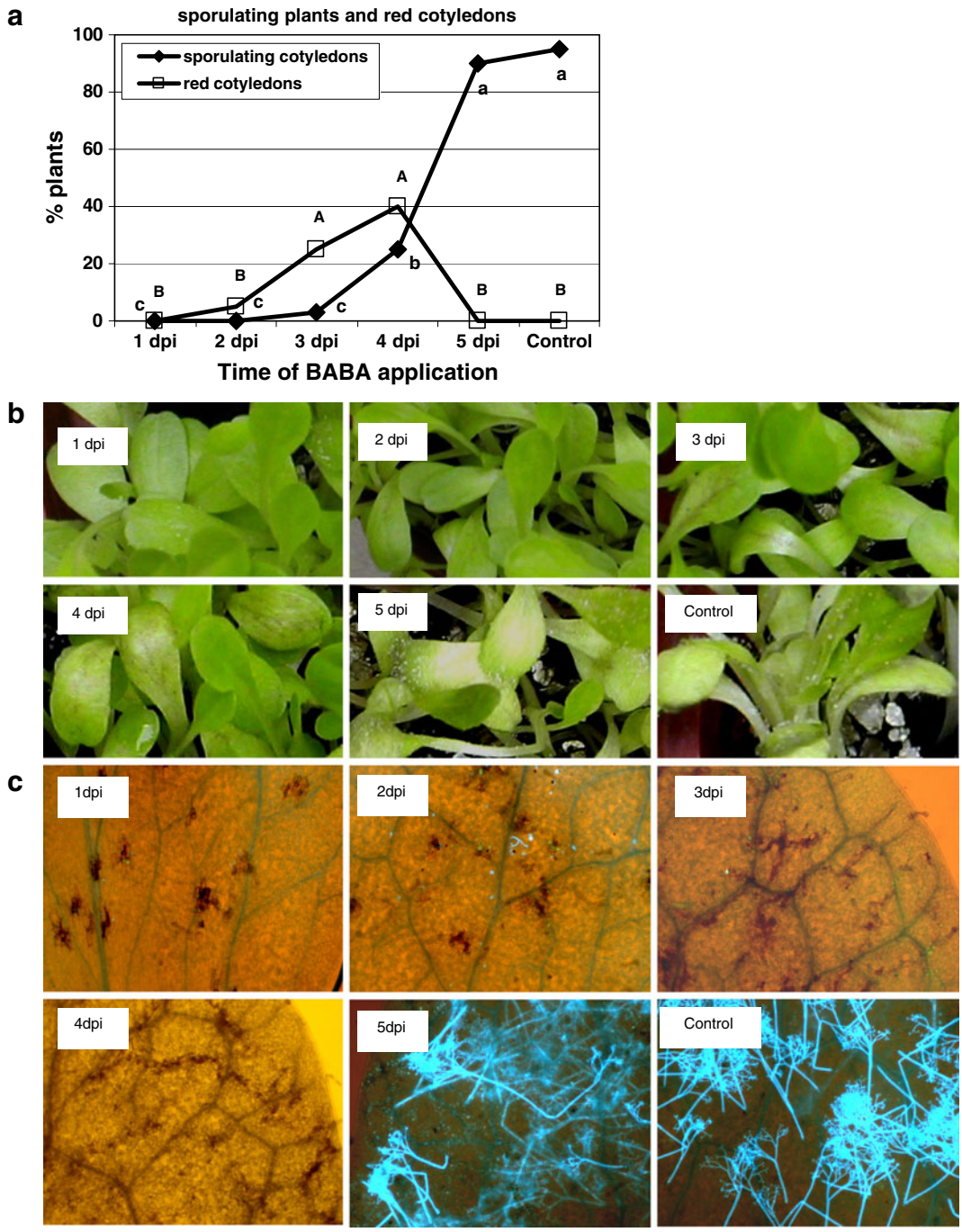

Fig. 2 BABA applied as a post-infection spray induced resistance against *Bremia lactucae* and red color in lettuce cotyledons. Seven days old seedlings were inoculated at time zero and spayed with 1,000 μg ml^{-1} BABA at 1, 2, 3, 4, or 5 dpi (n=5 pots with ~20 plants/ pot). **a** Percent plants showing sporulation and red color in cotyledons at 7 dpi. Different letters on figures indicate on significant differences between means (Tukey-Kramaer analysis, α=0.05). **b** Appearance of the plants at 8 dpi. Note sporulation in control and 5 dpi; also note the red color in cotyledons at 3 and 4 dpi. **c** Micrographs, taken at 8 dpi, of cotyledons after clarified with boiling ethanol and stained with calcofluor. Note red spots at 1 and 2 dpi, red strands in 3 and 4 dpi and blue sporophores at 5 dpi and control

treated at 3 dpi and 40% of the plants treated at 4 dpi developed red color in their cotyledons. Close-up photos show (Fig. 2b) no symptoms or sporulation in plants treated at 1 and 2 dpi; no sporulation but red colour produced in plants treated at 3 and 4 dpi; and, heavy sporulation with no red colour in plants treated at 5 dpi and in control plants. When examined microscopically (Fig. 2c), HR was seen in plants inoculated at 1 or 2 dpi, red mycelium runners in plants inoculated at 3 or 4 dpi, and profuse sporophores and spores (fluoresce blue) in plants inoculated at 5 dpi and the control plants. Our attempts to extract the red colour with water, boiling ethanol or methanol, or acidic methanol (Wu et al. 2009) have all failed, suggesting that this red colour is not anthocyanin.

