INDUCED SYSTEMIC RESISTANCE IN MUNG BEAN PLANT AGAINST ROOT-KNOT NEMATODE *MELOIDOGYNE JAVANICA* BY DL-β-AMINO BUTYRIC ACID

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Summary. The non-protein amino acid DL-β-amino butyric acid (BABA) has been used for inducing resistance against various pathogens in plants. In the present study, the potential of BABA as a resistance elicitor was assessed against an infestation of the root-knot nematode *Meloidogyne javanica* on plants of mung bean (*Vigna radiata*), cv. MN95. Pre-treatment with BABA were applied as a soil drenches of 50, 100 and 200 µg/ml aqueous solutions. Plants non-inoculated and not treated with BABA served as a positive control, while untreated plants inoculated with the nematode served as a negative control. Increasing concentrations of BABA were effective in providing resistance to mung bean plants against root-knot nematode. The aqueous solution of 200 µg/ml BABA significantly reduced the numbers of galls and egg masses of the nematode on the roots and numbers of eggs/egg mass. Growth of BABA-treated plants was enhanced compared to negative controls. Increased levels of chlorophylls and proteins were recorded after BABA treatments. The results suggest that the use of BABA has potential to control root-knot nematode infection in mung bean plants through induced systemic resistance.

Keywords: Chlorophyll content, control, protein content, *Vigna radiata*.

Root-knot nematodes (*Meloidogyne* spp.) are among the most destructive of plant pathogens and are responsible for approximately 50% of the overall damage caused by nematodes (Sasser and Freckman, 1987). Symptoms of root-knot infection are the formation of root galls, which result in reductions of growth and nutrient and water uptake, increased wilting, mineral deficiency and poor yield (Abad *et al*., 2003).

Plant treatments with various biotic and abiotic agents can induce resistance against subsequent pathogen attack (Walters *et al*., 2005). Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli (Van Loon *et al*., 1998). This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants and even insect herbivores (Vallad and Goodman, 2004). Systemic acquired resistance (SAR) is a form of induced resistance that can be triggered by exposing the plant to virulent, avirulent and non-pathogenic microbes, or artificially with chemicals (Sticher *et al*., 1997). The establishment of SAR is associated with the accumulation of pathogenesis-related (PR) proteins and salicylic acid (SA) throughout the plant (Vallad and Goodman, 2004).

The use of chemicals to activate SAR provides novel alternatives for disease control in plants (Neuenschwander *et al*., 1995). To be considered an activator of SAR, a chemical should exhibit three characteristics; first, the compound or its significant metabolites should not exhibit direct antimicrobial activity; second, it should induce resistance against the same spectrum of pathogens as in biologically activated SAR; and third, it should induce the expression of the same marker genes as evident in pathogen-activated SAR (Ryals *et al*., 1996).

DL-β-aminobutyric acid (BABA) is a non-protein amino acid which occurs rarely in nature. The only report in connection with plants describes its presence in root exudates of tomato plants grown in solarized soil (Gamliel and Katan, 1992). BABA plays a key role in plant defense responses against many disease-causing organisms such as bacteria, viruses and fungi, insect herbivores, and or abiotic stress (Cohen, 2002; Jakab *et al*., 2005). It has also been demonstrated to reduce attack by phytopathogenic nematodes in tomato and cereals (Oka *et al*., 1999; Oka and Cohen, 2001). A direct antimicrobial activity of this chemical has never been observed and it is not metabolized in tomato or in *Arabidopsis* (Cohen and Gisi, 1994; Jakab *et al*., 2001).

Therefore, the present study was conducted to investigate the effects of various concentrations of BABA on root-knot nematode infection and development, and on growth and physiological changes induced in mung bean (*Vigna radiata* (L.) Wilczek) cv. MN95 plants.

