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Seasonal variation in hydrochloric acid, malic acid, and calcium ions secreted by the trichomes of chickpea (*Cicer arietinum*)

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The concentrations of malate, calcium ions, hydrogen ions, and chloride ions were measured in secretions collected between June 1991 and May 1992 from the secretory trichomes of chickpea (*Cicer arietinum* L.). The average malate concentration was 114 m*M* and varied from 2 to 287 m*M*, the average chloride ion concentration was 177 m*M* and varied from 68 to 355 m*M*, and the average calcium ion concentration was 0.82 m*M* and varied from 0.24 to 1.97 m*M*. The variability in malate, chloride ions, and calcium ions closely followed the sinusoidal variation in the amount of solar radiation per day throughout the year (Riverside, CA, USA). Also, 77% of the variation in malate concentration, 51% of the variation in chloride ion concentration, and 67% of the variation in calcium ion concentration in collected secretions was directly dependent on the day of the year. In addition, the average hydrogen ion concentration was 299 m*M*, which corresponds to a pH of 0.52. Although the concentration of hydrogen ions varied from 195 m*M* (pH 0.71) to 417 m*M* (pH 0.38) throughout the year, the variation did not follow any clear pattern with respect to the day of the year. These data suggest that malate, chloride ion, and calcium ion secretion are linked to the amount of daily sunlight and to the day of the year while the pH of secretions is not directly linked to light level.

Key words – Calcium, chickpea, *Cicer arietinum*, Fabaceae, hydrochloric acid, malic acid, secretion, trichome.

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Introduction

In the Fabaceae, the presence of trichomes, both glandular and non-glandular, deters insect herbivory. This has been demonstrated in *Cicer arietinum* (Srivastava and Srivastava 1989), *Glycine max* (Lambert et al. 1992), *Medicago lupulina* (Goertzen and Small 1993), *Phaseolus vulgaris* (Quiring et al. 1992), and *Vigna unguiculata* (Oghiakhe et al. 1992). Only the glandular trichomes of chickpea (*Cicer arietinum*) secrete strong acids (Lazzaro and Thomson 1989), and there is a strong negative correlation among different cultivated varieties between the acidity of secretions and the degree of predation by *Heliothis armigera*, the main pest of *Cicer arietinum* (Srivastava and Srivastava 1989).

It has been reported that the trichomes of *Cicer arietinum* L. secrete organic acids, primarily malate, with concentrations ranging from 92 m*M* (Rembold and Weigner 1990) to 444 m*M* (Lauter and Munns 1986). In addition, secretions are reported to contain 231 m*M* chloride, with traces of calcium, magnesium, and potassium, while the pH of secretions is reported to be 0.25 (Lauter and Munns 1986). These secretory trichomes are multicellular, and are comprised of a basal cell, three stalk cells, and a cluster of 14 head cells. Above the head cells, a secretory chamber forms beneath the cuticle, and secretions collect in this chamber, exude from pores in the cuticle, and form a large drop on the tip of the trichome (Schnepf 1965, Lazzaro and Thomson 1989). At the ultrastructural level, numerous small vesicles are present along the edges of

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the head cells, and it has been proposed that these vesicles are involved in secretion (Lazzaro and Thomson 1989). Calcium is present within these vesicles, and these calcium containing vesicles fuse with the plasma membrane. In addition, calcium is present within the cell wall space around the head cells, in the secretion chamber, and in collected secretions (Lazzaro and Thomson 1992).

The study reported here quantitatively measures the amount of calcium ion in secretions, as well as the amount of malate, chloride ion, and the pH in collected secretions, and investigates whether the variation in secretion composition throughout the year is related to light level.

Materials and methods

Cicer arietinum L. cv. UC5 plants were grown in the glasshouse without any additional lighting. The glasshouse was vented to keep the temperature below 32°C, and heated to keep the temperature above 21°C. At the start of each collection period, 10–15 plants varying in age from 3 to 6 months were selected at random from a population of plants in the glasshouse. The branches of these plants were sprayed with deionized water to rinse off secretions already present on trichomes. Once washed, plants were watered daily with deionized water and given Hoagland's nutrient solution (Hoagland and Arnon 1950) every seventh day. This watering was done without touching the branches to prevent contaminating the secretion droplets. Secretions were collected after accumulating for 7 to 28 days on the trichomes. These collections were made between 1 June 1991 and 31 May 1992.

