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Rapid Communication

Rapid γ-Aminobutyric Acid Synthesis and the Inhibition of the Growth and Development of Oblique-Banded Leaf-Roller Larvae[1]

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The hypothesis that rapid γ-aminobutyrate (GABA) accumulation is a plant defense against phytophagous insects was investigated. Increasing GABA levels in a synthetic diet from 1.6 to 2.6 μmol g^{-1} fresh weight reduced the growth rates, developmental rates, and survival rates of cultured *Choristoneura rosaceana* cv Harris larvae. Simulation of the mechanical damage resulting from phytophagous activity increased soybean (*Glycine max* L.) leaf GABA 10- to 25-fold within 1 to 4 min. Pulverizing leaf tissue resulted in a value of 2.15 (±0.11 SE) μmol GABA g^{-1} fresh weight.

GABA is a nonprotein amino acid normally found as a major component of the free amino acid pool. Rapid and large accumulation of GABA in response to diverse environmental stimuli (Satyanarayan and Nair, 1990), including mechanical stimulation or damage of soybean (*Glycine max* L.) leaves (Wallace et al., 1984), is well documented. GABA synthesis is catalyzed by L-GAD (EC 4.1.1.15), a cytosolic enzyme (Breitkreuz and Shelp, 1995). Recent data indicate that GAD is activated by increases in the cytosolic concentration of either H^+ (Carroll et al., 1994; Crawford et al., 1994) or Ca^{2+} (Arazi et al., 1995; Snedden et al., 1995). The cytosolic concentration of these ions is normally around 100 nM and is much lower than the corresponding levels in the adjacent apoplast or vacuole (Bush, 1993, 1995). Mechanical stimulation is known to result in a rapid increase in cytosolic Ca^{2+} levels (Knight et al., 1991). In addition, any mechanical damage that ruptures the vacuolar membrane would result in increased H^+ and Ca^{2+} levels in the cytosol. Consequently, rapid GABA synthesis in response to mechanical stimulation or damage (Wallace et al., 1984) can be ascribed to increases in GAD activity mediated by increases in levels of cytosolic H^+ or Ca^{2+}.

In animals GABA is an inhibitory neurotransmitter. Here we investigate the hypothesis that the mechanical stimulation or damage resulting from the activities of phytophagous insects stimulates GABA accumulation, which in turn deters insect growth and development. This hypothesis was proposed previously (Wallace et al., 1984) but was not investigated.

MATERIALS AND METHODS

Plant Materials and Manipulation

Soybeans (*Glycine max* L. Merr. cv Corsoy 79) were grown in a growth chamber (16:8 h light:dark, 25°C) for 45 to 55 d. The third trifoliates, although still attached, were mechanically stimulated with six repetitions of rolling the leaflets, touching with a plastic rod, or stroking with a nylon brush. Each treatment lasted 30 s, and after an additional 30 s, samples were detached and placed immediately into liquid N_2. To simulate the mechanical damage caused by phytophagous insects, other samples were detached, crushed in a mortar for 30 s, and immersed in liquid N_2 after a subsequent 30-s period. This crush procedure was also carried out with terminal light-green leaves and mature dark-green leaves from the fifth node of apple (*Malus pyrus* cv MacIntosh) in a Vineland, Ontario, apple orchard in mid-June and mid-July, 1995. Control samples were collected directly into liquid N_2 without mechanical stimulation or damage.

GABA Extraction and Determination

Previously recommended procedures were adopted to minimize changes in GABA levels subsequent to the experimental manipulations. Samples (0.3–0.4 g) were pulverized in liquid N_2, and immediately after evaporation of the liquid N_2, they were treated with 5 mL of 100% methanol to inactivate enzymes (Wallace et al., 1984). Subsequent additions with stirring of 10 mL of chloroform and 5 mL of water were followed by centrifugation at 2800*g* for 10 min to separate aqueous and organic phases. In some experiments the pulverized powder was brought to 20°C for predetermined periods prior to methanol addition. The GABA-containing aqueous phase was removed, dried, redissolved, and passed through a 0.8 × 4 cm AG 50 W-X-8 (H^+) resin column (Bio-Rad). GABA was eluted with 4 N NH_4OH. The eluant from 2 to 6 mL inclusive was collected, dried, and redissolved in 0.1 M potassium pyrophosphate

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Abbreviations: GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; OBLR, oblique-banded leaf-roller.

buffer, pH 8.6. This procedure removed a pigment found in soybean and apple leaves that interferes with the 340-nm spectrophotometric-coupled enzyme assay system for GABA (GABAse, Sigma). The assay was described previously (Crawford et al., 1994).