MATERIALS AND METHODS

Plant material and chemicals

Seeds of mung bean cv. MN95, susceptible to *M. javanica* (Treub) Chitw., were surface-sterilized with 0.85% sodium hypochlorite, washed three times with a sterile solution of $MgSO_4$ (0.1M) and dried under a laminar flow hood. These seeds were used for the experiment in the green-house. DL-β-aminobutyric acid (BABA; purity 97%) was obtained from Sigma-Aldrich (Chemie GmbH, Riedstr. 2, D 89555 Steinheim, Germany). Distilled water solutions containing 50, 100 or 200 µg/ml of BABA were prepared.

Inoculum of root-knot nematodes

The *M. javanica* population was derived from a single egg mass and cultured on eggplant seedlings in a greenhouse. To extract eggs of *M. javanica*, the infected roots of eggplant were shaken for 3 min. in a 2% sodium hypochlorite (10% commercial bleach) solution, thoroughly washed under tap water and collected using the modified technique described by McClure *et al.* (1973). The egg suspension was then poured onto a just submerged cotton-wool filter and incubated at 28 ± 2 °C to obtain freshly emerged juveniles (J2). Only juveniles collected within 72 h were used as inoculum.

Effect of BABA on egg hatching

This test was performed to ascertain that BABA has no direct effect on egg hatch. Eggs of *M. javanica* were collected by the method of Hussey and Barker (1973) and suspended in distilled water. One ml of egg suspension (30-45 eggs/ml) and 1 ml of each aqueous solution concentration of BABA was poured into separate glass cavity blocks (diameter 2.5 cm) and kept at room temperature (29 ± 3 °C). Glass cavity blocks containing 1 ml egg suspension and 1 ml distilled water served as a control. Each treatment was replicated three times. After 48 and 96 h exposure, the number of hatched eggs was counted under a low power (6×) stereomicroscope and expressed as mean percentage of the total eggs incubated.

Effect of BABA on mortality of nematode juveniles (J2)

This test was conducted to be sure that BABA has no direct effect on juvenile viability. Eggs and egg masses of *M. javanica* were placed in distilled water and incubated at 28 ± 2 °C. After 72 h, emerged juveniles (J2) were collected and suspended in distilled water. One ml of freshly emerged juvenile suspension (40–50 juveniles/ml) was mixed with 1 ml of each of the three different concentrations of BABA solutions and transferred to glass cavity blocks kept at room temperature (29 ± 3 °C). Glass cavity blocks containing only the nematode suspension served as a control. Each treatment was replicated three times. After 48 and 96 h exposure, the numbers of dead juveniles were counted under a low power stereomicroscope and nematode mortality was expressed as percentage of the total nematodes incubated. Juveniles were considered dead if they did not move when probed with a fine needle (Cayrol *et al.*, 1989).

Greenhouse experiment

Effects of BABA on growth of mung bean and nematode infection and development. Groups of six seeds of mung bean were sown in plastic pots (8.1 cm diameter) containing 300 g of sterilized sandy loam soil. Before sowing, 3 ml aqueous cell suspension (2.6×10^4/ml) of *Bradyrhizobium japonicum* (strain 569Sm^r) was applied to each pot, including controls, for nodule formation. One week after germination, the seedlings were thinned to two of the same height per pot. BABA was prepared as aqueous solutions with final concentrations of 50, 100 and 200 µg/ml. Aqueous solutions of BABA (15 ml) were applied as soil drenches in four holes made around the roots of the plants. Five days after BABA treatment, about 2000 newly emerged second stage (72 h) juveniles (J2) were introduced in four holes made around the roots of the plants. Pots without nematode inoculum and BABA treatment served as positive controls, while pots with only nematode inoculum served as negative controls. Treatments and controls were replicated three times. The pots were placed in a completely randomized design on a bench in a green-house (temperature 28 ± 4 °C, average day length 13 $^1/_2$ h). Pots were watered daily. Forty days after nematode inoculation, plants were gently removed from the pots and the roots were carefully washed in running water. Data of root and shoot weight, shoot length, number of galls, egg masses, eggs per egg mass, number and size of root nodules were recorded. Number of galls, egg masses and nodules were counted under a stereomicroscope without staining the roots. For the estimation of eggs/egg mass, ten egg masses/treatment were randomly selected from the roots. Each egg mass was crushed in a drop of 0.01% sodium hypochlorite solution to dissolve the gelatinous matrix and examined under light microscope (de Leij, 1992). The sizes of root nodules (diameter) were measured under a stereomicroscope. For the estimation of chlorophylls and protein, 1 and 0.5 g samples, respectively, were randomly taken from both plants of the same pot and pooled. Whereas, for growth and infection parameters only one of the two plants in a pot was randomly chosen, to give three replicate plants.