The curative protective action of BABA in adult lettuce plants is shown in Fig. 3. Ten-leaf plants were inoculated with *B. lactucae*, sprayed with 1,000 μg ml^{-1} BABA at 1, 2, 3, or 4 days post inoculation, and placed at 7 dpi in 100% RH for 20 h in the dark to induce sporulation. The upper panel in Fig. 3a shows that sporulation of the pathogen occurred in the control but not in BABA-treated plants. Plants treated with BABA at 1 or 2 dpi were normal green whereas those treated at 3 or 4 dpi acquired red colour. The red colour was not removed from leaf discs after boiling in ethanol (Fig. 3a middle panel). Alive leaf discs infiltrated with DAB produced dark-brown colour in the mycelium runners (Fig. 3a lower panel) and in the mesophyll cells adjacent to the dark mycelium runners (Fig. 3b) suggesting H_2O_2 accumulation in the mycelium and in the adjacent mesophyll cells. UV microscopy showed that BABA applied at 3 or 4 dpi also induced encasement of the haustoria with callose (see below).

Protection by root application Figure 4 shows the results of a typical experiment in which BABA was applied to the root system of already-infected seedlings at various time intervals after inoculation. At 8 dpi, plants treated with BABA at 1, 2, or 3 dpi looked healthy whereas those treated later and controls looked affected (Fig. 4a). A close-up photograph (Fig. 4b) revealed that many cotyledons of plants treated at 3 and 4 dpi turned red. As shown in Fig. 4c the red colour originated from the mycelia colonizing in the mesophyll of these red cotyledons. Figure 4d provides data on the percentage of plants which developed red colour during the course of this experiment. After placing the plants in humid conditions to induce sporulation it became apparent that the degree of protection, as reflected by % plants showing sporulation and the number of sporophores produced per cotyledon, were directly related to the dose of BABA applied to the roots but inversely related to the time interval lapsed between inoculation and root treatment (Fig. 4e and f). Thus, plants treated at 1, 2 or 3 dpi were highly protected with all doses of BABA whereas plants treated at 4, 5, and 6 dpi were highly protected at BABA of 17.5, 35 and 70 μg ml^{-1} soil, respectively (Fig. 4f).

Similar results were obtained with the cultivar Strubelpeter inoculated with isolate CH suggesting that protection and red colour formation are not cultivar-specific or isolate-specific. The degree of protection as judged by sporulation of the pathogen at 9 dpi was positively related to the dose of BABA applied to the roots and negatively related to the time interval lapsed between inoculation and application. Thus, % sporulating plants among the plants treated with BABA of 17.5 μg ml^{-1} soil at 0, 1, 2, 3, 4, 5 and 6 dpi was 0, 0, 8, 20, 48, 60 and 100%, respectively; and, % sporulating plants among the plants treated with BABA of 35 μg ml^{-1} soil was 0, 0, 0, 4, 10, 44 and 95%, respectively. Red colour developed in cotyledons of plants treated at 3 dpi with BABA of all doses, at 4 dpi in cotyledons of plants treated with BABA of 35–140 μg ml^{-1} soil, and at 5 dpi in cotyledons of plants treated with BABA of 140 μg ml^{-1} soil.

Protection by isomers of BABA Isomers were tested for their capacity to induce curative resistance against *B. lactucae* in intact 7-days old lettuce plants as well as in detached cotyledons. Isomers were administrated at 1 dpi either by a spray of 1,000 μg ml^{-1}, soil drench to the root system of 35 μg ml^{-1} soil or 5 μg per cotyledon. None of the following compounds induced a significant protection: DL-2-aminobutyric acid; L-2-aminobutyric acid; DL-2-amino-*iso*-butyric acid; DL-3-amino-*iso*-butyric acid; 4-aminobutyric acid. The S-enantiomer of BABA was also inactive. Interestingly, these compounds also failed to compromise the curative protection induced by BABA (data not shown).

Microscopy of curative resistance induced by BABA The microscopic features expressed during the induction of curative resistance were dependent on the time after inoculation at which BABA was

