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The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* (Caryophyllaceae)

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Plants of the meadow annual *Agrostemma githago* (Caryophyllaceae) were grown in 1-m^2 field plots prepared with three fertilizer treatments as follows: (a) "low", no fertilizer, (b) "medium", 75 g of slow release fertilizer granules, (c) "high", 175 g of granular treatment. After sowing in spring the plants were left until flowering in late summer. Nectar was extracted using 5-µl glass microcapillary tubes. The material was frozen and sampled at a later date. The samples were analysed by high performance liquid chromatography (HPLC) using the AccQtag (Waters Corp.) system. Analysis showed that the total concentration of amino acids increased significantly with increasing fertilizer treatment ($P < 0.05$). Of the amino acids present glutamine showed a large and significant increase ($P < 0.01$), proline showed an increase ($P < 0.05$) and γ-amino butyric acid (GABA) showed a decrease ($P < 0.05$). No others showed a significant change. It is interesting to note that a common biosynthetic pathway (from α-ketoglutarate) links the amino acids that altered in concentration. Due to these changes the relative abundance of about half of the amino acids in the nectar was significantly altered. Glutamine showed a significant increase ($P < 0.001$) in percentage of the total with most of the remaining amino acids declining in relative abundance. The results show that, in contradiction to earlier work, soil conditions can affect the amino acid complement of nectar. This may have implications for plant-insect interactions, as local populations of pollinators may benefit from the increased amino acid content of the nectar and preferentially visit plants growing in high nutrient conditions.

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The presence of amino acids in the nectar of flowers has been known for some time. Early papers demonstrated that nitrogenous compounds were to be found in nectar (Ziegler 1956, Lüttge 1961). However, it was not until 1973 that amino acids were shown to be widespread (Baker and Baker 1973). A number of studies followed, advancing various hypotheses for the role of the amino acids and examining differences between and within plant species (Baker and Baker 1975, 1982, 1976, Gottsberger et al. 1984, 1989). Baker and Baker (1973), for example, showed that plants pollinated by butterflies contain a higher concentration of amino acids in their nectar than those plants pollinated by birds. A few studies have also explored intraspecific variation in nectar composition. An early study on intraspecific variation in nectar amino acids (Baker and Baker 1977) examined a number of plant species growing in different locations and concluded that nectar amino acid complement and composition was "remarkably constant" for any one species. Examples are cited of plants from several species growing in "strikingly different environments" yet displaying constancy of nectar composition (e.g. *Convolvulus arvensis*, *Geranium robertianum*). The method of analysis was thin layer chromatography (TLC) using dansylated samples (Baker and Baker 1976), in which concentration of

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individual amino acids was given as a relative colour scale, although in some cases only presence or absence was noted. No attempt was made to quantify the differences between environments.

A later study using more sophisticated methods of high performance liquid chromatography (HPLC) examined the variation in nectar composition at several levels: within a single plant, within a population and between populations of *Impatiens capensis* (Lanza et al. 1995). This study demonstrated, in contrast to the study of Baker and Baker, that nectar amino acid complement can alter between plants taken from different populations and that variations exist within a single population. Differences were recorded for total concentration and also for particular amino acids. No explanation was suggested for these variations.

Most recently the variation in nectar composition of five species in response to elevated CO_2 levels has been investigated (Rusterholz and Erhardt 1998). The study found that although total amino acid concentration did not vary, the composition of the nectar was altered by the treatment in all but one of the species analysed.

The present study is the first to determine intraspecific changes in nectar amino acid complement in response to an experimentally manipulated soil environmental variable. In this case a mixed (N:P:K) fertilizer treatment was applied. With modern farming methods relying heavily on fertilizers, changes in nectar composition could indicate that wild plants are affected by such practices. Altered nectar composition may impact upon nectar feeding insects that visit the flowers and so affect pollination.