Determination of chlorophyll content. Chlorophyll content was determined to assess the variability of chlorophyll levels under different stress conditions and to look for any relationship with plant growth. Chlorophylls were extracted from 1 g fresh leaves in 10 ml of 80% cold acetone. The extracts were centrifuged at 1,000 *g* three times for about 5 min. and each time a little acetone was added such that the final volume of the supernatant was 20 ml. Chlorophyll contents were estimated in accordance with MacLachlan and Zalik (1963). The absorbance of the supernatant was recorded at 645 and 663 nm on a Shimadzu UV-mini 1240 spectrophotometer for chlorophyll a and b contents, respectively. The amounts of chlorophylls were expressed as mg/g fresh weight. The coefficient of extinction used for chlorophyll a at 645 and 663 nm were 16.75 L/g/cm and 82.04 L/g/cm, respectively, and for chlorophyll b at 645 and 663 nm 45.6 L/g/cm and 9.27 L/g/cm, respectively.

Determination of total protein content. Protein was measured in leaves to assess its levels under (pathogen) stressed and non-stressed conditions and to relate it to plant growth. Fresh leaves (0.5 g) were plunged into hot 80% ethanol to kill the tissue quickly. After 5 min, the ethanol was removed and the leaf tissues were then

crushed in a mortar with 10 ml of 5% trichloroacetic acid (TCA) and centrifuged at 1,000 *g* for 5 min. The leaf tissues were then washed separately with 5 ml each of absolute ethanol, ethanol-chloroform mixture (3:1 v/v) and finally with ethanol-ether mixture (3:1 v/v). The washed residue was then incubated in 5 ml of 0.5N NaOH for 16 h at 37 °C. The sediment was removed by centrifuging at 4,000 *g* and washed once with 5 ml of 0.5N NaOH. The extract and wash were combined and made up to 10 ml with 0.5N NaOH. The total protein contents were estimated by the method of Bradford (1976) and expressed as mg/g fresh weight.

Statistical analysis

Data sets were subjected to either one-way analysis of variance (ANOVA) or factorial ANOVA depending on the experimental design. After ANOVA, Duncan's multiple range and Fisher's least significant difference (LSD) tests were performed (Sokal and Rohlf, 1995).

RESULTS

Effects on egg hatching and juvenile mortality

In *in vitro* tests (Table I), concentrations of BABA up to 200 µg/ml did not significantly alter egg hatching ($F = 0.383$, ns); likewise, BABA did not significantly influence juvenile (J2) mortality of *M. javanica* after 48 and 96 h exposure compared to controls ($F = 1.35$, ns).

Effects on nematode infection

Increasing concentrations of BABA reduced significantly ($P<0.001$) root-knot nematode infection in mung bean plants. The fewest galls per root system were observed in plants treated with 200 µg/ml of BABA, followed by 100 µg/ml of BABA compared to negative controls. Similarly, number of egg masses per root system and number of eggs per egg mass were also significantly reduced ($P<0.001$) in plants treated with various concentrations of BABA (Table II). However, the differences observed between the treatments with 100 and 200 µg/ml of BABA were not significant.