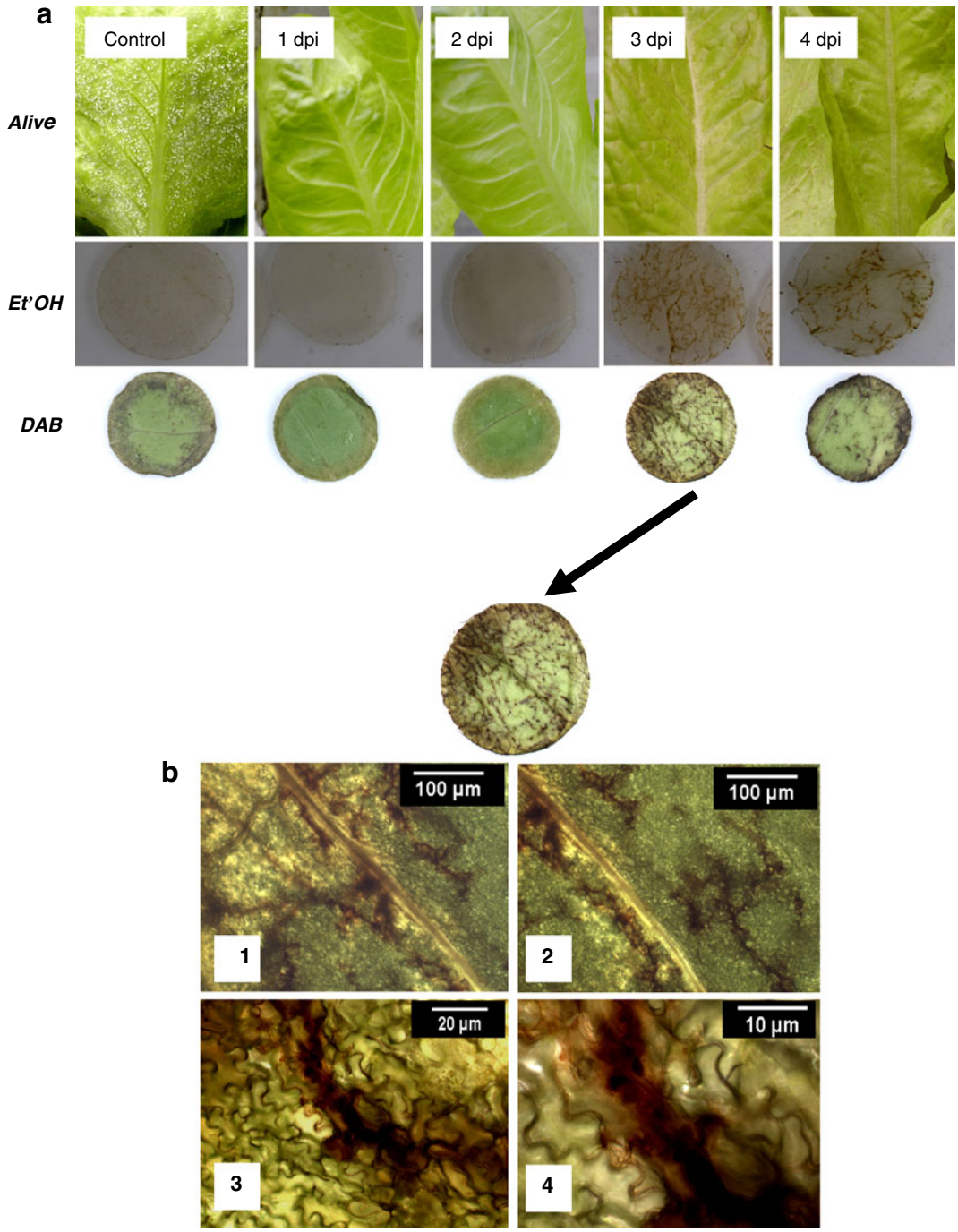

Fig. 3 Curative effects of BABA in adult lettuce plants: 10-leaf plants were inoculated with *Bremia lactucae* at time zero. At 1, 2, 3, or 4 dpi plants were sprayed with 1,000 µg ml^{-1} BABA. At 7 dpi, plants were incubated in a dew chamber for 20 h to induce sporulation of the pathogen and photographed. Leaf discs were then removed, clarified in ethanol, washed in water and photographed at ×10 with the aid of a stereomicroscope. Alive leaf discs were infiltrated with DAB and photographed at ×10. **a** Upper panel—intact leaves. Note sporulation in Control and light red color in 3 dpi and 4 dpi. Middle panel—leaf discs after boiling in ethanol (Et'OH), without staining. Note brown red trailing in 3 dpi and 4 dpi. Lower panel—alive leaf discs after staining with DAB for H_2O_2. Note heavy red-brown staining in 3 dpi and 4 dpi. **b** micrographs taken from the 3 dpi leaf disc after staining with DAB. Note DAB staining in running hyphae and in adjacent mesophyll cells

