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Sarah J. Doege
University of Arkansas, Fayetteville

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THE ROLE OF NATURAL CALCIUM OXALATE CRYSTALS IN PLANT DEFENSE AGAINST CHEWING INSECTS

By Sarah J. Doege
Department of Biological Sciences
Faculty Mentor: Kenneth L. Korth
Department of Plant Pathology

Abstract:
Calcium oxalate is the most abundant insoluble mineral found in plants and it is common among many plant families. Calcium oxalate crystals in plants can appear as irregular rectangles, spiked balls, or needles. The formidable appearance of these crystals has led to speculation that they might serve as a form of pest control by deterring chewing insects. We utilized mutant plant lines to assess the effects of plant calcium oxalate crystals on the survival and feeding habits of chewing insects. We have taken advantage of calcium oxalate-deficient (cod) mutants of the barrel medic, Medicago trunculata. Calcium oxalate crystals accumulate in wildtype M. trunculata leaves along the vascular strands of secondary veins. Results demonstrate that beet army-worm, Spodoptera exigua, larvae that feed on M. trunculata cod mutants with reduced levels of calcium oxalate crystals grow faster and larger than insects that feed on normal wildtype plants. Pupae formed by larvae raised on cod plants are significantly larger than those raised on the wildtype plants. The results of two-way choice tests indicate that older (4th instar or later) S. exigua larvae prefer to feed on leaves lacking calcium oxalate, whereas young larvae (2nd instar or earlier) show no feeding preference. This development-specific feeding preference is perhaps due to the feeding habits of the herbivore; young larvae typically feed between secondary veins, away from areas where the calcium oxalate crystals are localized. Accumulation of RNA transcripts encoding wound-inducible gene products is normal in the cod mutants, suggesting that these plants are not altered in their ability to sense or respond to wounding by insect herbivores. Because calcium oxalate crystals seem to serve as a feeding deterrent to insects, understanding how they are made and distributed could ultimately lead to novel, environmentally sound strategies for improving insect resistance in crop plant species.

Introduction:
All plants need mechanisms of defense to limit the amount of damage by herbivores. A plant with poor defenses faces the possibility of severe foliage damage, rendering it incapable of performing an adequate amount of physiological or reproductive measures to survive. Plants defend themselves using a variety of preformed and induced chemical and/or physical barriers. The tobacco plant, for example, uses the toxic chemical nicotine as a herbivore-defense. Thorns and trichomes are examples of physical defenses that plants might utilize as protection against herbivorous insects.

Crystals of calcium oxalate are prevalent in nature and can be found in over 215 plant families (McNair, 1932). Calcium oxalate is the most common insoluble mineral in plants. Among those that produce the crystals, calcium oxalate can account for up to 3-80% of a plant’s dry weight (Zindler-Frank, 1976; Libert and Franceschi, 1987). There are several hypotheses attempting to explain the role of calcium oxalate in plants - these include the regulation of bulk free-calcium, ion balance, light gathering and reflection, tissue support, and detoxification - but evidence supporting most of these hypotheses is insufficient (reviewed by Franceschi and Homer, 1980).

The placement, size, and shape of calcium oxalate crystals within some plant tissues suggest that they may play an important role in defense (Franceschi, 2001). In scanning electron microscope images, the more formidable crystals appear as multi-faceted spiked balls, or as needle-like with or without barbs. In some instances, the function of the crystals as defense mechanisms is clear, such as that of the stinging plant, Tragia ramosa (Thurston, 1976). The stinging hairs that cover its surface are conical cells, each containing a raphide (needle-shaped) crystal of calcium oxalate. Upon contact, this cell will break open, allowing the crystal to puncture the skin and administer a dermal irritant by way of grooves in the crystal. Calcium oxalate crystals in daffodil and agave are also known dermal irritants (Julian and Bowers, 1997; Salinas, et al., 2001). Many edible aroids contain the crystals in their leaves and corms, and as a result may cause swollen lips, mouth, and throat pain if prepared improperly for consumption (Bradbury and Nixon, 1998).