Measurement of the Growth and Development of *Choristoneura rosaceana* Harris (OBLR) Larvae

OBLR egg masses were provided by the Agriculture Canada Vineland Research Station (Vineland, Ontario) and were maintained on moist filter paper until they hatched. Individual 2-mm larvae were transferred with a fine paintbrush to 5-cm-diameter Petri dishes containing approximately 2.5 g of an artificial synthetic diet (Omnivorous Leaf Roller, Bio-Serv, Frenchtown, NJ). OBLR larvae were grown on diets containing different GABA levels. Without addition, the control diet had 1.6 μmol GABA g^{-1} fresh weight. GABA supplementation resulted in diets containing 2.6 and 11.6 μmol g^{-1} fresh weight. Levels remained stable for the duration of the experiment. In some experiments larvae were reared in 9-cm-diameter Petri dishes containing separate 2.5-g diets with two different GABA levels. On d 10, 17, 24, 31, 38, and 45 after hatching, larvae were immobilized by cooling on ice prior to transfer to a balance for weighing. Weight, percentage of survival, and days to pupation were recorded.

RESULTS

GABA supplementation reduced the survival rates of OBLR larvae, as measured by formation of a pupa, from 70 to 50 to 42%, and increased the days to pupation from 45 to 70 to 85 (Fig. 1). The Wilcoxon rank-sum test demonstrated that the difference in survival rates or days to pupation in the diets supplemented with GABA were significant ($P < 0.02$) compared with control values. The weights of surviving OBLR were measured at weekly intervals. Compared with control values, GABA at 2.6 or 11.6 μmol g^{-1} fresh weight reduced the growth of larvae. At 24 and 31 d the weights were reduced by 66 and 57%, respectively, relative

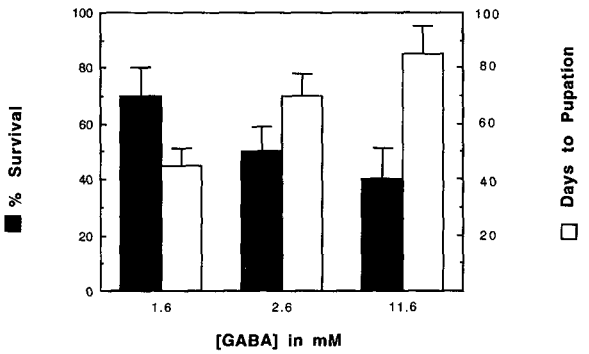

Figure 1. The survival and development of OBLR larvae reared on GABA-supplemented diets. Survival was measured as the percentage of larvae that formed pupae. Development was measured as the number of days required for pupation to occur. The experiment was repeated four times with 20 larvae in each treatment group. The mean ± SE is indicated.

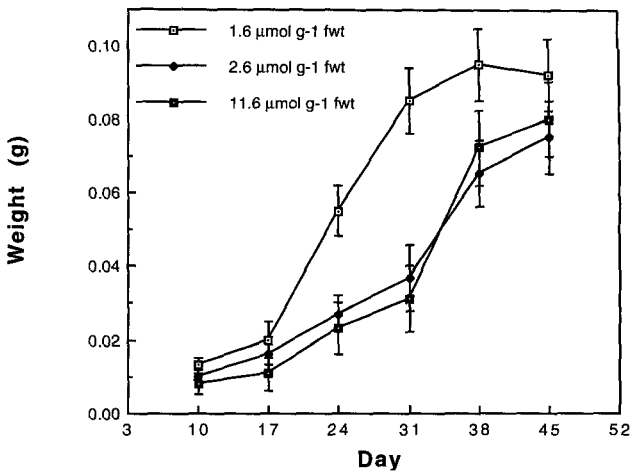

Figure 2. The growth of OBLR larvae reared on GABA-supplemented diets. Growth was measured by weighing larvae at weekly intervals for 45 d. The experiment was repeated four times with 20 larvae in each treatment group. The mean ± SE is indicated. fwt, Fresh weight.

to the weight of larvae grown on the control diet. At these times, the Wilcoxon test demonstrated that the difference in weight between larvae grown on control and supplemented treatments was significant ($P < 0.025$). Surviving larvae grown at 2.6 and 11.6 μmol GABA g^{-1} fresh weight gained weight rapidly after 31 d, and at 45 d their weight relative to controls was reduced by only 17%. The start of the pupation process in the control group was signaled by a slight weight loss between d 38 and 45 (Fig. 2).