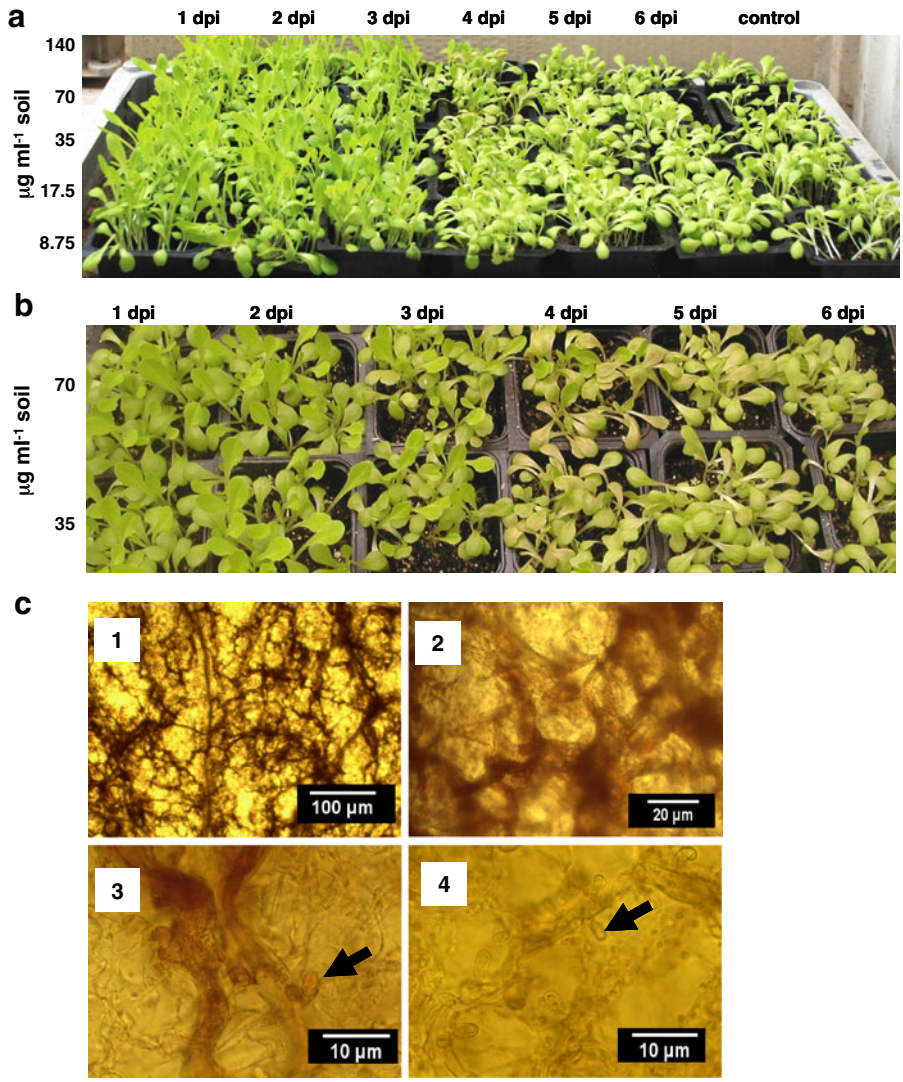

Fig. 4 BABA applied to the root system of lettuce plants provided curative protection against *Bremia lactucae*. Seven days old seedlings were inoculated at time zero. BABA was applied to the root system of potted plants as a single soil drench at 1, 2, 3, 4, 5, or 6 dpi to a final concentration of 8.75, 17.5, 35, 70, or 140 µg ml^{-1} soil solution. Control plants were treated with water. **a** The appearance of the plants at 8 dpi. Note that plants treated at 1, 2, and 3 dpi are bigger and greener than the others. A close-up photo of plants at 8 dpi. Note the green color of the plants treated at 1, 2, and 3 dpi; red color of the plants treated at 3 and 4 dpi, and chlorotic color of the plants treated at 5 and 6 dpi. **c** BABA (35 µg ml^{-1}) applied to the root system at 3 dpi induced a strong accumulation of red color in the mycelia of *Bremia lactucae* colonizing the mesophyll. Cotyledons were detached at 8 dpi, clarified in boiling ethanol, mounted in water on glass slides and examined with a dissecting microscope. **1**—bar=100 µm; **2**—bar=50 µm; **3** bar=10 µm; **4**—bar=10 µm. Note that the mycelia and haustoria in control plants did not turn red. The arrows point at haustoria. The red color was not removed after extraction with boiling methanol containing 1% HCl. **d** % red cotyledons at 8 dpi (n=25–30). **e** % sporulating plants at 9 dpi after placing the plants in a moist atmosphere in the dark for 20 h (control= 92.8±7.7%). **f** The number of sporophores produced per cotyledon at 9 dpi (n=5). Cotyledons were detached at 9 dpi, clarified in boiling ethanol, mounted in water on glass slides, stained with calcofluor and examined with a epi-fluorescent microscope. Values accompanied with different letters are significantly different (Tukey-Kramer test, α=0.05)

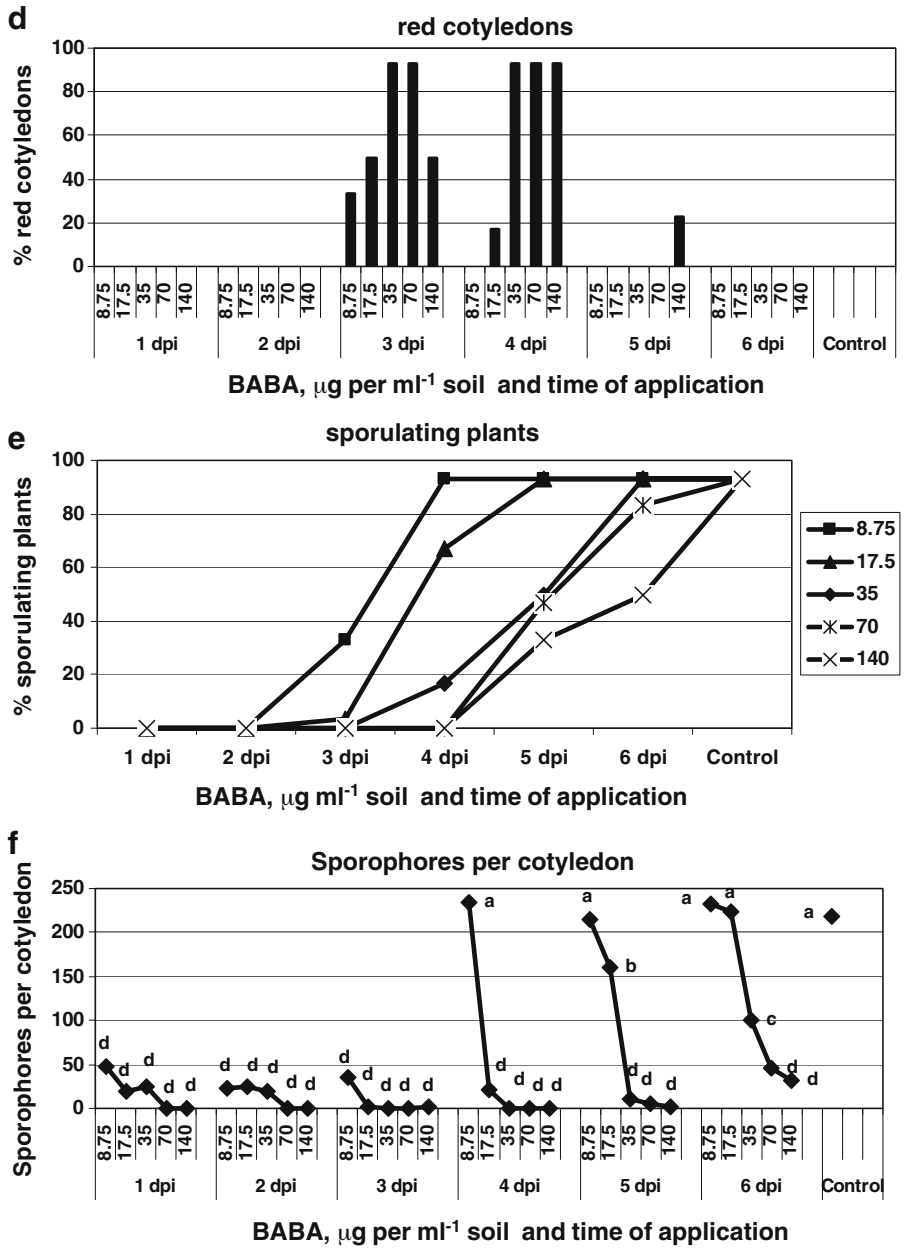

Fig. 4 (continued)