Research more specifically related to calcium oxalate crystals as herbivore defense reports that the dorcas gazelle, larvae of a lepidopteran species (Polytella clines), and a land snail (Eremina desertorum) all avoid feeding on certain tissues of a Negev desert lily (Pancratium sickenbergeri) that contain...
We utilized calcium oxalate deficient (cod) mutants of the barrel medic, *Medicago truncata*, to test the growth and survival of the chewing lepidopteran beet armyworm, *Spodoptera exigua*. *M. truncata* is an increasingly important model legume used in a variety of genetic and genomic studies. This diploid relative of alfalfa (*M. sativa*) is useful because of its short generation time, ease of transformation, its relatively small genome size, and the increasing number of mutant lines that are available (Cook, 1999). As in alfalfa leaves, calcium oxalate typically accumulates as prismatic crystals along the secondary veins of *M. truncata* leaves (Nakata and McConn, 2000).

Several *M. truncata* mutant lines have been identified with reduced levels of calcium oxalate or with changes in crystal morphology or accumulation patterns (Nakata and McConn, 2000; McConn and Nakata, 2002). The mutant line *cod5* completely lacks crystals, whereas *cod6* has drastically reduced calcium oxalate levels. Only one genotype isolated thus far, *cod4*, has elevated levels of calcium oxalate as compared to the wildtype control line, A17. Five of the cod mutants show a noticeable change in crystal shape (*cod1, cod2, cod3, cod6*, and *cod7*). Large, diamond-shaped crystals are seen in *cod1* and rectangular-kinked crystals are seen in *cod2*. Small, thin diamond-shaped crystals are found in *cod3*. Small, globular crystals are observed in *cod6*, and *cod7* accumulates crystals similar to those observed in wildtype leaves, but with unusual globular protrusions at the crystal surface. Although the overall levels of total calcium in the cod mutants is not different from those in wildtype *M. truncata*, the levels of calcium oxalate that accumulate can be very different. There are essentially no differences in phenotype at the whole plant level among the cod mutants, with the exception of *cod4* that is stunted and chlorotic in comparison to the wildtype. All of the cod mutants described in *M. truncata* are due to single-gene, non-allelic mutations (Nakata and McConn, 2000).

We report here the effects of calcium oxalate on *S. exigua* larvae, as measured by insect growth rates, weights, and feeding preferences on cod mutants as compared to plants with normal levels of calcium oxalate crystals.

**Materials and Methods:**

**Insect growth experiments**

*S. exigua* eggs were obtained from the Garst Rearing Laboratory (USDA-ARS, Starkville, MS) and allowed to hatch on artificial diet. The wildtype *M. truncata* A17, which was derived from the commercial cultivar "Jemalong", was the parental line in the mutagenesis that gave rise to the cod mutants and is used as the control treatment of all of our experiments. Plant cuttings from individual 5-week-old cod mutant lines were maintained hydroponically and placed into covered Petri dishes. At the initiation of the experiment to measure insect growth, five neonatal *S. exigua* larvae were placed in each dish and allowed to feed.

When sufficient plant material was available, three dishes per plant line were used. Two dishes were used for cod6 and cod4 measurements. Materials were kept in the growth chamber under a normal light: dark cycle (16:8 hr). As larvae grew to a size where it became practical to locate them among plant foliage, they were transferred to caged, intact plants maintained under the same conditions.

Larval weights were measured at intervals until pupation. Measurements ceased when larvae entered early stages of pupation, as they began to display significant weight loss associated with the process. This experiment was repeated a total of three times and in each case the larvae were allowed to pupate. Pupae were collected and length and dry weights were determined.

**Preference Tests**

Two independent two-way choice experiments were performed to measure insect preferences for cod mutant tissue. In the first experiment three 2nd instar, or alternatively three 4th instar, larvae were placed in a Petri dish containing leaf material from both of two genotypes. Larvae of similar size were chosen and allowed to feed overnight. Weight of leaf material was measured before feeding and then after the feeding period. Dishes containing plant material were kept in larger, humidified containers to prevent desiccation of the leaf material. Ratios of leaf material consumed of each phenotype were then calculated on the basis of the fresh weight of material consumed.

