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The Effect of Soil Nitrogen Levels on Thigmotropic Responses in the Venus flytrap, *Dionaea muscipula*

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Abstract

Venus flytraps are perhaps the world's most well-known carnivorous plant. They rely on small insects to acquire nitrogen, as the swampy environment that they inhabit in southeastern United States' Carolina coastal plains is insufficient in nitrogen. We studied the effect that soil nitrogen levels have on the flytrap's carnivorous behavior. We manipulated soil nitrogen levels to produce nitrogen-sufficient and nitrogen-deficient growth conditions for Venus flytraps. We then compared the closure time of the snap traps of the plants in nitrogen-sufficient and nitrogen-deficient soil, following touch stimulus. We found that flytraps in nitrogen-rich soil exhibited longer closure times. We concluded that, because the plants were grown in a nitrogen-rich environment, they did not require supplemental insect nitrogen, and were possibly conserving energy by responding less vigorously to touch stimulus.

Introduction

Venus flytraps, *Dionaea muscipula* - with their rapidly-closing snap-trap mechanisms for catching their prey - have mesmerized generations of observers, from Charles Darwin to today's preschool students[1]. *Dionaea's* indigenous habitat - swampy, low-lying regions in the Carolinas of the southeastern United States - contains fairly nitrogen-depleted soil. These conditions led to the evolution of carnivorous behavior for obtaining adequate amounts of nitrogen[1]. *Dionaea* captures its prey (small animals and insects) with a highly-sensitive and rapidly-moving snap-trap mechanism. The plant is separated into many leaves, each of which is divided into two parts that close together to form the trap[2]. The inner epidermis of the leaves contains three trigger hairs, that when stimulated, activate the closing of the snap-trap[2]. The stimulation of the trigger hairs generates action potentials that create an electrical charge to stimulate the motor cells of the leaf to close, interlocking the cilia on the edges of the leaves to prevent the prey from escaping[2].

We investigated whether relatively nitrogen-rich soil conditions might affect the kinetics of this mechanism. We exploited the thigmotropic characteristic of *Dionaea* (its movement in response to touch) to compare the time it took the snap-traps to close in response to consistent stimulus of the trigger hairs of plants in nitrogen-depleted soil versus those in soil treated with fertilizer (to increase the nitrogen content of the soil). We hypothesized that the speed of the thigmotropic response to touch stimuli would be reduced for *Dionaea* snap-traps growing in nitrogen-rich soil, as the high nitrogen content in the soil would satisfy the plant's need for nitrogen.

Materials and Methods

Growth plot preparation and maintenance

Four plastic aquarium containers measuring 23.2 cm long by 15.2 cm wide by 16.8 cm high were each filled with 7cm of commercially-available topsoil, a mixture of red sedge peat and sand. The topsoil initially measured nitrogen levels of "N0.5", ~7.5 parts per million (ppm), indicating nitrogen depletion; see below. One Venus flytrap (Carolina Biological Supply Company; Burlington, NC) was placed at the center of each of the four containers with the soil in which it was delivered left intact. Soil tests were performed on both the transplanting topsoil and the soil associated with the flytraps' roots to ensure that they had the same initial nitrogen concentrations (both N0.5, nitrogen-depleted; see below). Two containers were designated to be fertilized (for nitrogen addition), two designated to be nitrogen depleted. Peat moss (Carolina Biological Supply Company; Burlington, NC) was added to the second set of containers, surrounding each flytrap, to assist in the depletion of the nitrogen from the soil. The containers were fitted with plastic mesh covers and placed indoors by a window to be exposed to constant temperature and a natural light-dark cycle. For preparation of fertilization solution, 10 grams of commercial fertilizer (Carolina Biological Supply Company, Burlington, NC) were dissolved in a gallon of distilled water. Nitrogen-addition containers were watered with 100 mL of fertilizer water daily; nitrogen-depletion containers were watered with 100 mL of distilled water daily. *Soil nitrogen level testing*: A commercially-available soil testing kit (Model 5880, LaMotte; Chestertown, MD) was used to determine the relative nitrogen concentrations on a scale from N0 to N4. 0.5-g samples of soil were treated with nitrogen extraction solution (dilute HCl; order #5702, LaMotte) and nitrogen indicator powder (N-(1-Napthyl)ethylene diamine dihydrochloride, and sulfanilamide; order #5703, LaMotte) per the manufacturer's instructions. Following treatment, colorimetric analysis of the resulting solutions by comparison with standards afforded estimates of soil nitrogen content. N0 indicated nitrogen-depleted soil ≈ 0-7.5 ppm; N1, deficient ≈ 7.5-15 ppm; N2, adequate ≈ 15-22.5 ppm; N3, sufficient ≈ 22.5-30 ppm; and N4, surplus ≈ 30+ ppm. Soil tests were performed every other day, on average, for the duration of the experiment. *Measurement of closure times:* In order to measure the closure times of the Dionaea snap-traps, each open trap from each plant was mechanically stimulated on each trial day. Only fully-open traps were tested. To initiate the snap-traps' closure, one cm of graphite was extended from the tip of a mechanical pencil to stimulate the trigger hairs. Timing was started once stimulation began, and was stopped once the leaf of the flytrap stopped moving (reached full closure). Trigger