To collect trichome secretions, branches were excised in the morning and each compound leaf was dipped in a 15-ml conical centrifuge tube filled with light mineral oil, according to a method modified from Berry (1970). After all the leaves on a branch had been dipped, the oil/secretion droplet mixture was centrifuged at 1 300 *g* for 10 s, which forced the small secretion droplets, suspended in the mineral oil, to the bottom of the tube. This process was repeated with 2–3 branches from each plant until 100 to 500 µl of secretion were collected in the centrifuge tube. This aliquot was therefore a mixture of secretions from 10–15 plants. The tube was then centrifuged a final time at 1 300 *g* for 2 min, after which the mineral oil was drawn off and the secretions transferred to a microfuge tube and centrifuged at 10 000 *g* for 3 min. Finally, the secretions were drawn into a 50-µl syringe which had been passed through the thin oil layer in the microfuge tube while air bubbles were forced from the syringe's tip to prevent the movement of oil into the syringe.

Collected secretions were analyzed for malate, hydrogen ions, calcium ions, and chloride ions. Each assay was repeated 3 times on each aliquot, and average values are reported. Malate was assayed by reaction with malate dehydrogenase, which converts malate to oxaloacetate at a basic pH with excess NAD^+. The conversion of NAD^+ to NADH during this reaction was measured with a spectrophotometer at 340 nm (Williamson and Corkey 1969). Hydrogen ions were assayed by measuring the pH using an Acumet 910 high impedance (> 1 TΩ) pH meter (Fisher, Tustin, CA, USA) and a platinum pH microelectrode (MEPH1), in conjunction with a reference electrode (MERE1, both from World Precision Instruments, Sarasota, FL, USA) that was modified by substituting a glass micropipette pulled to a tip resistance of 15 MΩ when backfilled with 3 *M* KCl (R.A. Balsamo 1993, Thesis, Univ. of California, Riverside, CA, USA). The electrodes were tested and the pH meter calibrated with a 1.0 *M* pH standard (Fisher). A 20-µl drop of collected secretions was placed on Parafilm, and the measuring and reference electrodes were moved into the drop with micromanipulators. Readings were recorded after the pH stabilized, and measurements were repeated 3 times for each secretion sample. Calcium ions were assayed with the Total Calcium Test Procedure (StanBio Laboratory Procedure no. 0150, available from Fisher) and a spectrophotometer at 550 nm. This assay uses orthocresolphthalein complexone, which turns purple when bound to cal-

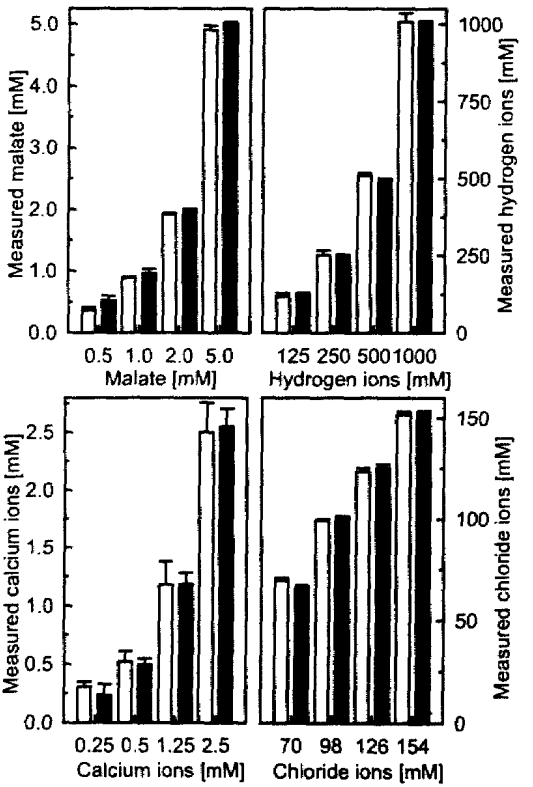

Fig. 1. Centrifugation through light mineral oil had no effect on the measured values of any standards compared to standards that were not centrifuged through mineral oil. Bars are means ± SD, n = 5 (except for calcium where n = 10).

Tab. 1. Contents of collected secretions. Secretions were collected 20 times between 1 June 1991 and 31 May 1992 and assayed for hydrogen ions, calcium ions, chloride ions, and malate. In addition, the average solar radiation (W m^{-2} day^{-1}) during each 7- to 28-day collection period was calculated. The average hydrogen ion concentration of 299 m*M* corresponds to a pH of 0.52. NM, not measured.