When larvae were grown in Petri dishes containing both control and GABA-supplemented diets (2.6 or 11.6 μmol g^{-1} fresh weight), the growth and developmental rates were similar to those for larvae grown exclusively on the control diet. Direct observation indicated that larvae spent more time on the control diet, indicating that they could discriminate between control and GABA-supplemented diets. Experiments with similar concentrations of L-Glu, L-Ala, L-Lys, and tryptamine did not reveal any significant inhibition of growth or development. Thus, inhibition in response to GABA cannot be attributed to a nonspecific toxic effect of elevated amino acid levels.

Soybean leaves were used to simulate the mechanical stimulation or mechanical damage arising from the movement or chewing activity, respectively, of phytophagous insects on leaf tissue. Unstimulated soybean leaf tissue had a GABA level of around 0.08 μmol g^{-1} fresh weight. Mechanical stimulation did not result in any observable damage to the leaf over the subsequent 24 h. Rolling, brushing, or touching the leaf for 30 s, followed by an additional 30 s at room temperature, resulted in a 9- to 11-fold increase in GABA levels. Periods longer than the additional 30 s did not result in further increases in GABA levels. After 24 h GABA levels had returned to unstimulated levels. When mechanical damage was simulated by crushing leaf tissue for 30 s, a surface electrode applied to the crushed leaf indicated a sap pH of 5.4. After crushing, samples were maintained at room temperature for 30 s and then placed in liquid N_2. This treatment resulted in an 18-fold increase to

1.54 μmol GABA g^{-1} fresh weight. If unstimulated tissue was frozen in liquid N_2, pulverized to a powder, and brought to 20°C, GABA levels rose to 0.71 μmol g^{-1} fresh weight after 2 min and to 2.15 μmol GABA g^{-1} fresh weight after 4 min (Table I).

In field observations of OBLR larvae on apple trees more than 90% were found on light-green expanding leaves at the terminal apex of the branches. The remainder were found on dark-green mature leaves farther back from the apex. The relationship between leaves frequented by larvae and their capacity to produce GABA when mechanically damaged was investigated. Both types of leaves had an unstimulated GABA level of around 0.10 ± 0.04 (SE) μmol g^{-1} fresh weight. After being crushed, light-green leaves synthesized 1.11 ± 0.09 (SE) μmol GABA g^{-1} fresh weight within 1 min. However, the corresponding figure for the dark-green, less-frequented leaves was 1.68 ± 0.24 (SE) μmol GABA g^{-1} fresh weight. Thus, it appears that larvae frequent leaves that have a reduced capacity to produce GABA when damaged. Evidence of phytophagous damage was found on both light-green and dark-green leaves. However, it was not clear at what stage in leaf development the damage had occurred, or which organism was involved.

DISCUSSION

Rapid GABA accumulation can now be attributed to increases in the cytosolic level of H^+ (Carroll et al., 1994; Crawford et al., 1994) or Ca^{2+} (Arazi et al., 1995; Snedden et al., 1995). At pH 7.0 in vitro GAD is stimulated 4-fold by the addition of optimal concentrations of Ca^{2+} and calmodulin. Compared with pH 7.0, activity at pH 5.8 is stimulated 12-fold and Ca^{2+} and calmodulin are not stimulatory (Snedden et al., 1995). In the present study, crushing resulted in a sap pH of 5.4. These data suggest that increased H^+ levels are primarily responsible for GAD activation and rapid GABA accumulation after crushing (Table I).

The hypothesis tested here is that phytophagous insect activity disrupts the normal cellular compartmentation, increasing H^+ and Ca^{2+} levels in the cytosol, which stimulates GABA synthesis, which in turn inhibits the growth and development of OBLR larvae (Fig. 3). In North America, the annual life history of the OBLR involves two cycles, with two populations of larvae resulting from eggs laid in June and August. Larvae feed on the expanding leaves and flower clusters of 50 to 80 plant species (Brunner and Beers, 1990) and pupate 40 to 50 d after hatching. In the present study, GABA supplementation of the diets inhibited the survival, development, and growth of OBLR larvae (Figs. 1 and 2). Development, as indicated by days to pupation, increased to 70 or 85 d, compared with values of 40 to 50 d for control larvae (Fig. 1) and larvae in the field (Brunner and Beers, 1990). The ability to complete the insects' annual life cycle may be compromised by this delay. At 45 d there is a 50% reduction in surviving larval biomass. A GABA-free diet was not developed, since diets with a completely defined chemical composition did not support larval growth, with or without GABA addition. Diets for the OBLR are plant-based, and in this study they contained wheat germ, the presumed source of GABA.