hairs were brushed continuously in the same direction for 60 seconds until closure began. If no movement was observed after 60 seconds of repeated stimulus, it was recorded that there was no closure. These non-closures were not included in the averages of the closure-time measurements for either nitrogen-sufficient or nitrogen-deficient conditions. *Data analysis:* All data collected was recorded and plotted in Microsoft Excel (Microsoft Corporation; Redmond, WA). Statistical analysis used either a Mann-Whitney U-Test (http://elegans.som.vcu.edu/), or a linear regression T-test available on the TI-84 Plus graphing calculator (Texas Instruments; Dallas, TX). All data are reported as mean + SEM.

Results

Soil nitrogen levels remained constant throughout testing

Throughout the experiment, we attempted to create and maintain two extreme cases of soil nitrogen levels: sufficient and depleted. We daily provided soluble fertilizer to two of the four containers in which flytraps were grown to increase nitrogen levels; we added peat moss and no fertilizer to the other two containers to deplete soil nitrogen levels. After transplantation of the *Dionaea* plants, soil testing (see Materials and Methods) was conducted every other day (on average) over 15 days to monitor nitrogen levels.

Colorimetric analysis indicated that each container had an initial soil nitrogen content of N0.5 (~ 7.5 ppm). The two containers treated with soluble fertilizer immediately (on the next testing day; two days later) displayed increased nitrogen levels of N3 (~ 22.5-30 ppm). Over this time, a scatter plot of the nitrogen content (on a 0-4 scale) versus day displayed relatively steep a slope of 1.25 (data not shown). Following this initial jump in soil nitrogen content, we monitored nitrogen levels in the nitrogen-sufficient containers for over one week. We began closure-time testing only after it was determined that the two fertilized containers had attained stable elevated soil nitrogen levels (average of N3; ~ 22.5-30 ppm). We made this determination by plotting measured nitrogen levels (0-4 scale) versus day after transplantation, now excluding the first day of initial low levels. Over a whole week, the slope of the resulting line reached a near-zero slope of -0.042 ($p = 0.60$, linear regression T-test); we thus deemed the nitrogen levels to be stable. These levels remained stable for the subsequent trials of closure-time testing.

Throughout the experiment, the soil containing peat moss, and watered without fertilizer, maintained a stable nitrogen content of N0.5 (~ 7.5 ppm), and did not deviate from this level. We began experimentation on plants in these containers only once the nitrogen levels in the nitrogen-sufficient containers had been deemed stable.

Dionaea snap-traps in nitrogen-sufficient soil close more slowly than those of Dionaea in nitrogen-deficient soil.

We performed closure-time testing to investigate the effect of soil nitrogen levels on the time it takes the snap-traps to close completely in response to a consistent stimulus. After stable nitrogen levels were achieved in each set of containers, closure-time testing was carried out only on the fully-opened *Dionaea* heads of each container for four out of five consecutive days. Closure time was recorded as the time from the initiation of the stimulus until the end of snap-traps' movement.

For calculation of average closure times and comparison of conditions, we pooled data from all tests performed on *Dionaea* from both of the nitrogen-sufficient containers; we similarly pooled data from both of the nitrogen-deficient containers. For nitrogen-deficient *Dionaea*, we measured a mean closure time over all trial days of 3.58 + 2.04 seconds (n = 25 trap closures), with a range of 1.68 to 9.09 seconds. Snap-traps of *Dionaea* grown in nitrogen-sufficient soil exhibited a significantly longer time to complete closure($p < 0.01$, two-tailed Mann-Whitney U test), with a mean of 16.12 + 7.42 seconds (n = 5 trap closures), and a range of 8.9 to 25 seconds (Figure 1). A smaller percentage of nitrogen-sufficient snap traps exhibit closure, as compared with nitrogen-deficient snap traps.