applied. Figure 5 presents a series of micrographs taken under combined incandescent and UV illuminations from 7-days old seedlings that were treated at 1–4 dpi with BABA (35 μg ml^{-1} soil) via the root system. We present four photos, each from a different plant, for each time point of application to illustrate the variety of responses associated with resistance. When applied at 1 dpi, BABA induced HR in the penetrated epidermal cells of the host (Fig. 5a–d). This HR was probably responsible for halting any further growth of the pathogen. Application at 2 dpi induced HR in epidermal cells and/or callose encasement of haustoria in the mesophyll cells (Fig. 5e–h). Haustoria encased with callose may fail to supply nutrients and water to the pathogen. Application of BABA at 3 dpi resulted in the accumulation of callose around the haustoria and/or red colour in the mycelia (Fig. 5i–l). BABA applied at 4 dpi induced a strong

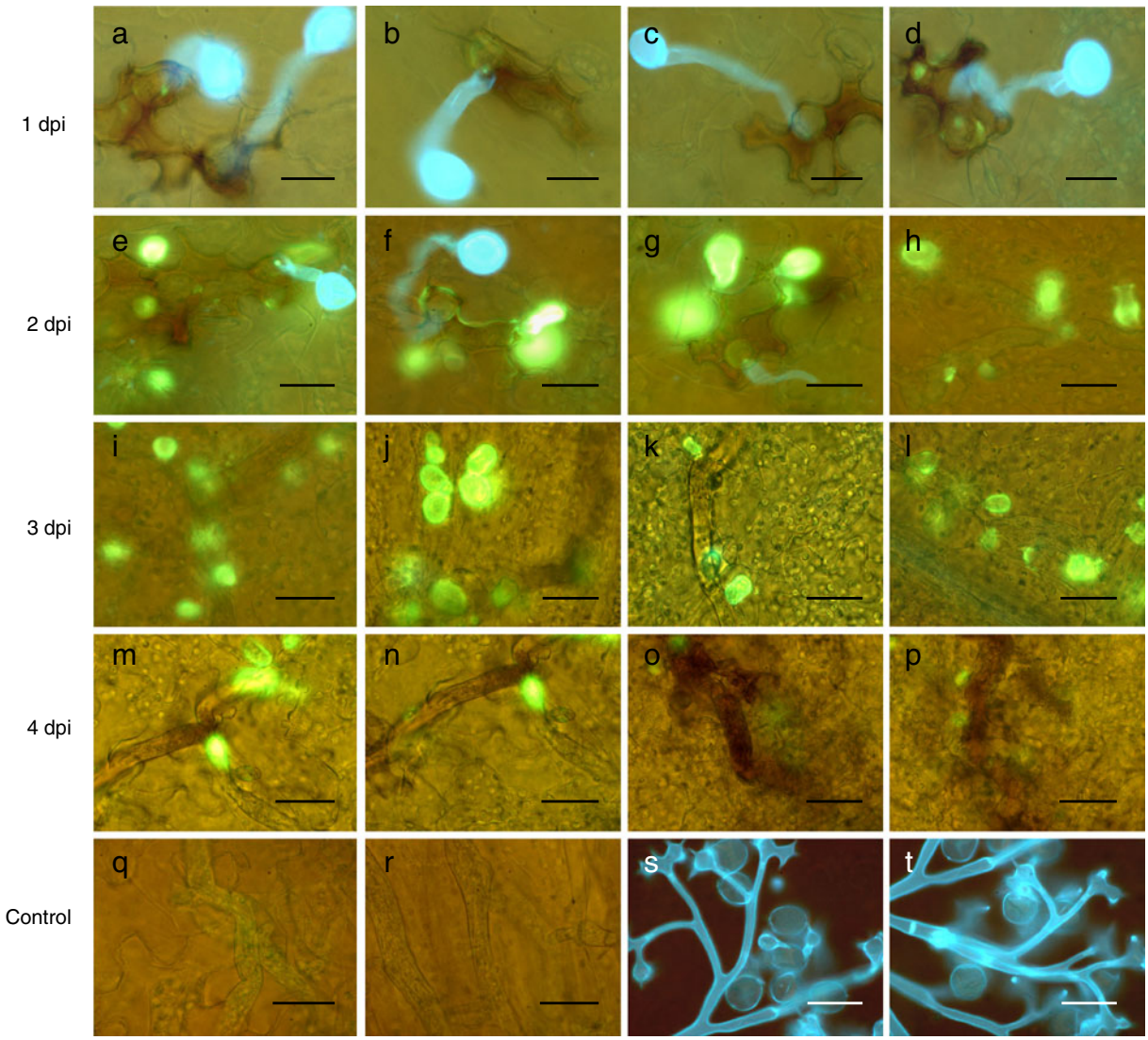

Fig. 5 BABA applied to lettuce plants at various time intervals after inoculation with *Bremia lactucae* induced different cellular responses leading to resistance. Seven days old seedlings were inoculated at time zero and BABA (35 μg BABA per ml soil solution) was applied to the root system as a single soil drench at 1, 2, 3, or 4 dpi. Control plants were left untreated. At 7 dpi, plants were placed at 100%RH for 20 h in the dark to induce sporulation and then, cotyledons (n=10) were detached, clarified in boiling ethanol, transferred to basic aniline blue for 24 h at 4°C, placed on glass slides, treated with 0.01% calcofluor and examined with aid of an epi-fluorescent microscope under mixed UV and incandescent lights. Spores, germ-tubes and sporophores fluoresce blue. Callose around haustoria fluoresces yellow. Epidermal cells showing HR stain brown. Hyphae undergoing discoloration stain red-brown. Bar=20 μm. **a–d** HR in epidermal cells of plants treated with BABA at 1 dpi. Note the association of HR with only penetrating germ-tube. **e–h** 2 dpi—enhanced callose accumulation around primary haustoria, some with HR. **i–l** 3 dpi—enhanced callose accumulation around haustoria; hyphae show slight red color. **m–p** 4 dpi—hyphae turn red. **q–r** control—normally-looking, hyaline hyphae. **s–t** control—sporophores bearing spores on leaf surface, fluoresce blue

accumulation of red material in the intercellular mycelia colonizing the mesophyll (Fig. 5m–p). Control inoculated plants produced hyaline hyphae in the mesophyll (Fig. 5q, r) and profuse sporophores and spores on the cotyledon surface (Fig. 5s, t).