In a second experiment, two-way tests were performed comparing insect preference of cod5 with A17, cod6 with A17, and cod5 with cod6, with five treatments per test. Larvae were maintained on wildtype plant material for several days before the experiment was initiated. One 3rd instar larva was placed into each dish and allowed to feed overnight. Similar amounts of leaf material were used in each treatment and the leaf material was weighed before and after feeding. The dishes were maintained as described above.

**RNA blot analysis**

Undamaged leaves from each genotype (A17 and cod1 -7) were collected, frozen immediately in liquid nitrogen and then transferred to a -80°C freezer. For collection of herbivory-damaged material, *S. exigua* larvae (3rd or 4th instar) were caged on plants for 21 hours and damaged leaves were collected. Total RNA, 10 μg per lane, was separated via formaldehyde agarose...
gel electrophoresis, transferred to nylon membrane, and hybridized with radio-labeled probes (Sambrook et al., 1989) to measure accumulation of the wound-inducible terpene synthase transcript (tps1). The tps1 gene was isolated from an insect-damaged M. truncatula leaf library and is strongly induced by methyljasmonate and insect damage (K. Korth, unpublished data).

Results:
Insect growth on cod plants.

Larvae of S. exigua that fed exclusively on the calcium oxalate deficient mutants cod5 and cod6 grew markedly larger than those raised on wildtype control (A17) plants. The larvae on cod5 and cod6 plants also started to pupate at least two days earlier than those reared on control tissue (Figure 1). Larvae raised on the other cod mutants also showed somewhat increased growth in comparison to the controls, but still much lower than the increase exhibited by cod5 and cod6. Larvae that fed on calcium oxalate-lacking mutants (cod5 and cod6) developed into significantly larger pupae than those that were reared on control tissue (Figure 2, A). Most of the other cod mutant-fed larvae developed into pupae that were slightly heavier than the controls. Interestingly, larvae that were fed on the cod2 mutant grew to an intermediate final pupal weight; cod2 contains a level of calcium oxalate that is intermediate between the levels in A17 and cod5. Larvae reared on the cod4 mutant, the genotype with the most calcium oxalate accumulation, showed the lowest pupal weight, although not significantly lower than controls.

Differences in pupal lengths exhibit the same trends as pupal weights on cod plants (data not shown). The difference between the pupal sizes of insects fed on A17 plants and those fed on cod5 and cod6 plants was visibly noticeable (Figure 2, B). A dramatic preference for the cod6 and A17 leaf material (Figure 3, B). A dramatic preference for the cod5 and cod6 plants in comparison to A17 was clearly apparent when older 4th instar larvae were tested (Figure 3, B and C).

In a second preference test, the insects again exhibit a preference for the calcium oxalate deficient mutants over the wildtype (Figure 4). In this experiment, a single 3rd instar larva was placed in each dish with the plant material. The value of the ratio of leaf material consumed in the cod5 vs. cod6 test is near to 1.0, indicating that there is essentially no preference exhibited for either of these genotypes in this test.

Wound responses in cod mutants

AD of the cod mutants were tested for their response to insect herbivory, and virtually all of the mutants showed a strong accumulation of wound-inducible tps1 transcript in response to insect feeding (Figure 5). The wounded cod6 mutant showed a low but positive degree of transcript increase over the untreated corresponding control, however the cod6 mutant plants had previously been subjected to mild thrip damage, which might explain the transcript accumulation in the untreated control. The experiment will need to be repeated to confirm these results.

Overall, these data suggest that the cod mutants are not altered in their ability to sense and respond to insect herbivory, as indicated by defense gene induction.