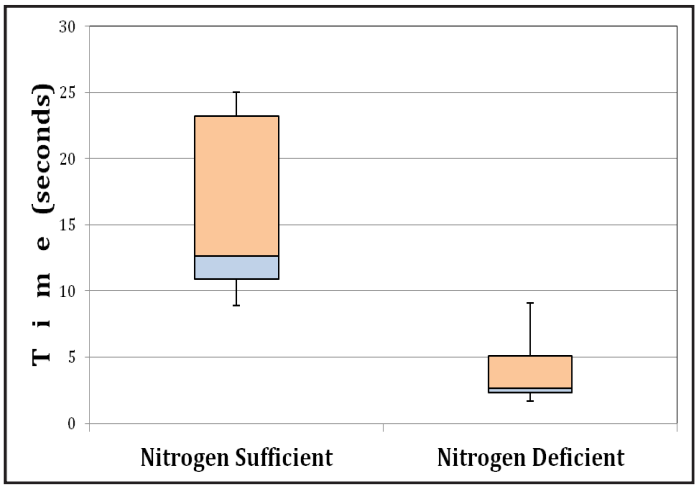

Figure 1. The closure time of *Dionaea* in response to a consistent stimulus at different soil nitrogen levels. A greater average closure time for snaps traps is found for *Dionaea* plants in nitrogen-sufficient (n =5 snap-traps) versus nitrogen-deficient soils (n = 25 snap traps). A significant difference was detected between times to closure under each condition ($p < 0.01$, Mann-Whitney U-test). Whisker ends demarcate maximum and minimum values; middle bar represents median values; orange boxes represent the second quartile of the data while the blue boxes represent the third quartile.

In addition to testing the effect of soil nitrogen levels on the closure time of *Dionaea* snap-traps, we also examined the relationship between soil nitrogen levels and the probability of the snap-traps' closing. We only performed closure-time testing on the traps open at the time of the experiment. Of these traps, some closed within 60 seconds of stimulation and some did not. Thus, for each day of testing, and for each condition (nitrogen-depleted or –sufficient), we calculated the fraction of snap-traps that closed, out of the total number open at the start of that day's trials.

We found, first, that the absolute number of open snap-traps decreased over time for the nitrogen-sufficient *Dionaea*. During the course of the experiment, progressively more previously-stimulated snap-traps from *Dionaea* plants in nitrogen-sufficient soil remained closed until the next trial (data not shown). We also found that for the nitrogen-sufficient *Dionaea*, the fraction of evoked snap-trap closures decreased over time, whereas for the nitrogen-deficient *Dionaea*, the fraction remained relatively stable

The Journal of Experimental Secondary Science

over time (Table 1). A significant difference ($p < 0.05$, two-tailed Mann-Whitney U-test) was detected between the fractions of evoked closures under the two conditions. No habituation to stimulation is evident in nitrogen-deficient snap-traps.

We also investigated whether there could be any habituation to touch caused by the frequent stimulation of the snap-traps in nitrogen-depleted soil. Out of the four separate trials of closure-time testing, a two-day gap existed only between the first and the second trial, while the rest of the trials were carried out on successive days. We observed the following mean values (+SEM) for time to closure over the course of four trials of closure-time testing: 3.54 + 0.74, 4.00 + 1.29, 3.13 + 0.65, and 3.74 + 0.84 seconds. A plot of the mean closure times versus trial day (Figure 2) revealed no clear trend of increasing or decreasing closure time of the nitrogen-deficient snap-traps over the course of daily stimulation. The line of best fit to the mean points had a slope of -0.0083 seconds/day; this slope was not significantly different from 0 ($p = 0.96$; two-tailed linear regression T-test). We did not conduct this analysis for the nitrogen-sufficient *Dionaea* because only five snap-trap closures were observed throughout the entire experiment.

Table 1. Fraction of closures of snap-traps.

Trial #	Fraction of closures; nitrogen - sufficient soil	Fraction of closures; nitrogen - deficient soil
1	0.50	0.61
2	0.33	0.71
3	0.00	0.75
4	0.00	0.67
MEAN +/-SEM	0.21 +/- 0.125	0.69 +/- 0.03

Fraction of snap-traps under each condition that closed in response to touch stimulus (on a trial-by-trial basis). A significant difference was detected between the fraction of closures under each condition (n = 4 trials for each condition; $p < 0.05$, two-tailed Mann-Whitney U-test).