Microscopy of resistance was also studied in detached cotyledons. BABA, applied as a single droplet of 10 μl per cotyledon to already-infected cotyledons was significantly efficient in inhibiting the development of *B. lactucae* as can be judged from the

number of spores produced per cotyledon at 6 dpi (Fig. 6). In two cultivars each inoculated with another isolate, percent inhibition of sporulation was positively related to the dose of BABA applied and negatively related to the time lapse between inoculation and application (Fig. 6). In cultivar Noga inoculated with isolate IL60, application of 5 μg BABA per cotyledon resulted in 100, 66, 57, and 37% inhibition of sporulation relative to control in cotyledons treated with BABA at 1, 2, 3 and 4 dpi, respectively. In cultivar Strubelpeter inoculated with isolate CH application of 5 μg BABA per cotyledon resulted in 100, 100, 98, 50, and 29% inhibition of sporulation relative to control in cotyledons treated with BABA at 0, 1, 2, 3 and 4 dpi, respectively. Figure 7 supplies the microscopic details of this resistance. Photos shown in Fig. 7a–h were taken with the aid of a stereomicroscope whereas those in Fig. 7i–p were taken with the aid of an epi-fluorescent microscope. Control inoculated cotyledons show abundant sporulation with no HR (Fig. 7a–d, i–l) whereas cotyledons treated with 5 μg BABA at 1 dpi developed strong HR with no sporulation (Fig. 7e–h, m–p).

Discussion

The striking finding of the present study is that BABA induced resistance against *Bremia lactucae* in already-infected lettuce plants. Resistance was almost

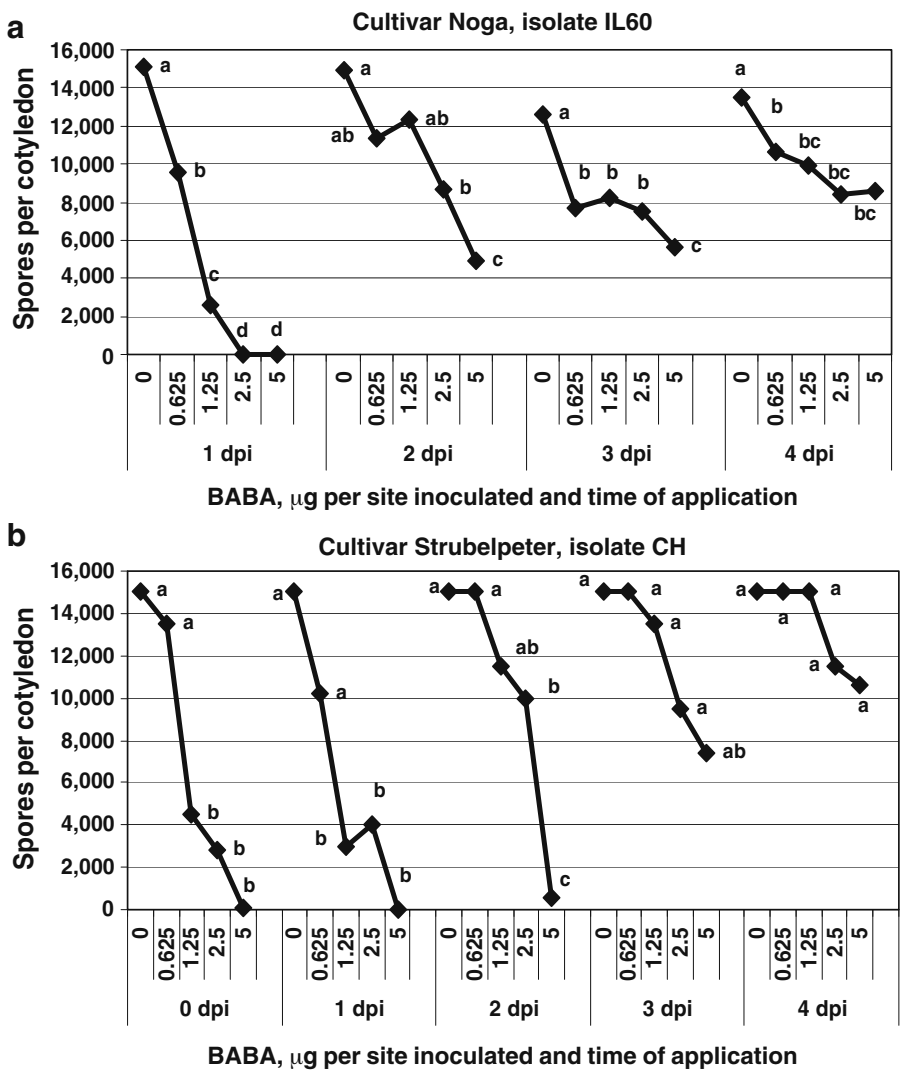

Fig. 6 Detached cotyledons (n=10) of lettuce cultivar Noga or Strubelpeter in closed Petri dishes were each inoculated with a 10 μl water droplet containing spores of *Bremia lactucae* isolates IL60 or CH, respectively. At 1, 2, 3, and 4 dpi or 0, 1, 2, 3, and 4 dpi, 10 μl droplets containing 0, 0.625, 1.25, 2.5, or 5 μg BABA were introduced into the inoculum droplets. At 6 dpi, cotyledons (n=5) were each placed in 2 ml of 50% ethanol and the number of spores discharged was counted with the aid of a cytometer. Values accompanied with different letters are significantly different (Tukey-Kramer test, α=0.05)