Materials and methods

Experimental design

Eighteen 1-m^2 plots were formed by removing the turf, to a depth of 10 cm, of the field site at the Open University campus in Milton Keynes, UK. The plots were arranged into six blocks of three treatments, each being 50 cm apart. The six blocks of plots were further split into two sets of nine treatments, treating each set as a Latin square with treatments being assigned randomly where permitted (Fig. 1). The plots were dug over and coarse sand and chipped bark were added to the heavy clay soil in order to improve drainage. The treatments applied were as follows: (a) no added fertilizer, (b) 75 g of slow release granular fertilizer (Bio Timed release capsules, pbi Home and Garden Ltd., N:P:K ratio 18:6:12) and (c) 175 g of granular fertilizer. The plant species chosen was corncockle, *Agrostemma githago* L. (Caryophyllaceae), a meadow annual formerly common across much of Europe but now in decline due to modern farming methods. This species was chosen as it was possible to grow and harvest quickly, it has a wide distribution and will grow well under a variety of conditions. Seeds (from Suttons seeds Ltd.) were sown directly onto the soil in early April and raked in. Germination was allowed to proceed naturally and no further care was necessary. By mid-July the plants were flowering and nectar collection commenced.

Nectar collection

Nectar was collected at the same time of day (14:00–16:00) over the period of the experiment and from flowers of approximately the same age. This was to minimize effects of flower ageing that have been shown to affect amino acid concentrations in nectar samples (Gottsberger et al. 1990). Newly opened flowers were covered with a fine net (dress net, 1-mm mesh size) to prevent visitation by insects and so possible contamination or nectar removal. The following day the nectar of those flowers was withdrawn using 5-µl glass graduated micropipette tubes, capillary action being enough to draw the nectar in. To minimize possible contamination with pollen the inflorescence was cut with sharp scissors, allowing the anthers to fall away, revealing the ovary. The volume of the samples was determined by measuring the fluid column in the pipette and each sample was aspirated into a glass chromatography vial (Chromacol #02-CTVG). After collection each plant was marked with a permanent marker pen to prevent re-sampling the same plant. The samples were frozen (at -40°C) shortly afterwards and kept until analysis by HPLC.

Analysis of nectar

The samples were thawed and the amino acids derivatized using the AccQtag protocol (Waters Corp.) (Cohen and Micheaud 1993) in a 0.02 M borate buffer (pH 8.59). HPLC was performed, with standards every four samples, using the following equipment: Waters 712 WISP autosampler, Waters 600 pump controller, Wa-

75	0	175
175	75	0
0	175	75

75	175	0
0	75	175
175	0	75

Fig. 1. Layout of 1-m^2 experimental plots. Each plot was sown with *Agrostemma githago* in spring with nectar being collected in late summer. Figures indicate g m^{-2} of fertilizer treatment added to each plot.

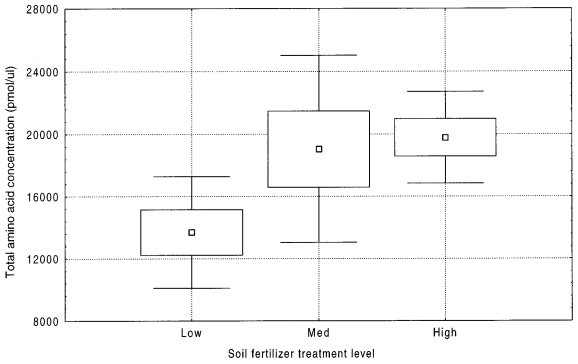

Fig. 2. Total concentration of amino acids in nectar of *Agrostemma githago* grown under three soil fertilizer conditions. Shown are: mean, standard error (box) and standard deviation (bar).

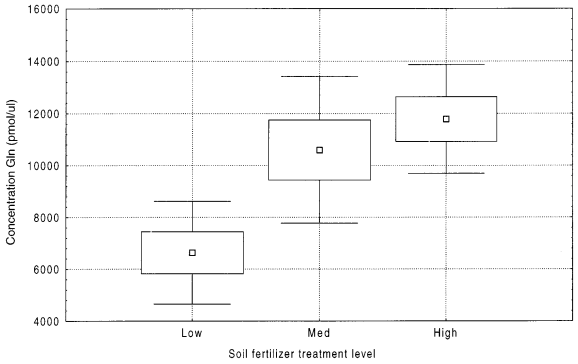

Fig. 3. Concentration of glutamine in nectar of *Agrostemma githago* grown under three soil fertilizer conditions. Shown are: mean, standard error (box) and standard deviation (bar).

ters 600 HPLC pump with 510 pump-heads. Separation was achieved using a Novapak C18 (15 cm × 4.6 mm) cartridge with guard column. The binary solvent system was a 6:4 acetonitrile/water mix and a TEA/phosphate (pH 5.04) buffer. Detection was via a Waters 474 scanning fluorescent detector (excitation at 295 nm and detection at 350 nm). The system was monitored and data collected using the Waters Millenium[32] software. Chromatograms were analyzed and compared to standards for identification of individual amino acids (see Fig. 6 for compounds identified). Peak areas were compared to standards to determine the concentration of individual amino acids. From these data the total concentration of all amino acids was determined and the proportion that each made to the total was also calculated as a percentage.