Effect on the growth of mung bean

Pre-treatment with BABA showed some significant ($P<0.001$) effect on the growth of mung bean following infection by *M. javanica*. Shoot length increased significantly ($P<0.001$) in plants treated with BABA compared to negative controls (with infection only) but not when compared to the positive control (without infection or BABA treatment). The effect of BABA on fresh shoot weight was minimal and a significant increase, compared to the negative control, was observed only at the rate of 100 µg/ml. Root fresh weight was largest in negative control and least in positive control plants. Increase in fresh root weight seemed to have been reduced with the increase of BABA from 50 µg/ml to 100 µg/ml. Only BABA at 100 µg/ml increased significantly the number of *B. japonicum* nodules on the roots, but the average size of the nodules was not affected by any of the treatments (Table III).

Table I. Effect of BABA on egg hatching and juvenile (J2) mortality of *Meloidogyne javanica*. Each value is the mean of three replicates.

Treatment	Hatching (%)		Mortality (%)	
	48 h	96 h	48 h	96 h
Control	21	52.3	3.3	6.7
50 µg/ml BABA	23.7	49.7	8.7	9.7
100 µg/ml BABA	21.7	48.3	5.7	8.3
200 µg/ml BABA	22.3	55.3	3.7	4.7
$LSD_{0.05}$	7.61		5.75	

Effects on chlorophyll and protein contents

Treatments with BABA also showed significant ($P<0.001$) effects on chlorophylls a and b and total protein contents compared to negative controls (Table IV). Chlorophyll contents were lower in negative controls, but there were no significant effects of other treatments. Similarly, chlorophyll a/b ratios were smaller in negative controls than in the other treatments. However, application of 50 µg/ml of BABA resulted in a significant ($P<0.05$) increase compared to other treatments with BABA and the positive controls. Total protein contents in leaves increased as the concentration of BABA increased. Lower amounts of protein were observed in negative and higher amounts in positive controls (Table IV).

Table II. Effect of BABA on infection of *M. javanica* in mung bean plant.

Treatment	Galls/root system	Egg masses/root system	Eggs/egg mass
Positive Control	0 a	0 a	0 a
Negative Control	57 d	24 d	351 d
50 µg/ml BABA	47 c	21 c	304 c
100 µg/ml BABA	35 b	15 b	246 b
200 µg/ml BABA	31 b	13 b	236 b
$LSD_{0.05}$	4.6	2.6	19

Duncan's Multiple Range test ($P = 0.05$): means followed by the same letters are not significantly different from each other.

Table III. Effect of BABA on growth of mung bean plants inoculated with *M. javanica*. Each value is a mean of three replicates.

Treatment	Shoot length (cm)	Fresh shoot weight (g)	Fresh root weight (g)	Nodules/root system	Average size of nodules (mm)
Positive Control	29.8 b	3.74 c	0.26 a	6 a	4 a
Negative Control	22.7 a	2.53 a	0.69 d	5 a	3 a
50 µg/ml BABA	28 b	2.52 a	0.54 c	5 a	4 a
100 µg/ml BABA	30 b	3.02 bc	0.40 b	9 b	4 a
200 µg/ml BABA	28 b	2.82 ab	0.40 b	5 a	4 a
$LSD_{0.05}$	2.68	0.30	0.06	2.5	1

Duncan's Multiple Range test (P = 0.05): means followed by the same letters are not significantly different from each other.

Table IV. Effect of BABA on chlorophyll contents and protein contents of mung bean plants infected by *M. javanica*.

Treatment	Chlorophyll a (mg/g fresh weight)	Chlorophyll b (mg/g fresh weight)	Chlorophyll a+b (mg/g fresh weight)	Chlorophyll a/b	Total proteins (mg/g fresh weight)
Positive Control	0.534 ab	0.372 ab	0.906 b	1.44 b	5.19 d
Negative Control	0.509 c	0.370 b	0.879 a	1.37 a	3.84 a
50 µg/ml BABA	0.538 a	0.367 c	0.905 b	1.46 c	4.26 b
100 µg/ml BABA	0.531 ab	0.373 a	0.904 b	1.42 b	4.76 c
200 µg/ml BABA	0.529 b	0.373 a	0.902 b	1.42 b	4.78 c
$LSD_{0.05}$	0.008	0.002	0.009	0.02	0.08

Duncan's Multiple Range test (P = 0.05): means followed by the same letters are not significantly different from each other.