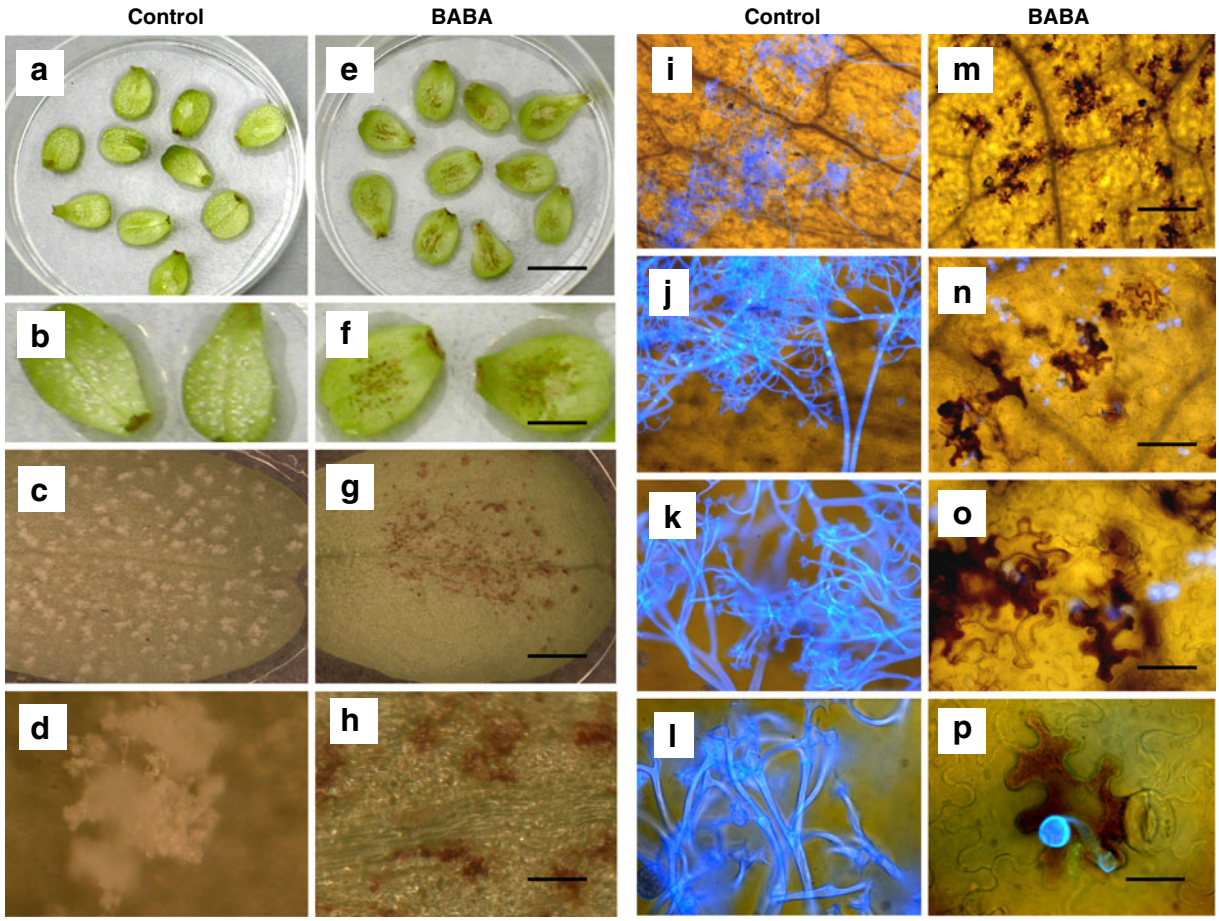

Fig. 7 Application of BABA (5 μg) to detached cotyledons at 1 day after inoculation with *Bremia lactucae* induced hypersensitive response in the invaded epidermal cell of lettuce. Detached cotyledons (n=10) in closed Petri dishes were each inoculated with a 10 μl water droplet containing spores of *Bremia lactucae*. At 1 dpi, a 10 μl droplet containing 0 or 5 μg BABA was introduced into the inoculum droplets. At 6 dpi, cotyledons (n=5) were clarified in boiling ethanol, stained with basic aniline blue and calcofluor and examined with a aid of a stereomicroscope equipped with incandescent light (photos **a**–**h**) or with a fluorescent microscope illuminated with mixed incandescent and UV lights (photos in **i**–**p**). Bar in **e**=13 mm; in **f**=6.5 mm; in **g**=1.6 mm; in **h**=0.5 mm; in **m**=400 μm; in **n**=160 μm; in **o**=80 μm; and in **p**=40 μm. Sporophores, spores and germ-tubes fluoresce blue. Epidermal cells undergoing HR are red-brown

complete in plants treated with BABA at 1–2 days post inoculation (dpi) and highly significant in plants treated at 3–5 dpi, depending on its dose. Such effective curative induced-resistance was not reported for any other plant immunizing agent. Curative protection is rare, even with systemic anti-oomycete fungicides such as metalaxyl/mefenoxam, whose curative efficacy lasts for 2 days (Cohen et al. 1979).