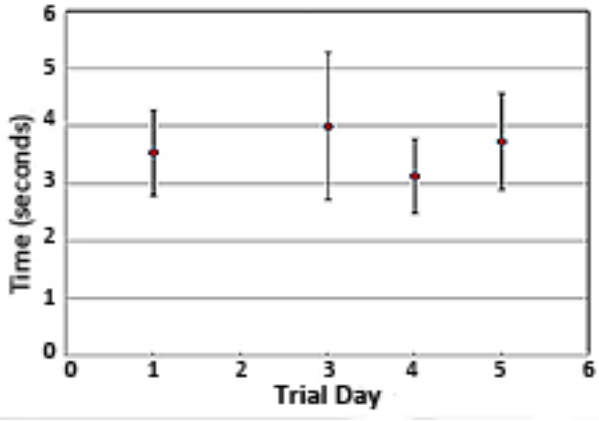

Figure 2. Average closure time, by trial, of the nitrogen-deficient *Dionaea*. Red points represent the mean closure times (+ SEM) for nitrogen-deficient *Dionaea* for each trial day. (Between the first and third day, no testing was performed.) No clear upward or downward trend is evident in the data; the line of best fit to the mean points had an equation of $y = -0.0083x + 3.63$.

Discussion

Nitrogen deficiency in Dionaea results in longer time to snap-trap closure.

By stimulating the head of the *Dionaea* and thus simulating the presence of an insect, we caused the leaves of the plant to close. In comparing the closure times between snap-traps of *Dionaea* in nitrogen-sufficient and nitrogen-deficient soil, we obtained results demonstrating that leaves of *Dionaea* close more quickly in nitrogen-deficient soil. These results suggest that there is little or no need for the *Dionaea* to 'snap' shut with the presence of nitrogen in the soil, as they did not have a need for prey-derived nitrogen. We inferred that the plants were most likely fully supplied with nitrogen from the fertilizer.

Additionally, we are able to conclude that the closure of the leaves within the nitrogen-deficient soil was not subject to habituation (Figure 2). Though this is a secondary conclusion, and solely based upon four trials, it suggests that *Dionaea* is not quick to habituate to repeated patterns of stimulation. This result additionally supports the notion that the reduced number of closures in nitrogen-sufficient snap-traps is not a result of habituation.

Possible Sources of Error and Additional Experiments

In order to prevent the plants from ingesting flies or other nitrogen-providing insects, we enclosed each plant in a plastic container with a lid that allowed air exchange through small slits. Very small insects may have been able to access the inside of the growth chamber through these slits, potentially affecting not only the number of open leaves but also the availability of nitrogen to the plants. However, the ventilation slits were sufficiently narrow that the number and size of insects able to enter through these holes would both be very small, and unlikely to have significant effects.

In conducting our experiments, we attempted to simulate the presence of a fly within the leaves of the *Dionaea*. In order to do so, we applied slight pressure within the leaf. Not knowing precisely where to target, we tried to stimulate lightly the entire leaf of each plant in the same way during each trial. While there was a chance for human error, we attempted to stimulate the leaves in a consistent manner. Future experiments may utilize an automated stimulating device to minimize human error.

Another issue to consider is that the fertilizer provided the *Dionaea* plants in nitrogen-sufficient soil with additional macronutrients that

were not present in the soil of those plants with depleted nitrogen levels. Additionally, peat moss in the nitrogen-deficient containers likely absorbed other nutrients besides nitrogen. These factors might have differentially affected the levels of non-nitrogen nutrients in the two soil conditions, introducing additional uncontrolled-for variables. While we are reasonably confident that the observed differences in thigmotropic responses were the result of different nitrogen levels, future experiments would necessarily involve testing for and carefully controlling for the presence of nutrients other than nitrogen.

Finally, testing snap-traps' closure responses in the presence of actual flies would be a logical extension of these investigations. Future experiments might examine whether plants in nitrogen-depleted soil would in fact prey upon more flies in order to gain nitrogen.

Could increased soil nitrogen levels affect native ecology via altered Dionaea behavior?

We can infer from our results that wild *Dionaea* growing with the presence of excessive nitrogen in the soil would be less quick to and less likely to 'snap' down upon flies.

Dionaea grow in very specific environments, limited to the coastal plains of the Carolinas[3], and the plants are already considered a threatened species by the National Red List. Studies have indicated a 400% increase in fertilizer usage in North Carolina since 19454, suggesting a likely increase in soil nutrients, nitrogen included, in those states where *Dionaea* are most prevalent. As nitrogen levels increase in the Carolina coastal plains, Venus flytraps may become less sensitive to prey. Over time, this could conceivably lead to loss of flytraps' carnivorous behavior.

A loss of *Dionaea's* carnivorous characteristics could affect more species aside from the plant itself. The Venus flytrap's natural prey - small insects and spiders[4] - would lose one of the main predators in the local microenvironment, possibly leading to overpopulation. An overabundance of small arthropods, particularly spiders, could upset the predator/prey balance of the microenvironment considerably, as spiders function in many ecosystems as predators themselves[6]. Future studies must be conducted to explore the long-term effects of fertilizers and other forms of human activity on local indigenous species, as an upset in the balance of species' behavior in established ecosystems could have lasting consequences.

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