Statistical analysis

Analysis was performed with Statistica (StatSoft Inc.) using two-way ANOVA with factors of block and soil nitrogen treatment. The blocks were considered as random effects and the mean-square ratio was therefore the treatment effect over the error (Sokal and Rohlf 1981). Data from different plants in a given plot were averaged to avoid pseudo-replication (between five and nine samples per plot). Percentage data were arcsine transformed before analysis.

Results

The total concentration of amino acids increased significantly with soil fertilizer treatment ($F_{2,10} = 4.80$, $P < 0.05$, Fig. 2). This increase was largely due to significant increases in glutamine ($F_{2,10} = 13.67$, $P < 0.01$, Fig. 3) and proline ($F_{2,10} = 7.52$, $P < 0.02$, Fig. 4). One amino acid, γ-amino butyric acid (GABA), showed a decrease ($F_{2,10} = 4.44$, $P < 0.05$, Fig. 5) whilst of the remaining amino acids detected only asparagine showed a significant response ($F_{2,10} = 4.50$, $P < 0.05$) but this amino acid was found in only small quantities (Fig. 6).

Since some amino acids showed a significant response to the soil fertilizer treatment with others showing little change, the relative abundance of amino acids changed between treatments. Nearly half of the amino acids show a significant change in their relative proportions in the nectar (Fig. 7) with glutamine increasing (Fig. 8) and many of the others decreasing (e.g. valine, Fig. 9).

Discussion

Most of the amino acids showed no significant response to the soil fertilizer treatment. Of those that did the responses were variable. Glutamine and proline both showed increases in response to the fertilizer treatment but exhibited different threshold levels. Glutamine concentration rose sharply with the "medium" treatment

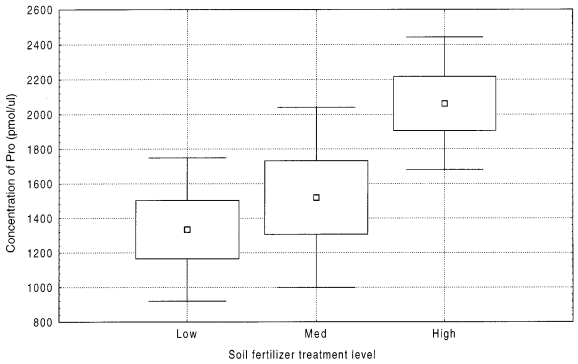

Fig. 4. Concentration of proline in nectar of *Agrostemma githago* grown under three soil fertilizer conditions. Shown are: mean, standard error (box) and standard deviation (bar).

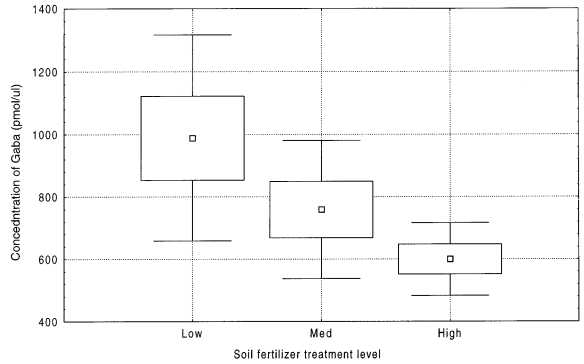

Fig. 5. Concentration of GABA in nectar of *Agrostemma githago* grown under three soil fertilizer conditions. Shown are: mean, standard error (box) and standard deviation (bar).

but only rose modestly beyond that. Proline concentration, on the other hand, showed a small increase in the "medium" treatment and a sharp rise in the "high" treatment. GABA showed a decrease in response to increasing soil fertilizer whilst the other amino acids showed no significant response in absolute concentration. The changes that did occur were sufficient to produce changes in the relative abundance of nearly half of the amino acids detected in the nectar.