Inoculated plants protected by BABA developed various types of symptoms, depending on the time-lapse between inoculation and treatment. Intact plants treated with BABA at 1 or 2 dpi developed no visual symptoms but showed HR when examined microscopically; plants treated at 3 or 4 dpi developed red leaves and produced only a few sporophores with spores; and, plants treated at 5 or 6 dpi developed chlorosis and produced reduced number of spores relative to control-inoculated plants. Detached leaves treated with BABA at 1 dpi developed a strong hypersensitive response (HR), and those treated at 2, 3 or 4 dpi produced reduced number of sporophores and spores.

These responses are different from those described for plants treated with BABA *prior* to inoculation (Cohen et al. 2010). Pre-infection application of BABA inhibited symptom development, and hence

sporulation, due to lack of colonization. BABA applied preventively allowed the spores to germinate, produce appressoria and penetrate into the treated leaf tissue without inducing HR. Its major effect when applied *prior* to infection was to induce encasement of the primary infections structures with heavy callose deposits. This callose prevented hyphae and haustoria formation and therefore abolished disease development (Cohen et al. 2010).

Here we show that post-infection application of BABA induced various types of host and pathogen responses, depending on the developmental stage the pathogen has reached at time of application. At 1 dpi, BABA induced HR in the invaded epidermal cells. At 2 dpi, it induced heavy callose encasement of the haustoria. Treating the infected plants at 3 or 4 dpi, a stage at which heavy colonization has already taken place, induced the accumulation of red material in the mycelia giving red appearance to the leaves. DAB staining showed that this discoloration was associated with the accumulation of H_2O_2 in the mycelia and adjacent mesophyll cells. The red material accumulated in the mycelia was not anthocyanin, as it did not dissolve in HCl-methanol, as it did in *Arabidopsis* treated with BABA (Wu et al. 2009). Application of BABA to lettuce plants at 5 or 6 dpi produced no microscopic changes in the infected leaves as compared to control-inoculated plants.

In a recent study (Cohen et al. 2010) and in this one we analyzed the structure-activity relationships of amino-butyric acids in induced resistance. Unlike BABA, the isomers 2-amino-butyric acid and 4-amino-butyric acid were ineffective, suggesting that the 3-(β) position of the amino group is crucial for activity. The *iso* (branched) isomers of 2-amino-butyric and 3-amino-butyric were infective, suggesting that a strait molecule only is recognized by a putative plant receptor. The S-enantiomer of BABA was ineffective while the R-enantiomer was effective suggesting that a specific stereoscopic arrangement of the amino group on carbon 3 is critical for binding and activity. β-alanine (3-aminopropanoic acid) was inactive, teaching that a terminal methyl group is required for binding to the target molecule. Current results (Vaknin and Cohen, *unpublished data*) showed that 3-aminopentanoic acid and 3-aminohexanoic acid are active whereas 3-aminoheptanoic acid and 3-aminooctanoic acid are not, suggesting that binding is confined to 4-, 5-, and 6-carbon β-amino acids.

These data strongly support the idea that BABA binds in a stereo-specific manner to a receptor protein. Such receptor may occur in the host and/or the pathogen. Our previous data (Cohen and Gisi 1994) showed that ^{14}C-BABA binds to protein(s) located in the cell wall of tomato, tobacco and potato leaves but not in their cytoplasm. To serve as a receptor a protein must carry a pocket that recognizes the size and stereo-structure of BABA. Such specific pocket was reported in the enzyme β-transaminase from *Mesorhizobium* sp. which carries an enantio-selective recognition mode of β-aminocarboxylic acids (preferably BABA) in its active site (Kim et al. 2007). Extensive efforts are currently invested in finding proteins which bind to BABA in a specific way.

We propose here several mechanisms, some are hypothetical, that might explain, alone or together, the induced preventive and curative protective activity of BABA against disease, taking into account that it has a broad spectrum of protective activity in many pathosystems:

1. BABA induces the production, in the host and/or the pathogen, of compounds toxic to the invading pathogen, e.g. H_2O_2 by enhancing the activity of peroxidase and/or NADH-oxidase.
2. BABA induces the production of callose around primary infecion structures and haustoria by accelerating the activity of callose synthase directly or indirectly. Callose encasement may:

(a) block the influx of nutrients and water from the host into the pathogen, causing starvation and growth cessation of the pathogen.

(b) block the secretion of effector proteins from the haustoria into the extra-haustorium matrix (apoplastic effectors) or into the host cell (cytoplasmic effectors). Effector proteins are secreted all along the colonization period as they are required for compatibility (Schornack et al. 2009).

3. BABA binds to one or more effector proteins while harboring the haustorium or after their secretion, altering their function.
4. BABA binds to translocator proteins located on the host plasma membrane, turning them non-functional.

The post-infection immunizing capacity of BABA presents a challenge to the prevailing dogma of priming. Priming is often used to explain the mode

of operation of SAR compounds in general and BABA in particular. SAR elicitors, including BABA, are normally applied 2–4 days *before* inoculation to achieve best protection. The period lapses between the induction treatment and challenge-inoculation is required for "priming". Priming refers to a phenomenon where plants are sensitized to stress by displaying faster and/or stronger activation of various defense responses. BABA was shown to prime *Arabidopsis* to respond faster and stronger to biotic and abiotic stresses (Conrath et al. 2002, 2006; Van der Ent et al. 2009; Wu et al. 2009). The data presented here show that no priming involves in the curative-protective activity of BABA against *B.lactucae* in lettuce because priming requires *bona fide* a lapse period of a few days to establish and this time is not available in already-infected plants.

Taken together, BABA is shown here to provide strong protection against downy mildew in lettuce even when applied to plants that harbour a progressive colonization with *B.lactucae*. This explains the fact that two treatments with BABA were sufficient to suppress downy mildew development in field-grown lettuce crop (Cohen et al. 2007).

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