Discussion:

Although it has been suggested before as a possibility (Ehrlich and Raven, 1965), there is only limited suggestive evidence that plant-derived calcium oxalate crystals serve to deter chewing insects. A specialist leafminer on American holly avoids feeding on cell layers that contain calcium oxalate crystals, and it was proposed that the crystals might serve as a mechanical barrier to feeding, although these insects were shown to feed at lower levels on crystal-containing cells in some tissues (Kimmerer and Potter, 1987). A plant low in calcium oxalate (Sinapis alba) was shown to be a more suitable aphid-host than one with higher levels (Chenopodium quinoa); the higher level of plant resistance was attributed partially to higher levels of oxalate (Liebig, 1979), but there might be other significant biochemical differences between the plant species. In contrast, one study showed that the preferred host plant for beetle (Lysathia ludoviciana [Fall] Coleoptera: Chrysomelidae) larvae was very high in calcium oxalate crystals compared to alternative hosts that were offered to the insects. The authors suggested that this host specificity might be attributable to a positive preference for the crystals by the larvae (Campbell and Clark, 1983).

We present evidence that strongly supports the hypothesis that calcium oxalate crystals are effective deterrents of chewing insect herbivory. S. exigua grew distinctly larger and pupated more quickly on plant lines with decreased levels of calcium oxalate, cod5 and cod6. Larvae reared on the other plant lines
tested also showed some increase in growth (but far from the level of cod5- and cod6-fed insects), and this may have been due to the decreased calcium oxalate in these plants or altered crystal morphology, depending on the genotype. Similar trends for larval growth were also seen in other feeding experiments using the cod mutants (data not shown). Our conclusions are strengthened by the fact that cod5 and cod6, although they are nonallelic, independent, single-gene mutations, have very similar phenotypes in terms of calcium oxalate, accumulation and in terms of how lepidopteran larvae respond to them as a food source.

We confirmed that mutant lines were not altered in their abilities to respond to wounding by insect pests. If plants were impaired in their wound-response capabilities, then greater insect growth may have been due to this and not to the absence of calcium oxalate crystals. A gene for a terpene synthase enzyme involved in the wound response pathway is expressed at low levels in undamaged A17 M. truncatula, but is highly up-regulated in insect damaged leaves. Our studies show that all of the mutant cod lines, perhaps with the exception of cod6, expressed normal levels of the terpene synthase transcript, ips1, in non-wounded and wounded leaves. This means that these plants are most likely not altered in their ability to respond to insect damage. Results for cod6 were not as clear as for the others; there was only a slight increase in expression of the gene in insect damaged leaves. Examination of ips1 transcript induction in the leaves of cod6 will be confirmed.

Not only do calcium oxalate crystals have negative effects on the growth of S. exigua, but the larvae also prefer to feed on M. truncatula lacking calcium oxalate. In a preference test, 1st and 2nd instar larvae showed no preference for the cod mutants over the control A17. This may be due to the fact that younger larvae tend to feed around the secondary veins, normally avoiding the tissue that contains the calcium oxalate crystals. Because of this feeding pattern, it is likely that young larvae would have no preference for either genotype because they would not be ingesting large amounts of calcium oxalate in either case. Older larvae (4th instar and up), which usually feed on whole leaves (secondary veins and all), show much greater preference for the calcium oxalate deficient mutants. Insect preference for the cod5 and cod6 mutants was not as dramatic as in a second experiment (Figure 4) as compared to an earlier measurement (Figure 3), but experimental procedure was slightly different each time. In the second experiment, only one insect per plate was used and each insect had a relatively larger amount available to be eaten. Still, preference for the cod5 and cod6 mutants was two-fold higher than that of the control plants.

Further studies in food utilization need to be applied to determine if decreased insect growth on wildtype A17 plants is due to less feeding by the insect (due to presence of an anti-feedant), or because of decreased nutritional value of the A17 plant. Identification of the genes involved in calcium oxalate crystal formation in plants might eventually allow for the targeted alteration of crystal levels, distribution and/or shape within plant tissues and contribute to the development of crops with better resistance to herbivorous insects.