Glutamine is well known as an important amino acid in nitrogen metabolism and it is conceivable that the observed increase in glutamine may be a mechanism of shunting some excess nitrogen out of the cells. However, since nectar is produced in small volumes and over a short period (often just a few days) it seems unlikely that the presence of glutamine could be an important mechanism of excretion. Glutamine is implicated in many biosynthetic pathways as a donor of amide groups such as in the formation of purine nucleotides and the neurotransmitter serotonin (Stryer 1988). It is highly abundant in muscle and liver, where it may be used in gluconeogenesis (Mouterde et al. 1992, Nurjhan et al. 1995) and would most certainly be a beneficial addition for the energetically expensive process of flight in insects.

Proline is on the same biosynthetic pathway as glutamine (from α-ketoglutarate and glutamate, Fig. 10) and its increased abundance may be a by-product of the extra glutamine synthesis. The structure of proline is such that, when incorporated into protein chains, it causes a bend to appear. Like the majority of amino acids, proline can be used in energy production.

GABA is known as a neuro-transmitter. It acts to increase the permeability of post-synaptic membranes to chloride ions and so acts as an inhibitory transmitter. GABA can be synthesized from glutamate (catalysed by glutamate decarboxylase) and so is also linked to the glutamine synthesis pathway (Fig. 10). Glutamate (an excitatory neuro-transmitter) is easily formed from glutamine (by glutaminase). The function of such a non-protein amino acid in the nectar is unclear. It is conceivable that its presence affects the neurobiology of appetite (of visiting insects) in some manner, with different levels leading to different durations of feeding at the plant.

If local soil conditions favour higher amino acids in the nectar then local populations of (insect) pollinators may derive certain benefits. Adult feeding on amino acid rich food (e.g. pollen) has been shown no increase longevity and reproductive ability in certain heliconine butterflies (Gilbert 1972, Dunlap-Pianka et al. 1977). A later study on a temperate species (*Euphydryas editha*) showed that amino acids in the adult diet led to heavier eggs (Murphy et al. 1983). Further studies have produced more equivocal data (e.g. Moore and Singer 1987, Hill and Pierce 1989). This perhaps shows that insect life histories are varied and that some adaptations to adult feeding have occurred in some species and not in others. A larger population of pollinators

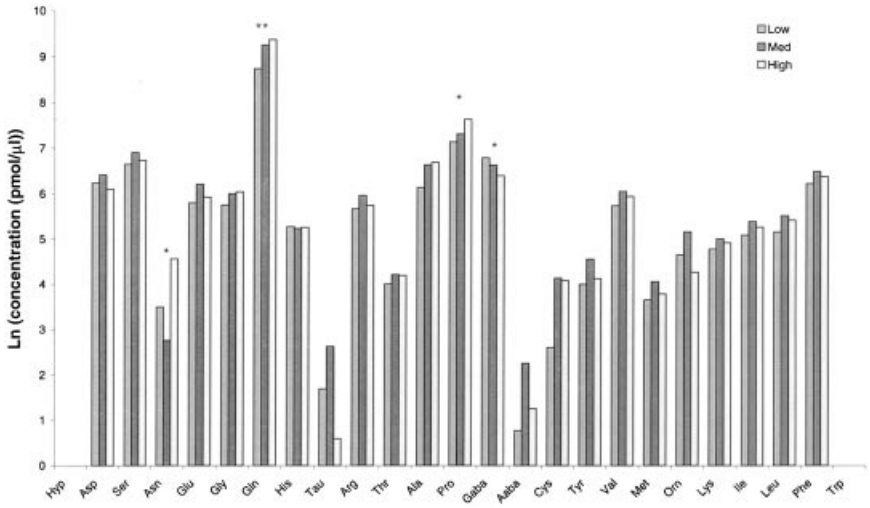

Fig. 6. Total concentration of amino acids (pmol μl^{-1}) in the floral nectar of *Agrostemma githago* when grown under three soil fertilizer treatments (low, medium and high). Concentration is given as natural logarithm for display purposes. All amino acids analysed are shown with hydroxyproline (hyp) and tryptophan (trp) not being detected in any samples. Significant results indicated thus: * $P < 0.05$, ** $P < 0.01$.