Date of collection	H^+ (m*M*)	Ca^{2+} (m*M*)	Cl^- (m*M*)	Malate (m*M*)	Light (W m^{-2} day^{-1})
June 14	NM	1.03	NM	280	292
June 21	NM	1.69	NM	280	283
June 28	351	1.84	NM	179	286
July 5	NM	1.97	NM	271	294
August 21	NM	0.96	106	82	277
August 28	NM	1.03	146	107	277
September 4	417	1.39	197	196	273
September 11	231	1.07	162	41	262
October 8	195	NM	109	6	218
October 23	344	NM	166	34	198
December 17	319	0.54	164	29	122
January 25	227	0.70	90	9	135
February 4	321	0.41	163	11	157
February 13	200	0.49	68	2	169
March 2	222	0.27	128	20	133
March 16	344	0.32	220	65	166
March 23	271	0.24	189	43	164
April 1	282	0.40	190	47	145
April 15	389	0.42	355	181	174
April 30	349	0.42	221	287	281
May 14	321	0.31	334	216	258
Average	299	0.82	177	114	
SD	68	0.56	77	105	

cium, and whose absorbance at 550 nm is proportional to the calcium concentration (assay kit no. 0150 instructions). Chloride ions were assayed with the Direct Chloride Procedure (StanBio Laboratory Procedure no. 0210, available through Fisher) and a spectrophotometer at 500 nm. In this assay, chloride reacts with mercuric isothiocyanate to release thiocyanate ions. These ions react with ferrous ions to form ferric thiocyanate, a red compound which is measured at 500 nm (assay kit no. 0210 instructions). Daily solar radiation readings (W m^{-2}) were collected from station no. 44 (Agricultural Operations, Univ. of California, Riverside, CA) of the California Irrigation Management Information System. This station was about 1000 m from the glasshouse where the chickpea plants were grown.

To be certain that the method of collecting secretions was not affecting the measured values of malate, hydrogen ions, calcium ions, and chloride ions, several 10-µl drops of standard solutions of each compound were placed on the surface of a tube of mineral oil and collected in the same way as the secretions. There was no difference in the measured values of any of these standards before and after collection through mineral oil (Fig. 1).

Polynomial regressions of data sets were performed with Sigma Plot 4.16 (Jandell Scientific, San Francisco, CA, USA) and the pairwise *t*-tests on regression coefficients were performed with Excel 4.0 (Microsoft, Seattle, WA, USA) following the statistical theory on polynomial regression by Zar (1984).

Results

Secretions were collected 20 times between 1 June 1991 and 31 May 1992, and measured for malate, hydrogen ions, calcium ions, and chloride ions (Tab. 1). Throughout the year, the malate concentration in secretions ranged from 2 to 287 m*M* (mean = 114 m*M*). The concentration of chloride ions in secretions varied as well, from 68 to 355 m*M* (mean = 177 m*M*). In addition, the calcium ion concentration in secretions ranged from 0.24 to 1.97 m*M* (mean = 0.82 m*M*). Finally, the hydrogen ion concentration in secretions varied from 195 (pH 0.71) to 417 m*M* (pH 0.38). The mean hydrogen ion concentration was 299 m*M*, which corresponds to a pH of 0.52 (Tab. 1).

To account for some of the variability in the concentrations of malate, chloride ions, calcium ions, and hydrogen ions, we plotted the concentration of each component as a function of the day of the year when secretions were collected (Fig. 2A–D). In addition, we plotted the daily solar radiation level in Riverside, CA, as a function of the day of the year (Fig. 2E). Polynomial equations, increasing sequentially from first order to sixth order, were regressed independently through each of these plots, and the third order polynomial equations were the highest order in which the final coefficient (m_3 of $y = b + m_1x + m_2x^2 + m_3x^3$) was statistically significant (Tab. 2). The coefficients of determination (r^2) for each of these regressions indicated that 77% of the variation in malate concentration in secretions was due to the day of the year on which secretions were collected (Tab. 3). In addition,