DISCUSSION

High levels of BABA (100 and 200 µg/ml aqueous extract) applied as a soil drench reduced nematode infestation of mung bean plants. It is known that BABA has the ability to induce resistance in plants against various plant pathogens, such as fungi (Kamble and Bhargava, 2007; Olivieri *et al*., 2009), bacteria (Pajot and Silué, 2005; Baysal *et al*., 2007), viruses (Lazzarato *et al*., 2009) and nematodes (Oka *et al*., 1999; Oka and Cohen, 2001). Our results corroborate these findings. Pretreatment of mung bean with BABA reduced the number of galls, egg masses and eggs/egg mass of the nematode, which suggests that induction of resistance against root knot infection in BABA-treated plants had occurred. In particular, treatment with 100-200 µg/ml aqueous extract of BABA provided greater resistance to root-knot infection. These findings agree with those by Oka *et al*. (1999), who found reductions in the galling index and number of eggs of *M. javanica* in root-knot infected tomato plants after pre-treatment with BABA. Similarly, Oka and Cohen (2001) also reported that wheat plants treated with BABA as a soil drench resulted in fewer egg masses of an unidentified *Meloidogyne* sp. However, at the time the experiment in pots was discontinued, the number of egg masses of the nematode per treatment was smaller than the number of galls. Also, numbers of eggs per egg mass was rather small, even in the negative control. A later observation would probably have discriminated more between the treatments. The inhibition of infection may be due to changes in plant metabolism or by making the plant cell wall physically harder for nematodes to penetrate (Oka *et al*., 1999). It is also reported that BABA is not metabolized in plants and it is thought to bind to cell wall proteins, making them more resistant to pathogen attack (Cohen and Gisi, 1994). Higher peroxidase activity also accelerates lignin synthesis in roots of BABA-treated tomato and cucumber plants compared with untreated plants (Oka and Cohen, 2001). Lignin formation at pathogen infection sites would also inhibit the entry of nematodes into the roots. Increase in shoot weight and shoot length would indicate an improvement of growth of BABA-treated plants, which, however cannot be attributed entirely to nematode control at least at the least rate of application. However, BABA does not improve plant growth directly as it does not interfere in plant metabolism. It was also observed that increased concentrations of BABA did not significantly increase plant growth compared with the positive control.

Chlorophylls and protein contents were also affected in infected plants compared to uninfected control plants. These are related to plant metabolic activities and the plants with improved growth showed increased chlorophyll and protein contents. Increase of chlorophyll and protein contents in BABA-treated plants compared with the negative control can be attributed to alleviation of root-knot nematode stress. Chlorophyll a/b

ratio is less in negative control plants, because the degradation pathway of chlorophyll a is somewhat different from that of chlorophyll b. Chlorophyll a may be degraded first, prior to degradation of chlorophyll b and the inhibition of chlorophyll synthesis may be due to stress (Kariola *et al*., 2005). Likewise, protein contents increased significantly in treated plants compared with negative controls, though the maximum level of protein was found in positive controls. It is pertinent to note that protein content was reported to increase as a result of inhibition of root-knot nematode infestation in okra and brinjal plants (Abbasi *et al*., 2008). The developing giant cells and galls in root-knot infected plants represent the major sinks for nutrients and amino acids (Hoth *et al*., 2005), which results in a lower availability of amino acids for protein synthesis. However, BABA treatment impairs this mechanism by reducing nematode infestation. BABA has also been shown to induce accumulation of pathogenesis-related (PR) proteins and deposition of callose and lignin, and has been reported to act through jasmonic acid, ethylene and abscisic acid defense pathways (Cohen *et al*., 1994; Hamiduzzaman *et al*., 2005). BABA did not affect egg hatching or mortality of juveniles of *M. javanica*. Therefore, the reduction of nematode infection and development in mung bean results from defense mechanisms activated by the plants following uptake of BABA.

Based on these results, BABA appears to have potential to be used as an agent for inducing systemic resistance against *M. javanica* in mung bean.

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