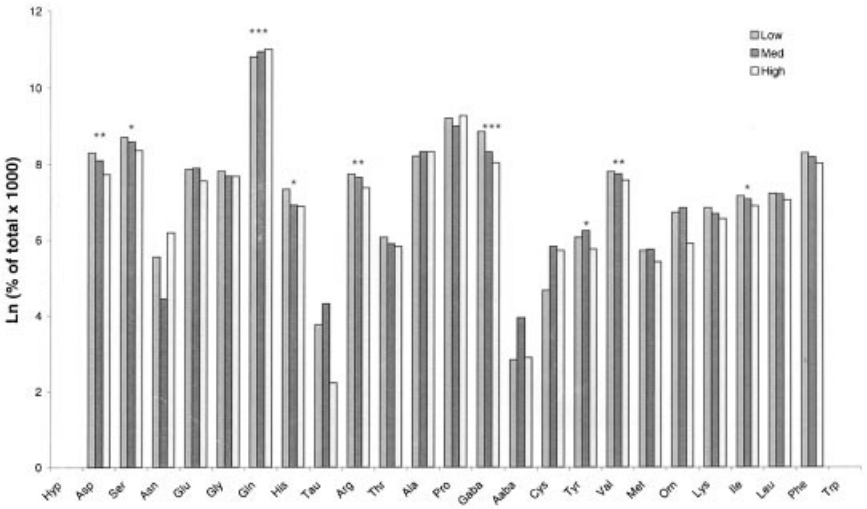

Fig. 7. Relative abundance of amino acids in floral nectar of *Agrostemma githago*, presented as percentage of the total, when grown under three different soil fertilizer treatments (low, medium and high). For display purposes the natural logarithm is shown but to prevent negative results each percentage was multiplied by 1000 first. Significant results are marked thus: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

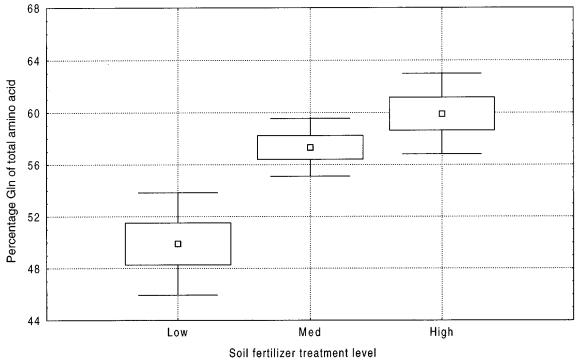

Fig. 8. Percentage of glutamine in nectar of *Agrostemma githago* grown under three soil fertilizer conditions. Shown are: mean, standard error (box) and standard deviation (bar).

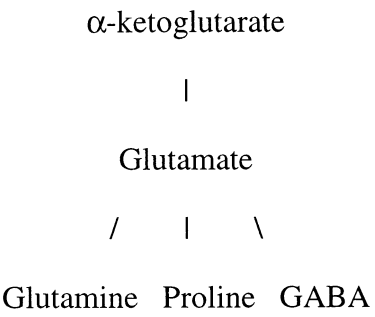

Fig. 10. The biosynthetic pathway involving glutamine α-ketoglutarate can be fed into the citric acid cycle to provide energy.

may lead to higher cross-fertilization of the plants and therefore reduce the level of selfing in previously pollinator-limited plant populations. If amino acid concentration is increased and as a result pollinators show local increases in population size, then this may have important consequences for plant genetic diversity.

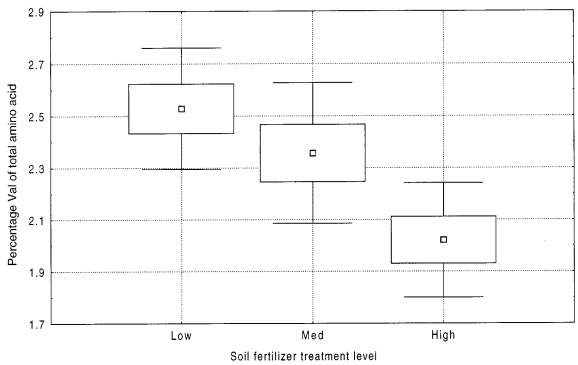

Fig. 9. Percentage of valine in nectar of *Agrostemma githago* grown under three soil fertilizer conditions. Shown are: mean, standard error (box) and standard deviation (bar).

Field boundaries are an important habitat for many plant species. For animals they provide food, shelter and corridors facilitating movement. Modern farming methods rely heavily on nitrogenous fertilizer. Drift from application techniques and run-off from agricultural land can easily lead to nutrient rich environments for wild populations of plants, particularly around field margins (Kleijn and Snoeijing 1997). As this study has shown, the increased soil nutrient status of field margins may in turn impact upon nectar composition and therefore the activity of pollinators. Further work is planned to look at the responses of other species to increased soil fertilizer treatments.

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