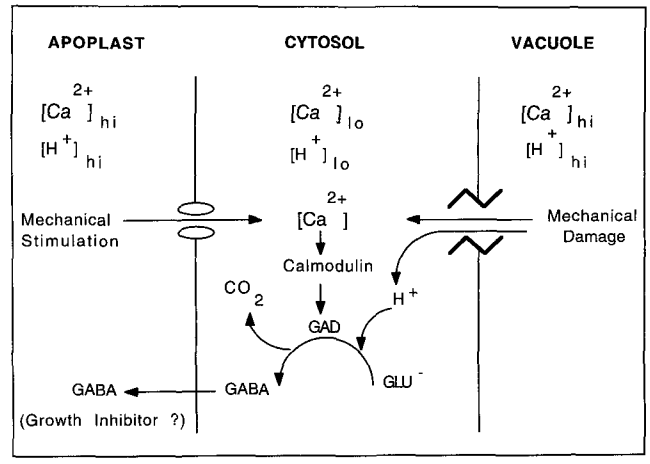

Figure 3. Mechanisms by which GABA synthesis may be increased when phytophagous insect activity results in damaging or nondamaging mechanical stimulation of leaf tissue.

Pulverizing leaf tissue most closely simulates the tissue damage that results from larval feeding, and this treatment resulted in values of 2.15 μmol GABA g^{-1} fresh weight (Table I). Significant inhibition of larval survival, growth, and development was observed when GABA concentrations were increased from 1.6 to 2.6 μmol GABA g^{-1} fresh weight (Figs. 2 and 3). Thus, pulverizing tissue resulted in GABA levels that were within the concentration range at which GABA becomes inhibitory. Larvae confined to a Petri dish were unable to avoid high GABA levels; however, in the field OBLR larvae dispersed rapidly after hatching from the egg mass (AliNiazee, 1986; Carrière, 1992b). They may avoid noxious or toxic levels of GABA by migrating to other tissues or plants (Carrière, 1992a, 1992b) with a reduced capacity to synthesize GABA. Reports that 90% of OBLR larvae are found on expanding terminal apple leaves, which, when crushed, generate lower GABA levels, support this suggestion. OBLR larvae feed on expanding immature leaves (Brunner and Beers, 1990), and previous work has demonstrated higher GABA levels in mature leaves from a variety of plants (Lähdesmäki, 1968).

Table I. *Mechanical stimulation of GABA synthesis*

Non-damaging (roll, brush, touch) or damaging (crush) mechanical stimuli were applied to soybean leaves for 30 s. Following a subsequent 30 s period, tissue was immersed in liquid N_2, and GABA was determined. In some experiments unstimulated tissue was pulverized in liquid N_2 prior to a 4-min exposure of the powder to 20°C. Each value is derived from a minimum of three experiments with duplicate GABA determinations. The mean ± SE is indicated.

Treatment	μmol GABA g^{-1} fresh wt	Percent Increase
Control	0.08 ± 0.02	0
Roll	0.99 ± 0.08	1137
Brush	0.90 ± 0.07	1025
Touch	0.83 ± 0.06	938
Crush	1.54 ± 0.12	1825
Pulverize	2.15 ± 0.11	2587

It is not clear whether leaf tissue stimulation resulting from larval locomotion can increase GABA synthesis or whether any such increase functions in plant defense. It is clear, however, that nondamaging stimulation resulting from rolling, brushing, or touching leaf tissue does cause a rapid and large increase in GABA levels (Table I).

The ability of insect larvae to grow rapidly after a period of growth inhibition (Fig. 2) has been noted before (Carrière, 1992a; Keyserlingk and Willis, 1992). However, the mechanism by which elevated GABA levels inhibit larval growth and development has not been established. If ingested GABA is absorbed into the larval hemolymph, the neuromuscular junctions of body wall muscles are exposed to this inhibitory neurotransmitter. These junctions are not protected by a covering of glial cells, so injection of physiological concentrations of neurotransmitters into the hemolymph causes reversible paralysis (Irving et al., 1976, 1979). In vertebrates GABA receptors are restricted to the brain and are protected by the blood-brain barrier. Many commercially employed insecticides are agonists or antagonists of the GABA-gated Cl^- current, and are thought to cause paralysis through disruption of normal neuromuscular activity (Keyserlingk and Willis, 1992; Casida, 1993). GABA ingestion that raises levels in the hemolymph may similarly disrupt normal neuromuscular activity.

The data support the hypothesis initially proposed by Wallace et al. (1984) that GABA accumulation may be a defense against phytophagous insects. However, it should be recognized that rapid GABA accumulation may represent one of many chemical defense systems that immobile plants deploy against phytophagous invertebrates. The existence of transgenic tobacco plants overexpressing GAD activity (Baum et al., 1995) raises the possibility of genetically engineered crop plants more resistant to phytophagous attack.

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