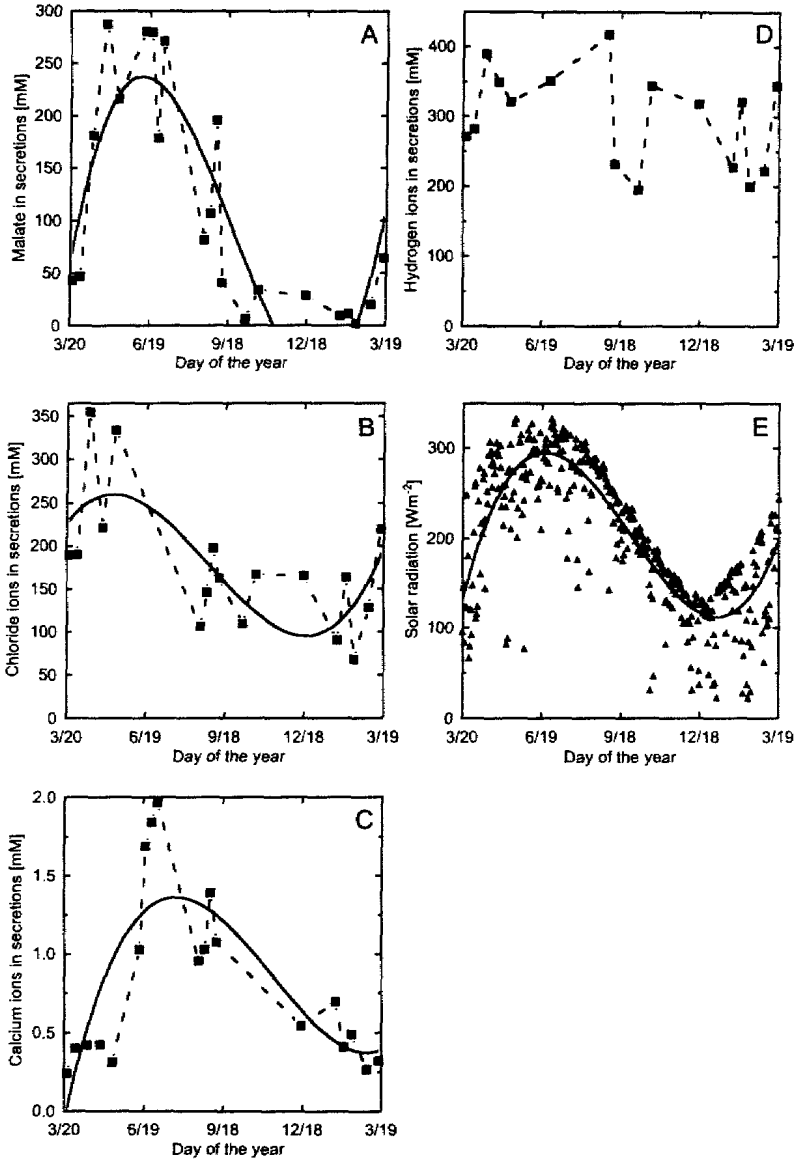

Fig. 2. A. The concentration of malate in secretions varied sinusoidally throughout the year, having a maximum in June and a minimum in December. B. The concentration of chloride ions in secretions also varied sinusoidally throughout the year, having a maximum in June and a minimum in December. C. In addition, the concentration of calcium ions in secretions varied sinusoidally throughout the year, having a maximum in June and a minimum in February. D. Although the concentration of hydrogen ions in secretions varied throughout the year, there was no significant pattern to this variation. E. Finally, the amount of solar radiation (W m^{-2}) available to the chickpea plants varied sinusoidally throughout the year with a maximum in June and a minimum in December, identical to the plots of malate and chloride ions, and similar to the plot of calcium ions. The x-axes in A–E start at 20 March to follow the cycle of the seasons, beginning with spring (20 March) and progressing through summer (19 June), autumn (18 September), and winter (18 December). The dates for the onset of each season are derived from dividing the calendar year into four 91-day segments, with 20 March set as day zero.

51% of the variation in chloride ion concentration in secretions, 67% of the variation in calcium ion concentration in secretions, and 69% of the variation in solar radiation in Riverside, CA, was due to the day of the year (Tab. 3). However, only 22% of the variation in hydrogen ion concentration in secretions was due to the day of the year (Tab. 3).