Acknowledgements:

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Literature Cited:


Figure 1. A. Representative Spodoptera exigua larvae after feeding for an equal time period on wildtype A17 or cod5 plants, as indicated. B. S. exigua larvae grow larger and pupate earlier on plants with lower levels of calcium oxalate crystals. Neonates were caged on plants of each genotype, and larval fresh weights were determined at the times indicated. Experiments were carried out with five larvae per plant and three plants of each genotype per treatment. Plants were replaced as needed. Determination of weights was halted when larvae entered early stages of pupation.

Figure 2. Lepidopteran insects feeding on cod mutants lacking oxalate crystals develop into larger pupae. A. Dry weight of pupae reared on plants with lower levels of calcium oxalate (cod5, cod6) is significantly higher (p<0.01) than those reared on normal plants (A17). Groups not showing significant difference in dry weights are indicated by like letters on graph bars; error bars indicate standard deviation (S.D.). B. Representative pupae from insects feeding on each type of plant.
A. Typical feeding pattern of 1st-2nd instar larvae on M. truncatula. B. Fold-difference in relative amounts of fresh leaf tissue consumed when 2nd instar or 4th instar larvae were tested in two-way choice tests comparing cod mutants and A17. A value of 1.0 indicates that larvae consumed equal amounts of tissue from each genotype. Two larvae per plate were caged overnight with equal fresh weights of tissue. C. A typical result showing the clear insect preference for cod mutant tissue.

Figure 4. S. exigua larvae prefer cod tissue over wildtype A17 tissue, but show no preference for one mutant over another. Fold-difference in relative amounts of fresh leaf tissue consumed when 3rd instar larvae were tested individually in two-way choice tests comparing cod mutants with each other and against A17. A value of 1.0 indicates that larvae consumed equal amounts tissue from each genotype.
Figure 5. RNA blot showing normal accumulation of a wound-induced transcript in herbivore-damaged cod mutants. Leaves were fed upon by S. exigua larvae for 21 hours and accumulation of a characterized wound-inducible transcript (tps1), encoding terpene synthase, was analyzed. Transcript levels in leaves subjected to herbivory (+) were compared to untreated controls (-). The data indicate that wound signaling pathways and inducible gene expression are functional in all of the cod mutants.

Faculty Comment:

Professor Kenneth L. Korth, Ms. Doege’s mentor made the following comments about her work:

Ms. Doege has worked in my lab since the fall of 2001. She is a tremendously fast-learner, a careful and meticulous student, and pleasant co-worker in the lab. Her academic successes and the pace of her studies are notable. I have been impressed by her ability to grasp the biological significance of the experiments that she performed and other projects that are ongoing in my lab. I am even more impressed by the level of the questions she asks regarding her research, indicative of her understanding of the topics we are studying. Sarah has shown great dedication to her work, spent many long and sometimes boring hours in the lab, and has completed every task that was put before her.

With a minimal amount of guidance from myself, Sarah wrote a short proposal for the project that she has worked on. Based on that proposal, she was awarded an Arkansas SILO-State Undergraduate Research Fellowship. Funds from the fellowship were applied to the research project and also allowed Sarah to attend and present a poster at the annual meeting of the American Society of Plant Biologists in Denver, Colorado. In addition, she was awarded an Adair Scholarship to conduct research in the Department of Plant Pathology during the summer of 2002. The research project has allowed Sarah to answer some important biological questions, to learn several molecular biological techniques, to apply statistical analyses, and to get a solid introduction into lab-based biological research.

The work that Sarah completed has successfully addressed a long-held hypothesis that has, until now, been very difficult to dissect. Namely, that the calcium oxalate crystals normally found in the leaves of many plant species can serve as feeding deterrents to prevent damage by chewing insects. Scientists studying plant-insect interactions have largely overlooked the role of these common insoluble crystals in insect defense. These findings have important implications for novel mechanisms of insect control in crop plants, as the genes controlling formation of calcium oxalate might provide valuable tools for improving insect resistance. Sarah will be the first author listed on a manuscript that will soon be submitted to a peer-reviewed scientific journal.