Since the day of the year accounted for such a large percentage of the variability in the levels of malate, chloride ions, calcium ions, and solar radiation, the third order polynomials regressed through these data sets were plotted in Fig. 2A–C and E. The plot of malate concentration versus day of the year had a sinusoidal pattern, with a maximum in June and minimum in December (Fig. 2A). In addition, the plot through chloride ion concentration versus day of the year also had a maximum in June and a minimum in December (Fig. 2B). A less pronounced sinusoidal pattern emerged from the plot of calcium ion concentration versus day of the year, with a maximum in June and a minimum in February (Fig. 2C). Since only 22% of the variation in hydrogen ion concentration in secretions was due to the day of the year (Tab. 3), the

Tab. 2. Statistical test of polynomials regressed through plots of secretion contents or solar radiation versus day of the year. Sequential regression analyses were performed independently through plots of malate, chloride ions, calcium ions, hydrogen ions, or solar radiation versus day of the year. The highest order regression coefficients (m_n) from the analyses were pooled and tested for being significantly different from zero using a 2-tailed, pairwise t-test at $\alpha = 0.025$ with 4 degrees of freedom. This indicates that the third order regression coefficient (m_3) is the highest significant coefficient, and that the third order polynomial is a valid prediction of the relationship between secretion content or solar radiation and day of the year. *, Significant; NS, not significant.

Polynomial regressed through data	Tested coefficient	t-Value	Significance
$y = b + m_1x$	m_1	3.13	*
$y = b + m_1x + m_2x^2$	m_2	0.72	NS
$y = b + m_1x + m_2x^2 + m_3x^3$	m_3	2.98	*
$y = b + m_1x + m_2x^2 + m_3x^3 + m_4x^4$	m_4	1.58	NS
$y = b + m_1x + m_2x^2 + m_3x^3 + m_4x^4 + m_5x^5$	m_5	2.05	NS
$y = b + m_1x + m_2x^2 + m_3x^3 + m_4x^4 + m_5x^5 + m_6x^6$	m_6	1.56	NS

third order regression was not plotted in Fig. 2D. Finally, the sinusoidal pattern in the plot of solar radiation versus day of the year had a maximum in June and a minimum in December (Fig. 2E). This pattern was identical to the plots of malate and chloride ion secretion throughout the year and similar to the plot for calcium ion secretion. However, there was no similarity between the plots of hydrogen ion concentration and solar radiation during the year.

Discussion

The concentrations of malate, chloride ions, and calcium ions all vary sinusoidally throughout the year, and this variation parallels the change in light level. This suggests that the secretion processes for malate, chloride ions, and calcium ions are light sensitive. It is logical to assume that the secretion of an organic acid by a plant will depend directly on sunlight, since the carbon source for the organic acid must ultimately come from photosynthesis. In addition, Koundal and Sinha (1983) demonstrated that the amount of titratable acid in secretions was greater during daylight hours over a 72-h diurnal cycle. They assumed that all the titratable acid was malate, but this assumption is erroneous, since substantial chloride ions are also present in secretions as hydrochloric acid (Lauter and Munns 1986).

Tab. 3. Analysis of third order polynomial regressions through plots of secretion contents or solar radiation versus day of the year. The correlation coefficients (r) indicate that the third order polynomials regressed through plots of malate, solar radiation, calcium ions, or chloride ions versus the day of the year fit well. Consequently, these regressions were plotted in Fig. 2A–C,E. Since the regression through the plot of hydrogen ions versus day of the year fit poorly (0.472), it was not plotted in Fig. 2D. In addition, the coefficients of determination (r^2) indicate that a large percentage of the variation in malate (77%), calcium ions (67%), and chloride ions (51%) in secretions, as well as 69% of the variation in the solar radiation levels, was dependent on the day of the year. However, only 22% of the variation in hydrogen ion concentration in secretions was dependent on the day of the year.

Dependent variable	r	r^2
Malate	0.876	0.768
Chloride ions	0.715	0.511
Calcium ions	0.817	0.667
Hydrogen ions	0.472	0.223
Solar radiation	0.831	0.691

The similar variation in malate, chloride ion, and calcium ion concentrations in chickpea secretions suggests that the secretion of these compounds may be linked, although the average malate and chloride ion concentrations were 139 and 216 times greater, respectively, than the average calcium ion concentration. Ultrastructurally, calcium is localized within small vesicles along the edges of the head cells, and these vesicles fuse with the plasma membrane. Calcium is also localized within the cell wall space, secretion chamber, and within collected secretions, demonstrating that the head cells are the site of calcium secretion (Lazzaro and Thomson 1992). It is possible that malate and chloride ions may be secreted from the head cells via the same pathway as calcium ions in these trichomes.

Although the concentrations of hydrogen ions varied in secretions, this variation did not have any clear pattern with respect to the day of the year or the amount of solar radiation. This suggests that hydrogen ions are secreted by a different pathway than malate, chloride ions, and calcium ions, and that the concentrations of secreted hydrogen ions, and subsequent pH of secretions are not directly dependent on sunlight. Hydrogen ions may be transported directly across the plasma membrane by a proton pumping ATPase (Sze 1984) in contrast to the apparent loading of calcium into secretory vesicles observed in earlier studies (Lazzaro and Thomson 1992).

We measured 177 m*M* chloride ions, 114 m*M* malate, 299 m*M* hydrogen ions (pH 0.52), and 0.82 m*M* calcium ions in secretions. Lauter and Munns (1986) report that *Cicer arietinum* secretions contain 231 m*M* chloride ions, 444 m*M* malate, 563 m*M* hydrogen ions (pH 0.25), and less than 9 m*M* calcium, magnesium, and potassium ions combined. The reported chloride ion concentration (Lauter and Munns 1986) is within the range of our data, but the malate concentration is greater than the maximum values in our study. In addition Rembold and Weigner

(1990) report that secretions contain 92 m*M* malate, which is within the range of our measurements. Each of these malate concentrations (444, 114, and 92 m*M*) come from a different variety of *Cicer arietinum*, and there is evidence that malate content does significantly differ between varieties, while chloride ion content does not (Lauter and Munns 1986). The hydrogen ion concentration measured by Lauter and Munns (1986) is also outside the range of our measurements, probably due to the increased malate levels reported in their study.

The total number of cations does not equal the total number of anions presented in Tab. 1. This is probably due to the fact that not all the organic acids present in the secretions are being measured. The largest unmeasured component is oxalate. Lauter and Munns (1986) found that secretions contain 174 m*M* oxalate, in addition to 444 m*M* malate. Rembold and Weigner (1990) found that secretions contain 43 m*M* oxalate, 8.3 m*M* glucose 6-phosphate, 3.8 m*M* citrate, and traces of succinate, malonate, oxaloacetate, and fumarate in addition to 92 m*M* malate. In addition, older trichomes of the UC5 cultivar of *Cicer arietinum* (the variety used in this study) form calcium oxalate crystals in the cell wall space around the head cells (Lazzaro and Thomson 1989), so it is possible that we did not measure all the oxalic acid in the secretions. This is also likely because the variation in pH does not follow the variation in malate and chloride, the only counter ions measured.

The proton concentration in *Cicer arietinum* secretions (pH 0.52 in this study and pH 0.25 in Lauter and Munns 1986) is one of the highest in plant material, and is at least 30 times greater than the proton concentrations measured in the tank fluid of carnivorous plants (pH 2–9, Juniper et al. 1989). In addition, the secretion of high concentrations of organic acids is a unique feature of these trichomes (Lazzaro and Thomson 1989), although trace amounts of organic acids are sometimes present in secretions from nectaries (Findlay 1988). Secretion of calcium ions and chloride ions also occurs in secretory trichomes of halophytes. *Tamarix aphylla* secretes 77 m*M* calcium and 9 m*M* chloride when watered with nutrient solution (Berry 1970). Epidermal peels of *Avicennia germinans* secrete 60 m*M* chloride when floated on distilled water and up to 355 m*M* chloride when floated on 300 m*M* NaCl (Dschida et al. 1992). Isolated leaves of *Limonium perezii* placed in distilled water secrete 0.3 m*M* calcium and 3.7 m*M* chloride, but these values rise to 1.6 m*M* calcium and 300 m*M* chloride when the leaves are placed in a solution of 300 m*M* NaCl (Faraday et al. 1986). In addition, the counter ion for this large chloride secretion is sodium (Faraday et al. 1986), while in chickpea secretions the counter ion is hydrogen (Lauter and Munns 1986).

In summary, the pH in secretions from *Cicer arietinum* trichomes does not vary with the amount of sunlight, while the secretion of malate, chloride ions, and calcium ions does. In addition, a significant percentage of the variation of malate, chloride ions, and calcium ions in collected secretions is directly dependent on the day of the year. The secretion of such a strong acid (pH 0.52), and the secretion of a high concentration of malic acid (113.7 m*M*) are both unique features of these secretory trichomes.

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