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On the Variability of Chlorogenic Acid Concentration

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Summary. The plasticity of phenolic acid concentration in plants under various physiological stress situations raises questions concerning the ecological significance of such behavior. *Helianthus annuus* L. is remarkably plastic with regard to chlorogenic and isochlorogenic acid concentrations when subjected to nitrogen deficiency, NaCl-stress, short exposures to UV radiation or to combinations of these stresses. Stress due to NaCl has not previously been reported to produce this effect.

I propose that chlorogenic acid is representative of a group of chemicals which have originated as regulators of various metabolic systems under stress and have subsequently acquired allelochemic properties against pathogens, herbivores, and competitors. If this hypothesis is correct then specific patterns of distribution with regard to concentrations of phenolic acids should be found. Some such predictions are discussed and means of distinguishing between various selective agents are explored. Generally, if natural environmental stress stimulates phenolic acid concentrations and hence more efficient growth patterns, then for several kinds of variables, higher concentrations should be found in more rather than less stressful habitats. If biological agents are significant agents of natural selection for this group of substances, such a pattern should not occur in some cases.

Introduction

Whittaker (1970) proposed the term *allelochemic* to refer to any substance that can impose structure upon a biotic community and apply selective pressures. Anti-herbivory, antibiosis, and allelopathy are some allelochemic processes.

In theory it is possible to distinguish three classes of such compounds on the basis of chemical origins. An *end product* results from a specific sequence of enzymatic reactions and is not involved in any but allelochemic processes. Many alkaloids, such as nicotine, exemplify this group. Ehrlich and Raven (1965) emphasized these compounds in their discussions of the coevolution of plants and butterflies which demonstrated that herbivore pressure can select for plant-produced poisons. A *by-product* results from metabolic activities or is a non-essential chemical relative of metabolically important chemicals. These have been termed "wastes" by Muller (1969, 1970) and include such substances as tannins, rubber, and perhaps some terpenoid compounds. Muller (1969) and Whittaker (1970) suggested that these compounds originate as wastes and may acquire allelochemic functions subsequently. The third putative class of allelochemics is termed *metabolites*. These are involved in normal cellular activities, but as a consequence of several distinct selective pressures have acquired additional functions. Many of the simpler aromatic compounds may be of this sort.

Muller (1969) distinguished between the *primary* and the *principal* functions of chemicals. The primary function of any chemical is that function for which it was initially synthesized. Subsequent natural selection may lead to partial or complete functional changes and these in turn may result in quantitative changes in the concentration of the chemical. This newer role may become the principal function of the chemical. For many types of chemicals there is no assurance that primary and principal functions will be identical. For example, terpenes may have originated as waste products while they are now functional as allelopathic agents or insect attractants.

One purpose of this paper is to demonstrate that the metabolitic allelochemics exist and that they have been subjected to several forms of natural selection. It should be recognized that distinctions between classes are artificial and that the groups form a continuum. Further, while allelochemic members of particular chemical groups often fall into the same category, the simple aromatics, which are the subject of this paper, may be either by-products or metabolites.

Previous studies of the variability of phenolic acid concentration have stressed biochemical aspects, mentioned ecological aspects, and have left evolutionary considerations largely unexplored. In this paper I will review and expanded upon what is known about phenolic acid variability and explore its ecological and evolutionary aspects.

The kinds of selective agents acting upon a particular compound are a function of its origin. End-products, by-products, and metabolites, in that order, should be influenced by an increasingly complex array of forces. End-products may be a response solely to selective exerted by herbivores or competitors. It is a rather simple task to make predictions concerning the distribution, concentration, and type of chemicals to be expected under different conditions if only one type of pressure is exerted. By-products are subject to more complex sets of selective pressure. Because they are produced by the normal cellular machinery, compartmentalization is more difficult than in the case of end-products. Thus evolution of less toxic by-products for the avoidance of self inhibition is balanced by selection for increased toxicity of these chemicals under herbivore or pathogen pressure. Therefore, toxicity and concentration of particular by-products represents a compromise between opposing selective agents and the degree of toxicity can theoretically be used to determine the relative importance of these agents.

Metabolic compounds with allelochemic properties are influenced not only by selection due to herbivores, pathogens, and competitors, and by the ability of the plant to tolerate or compartmentalize potentially autotoxic compounds, but also by the requirements dictated by metabolic processes for which the compounds were initially synthesized. Thus at least three types of selection are acting on metabolites and quantitative determination of the effects of natural selection on any particular system will be simplest for end-products but more difficult for metabolites (G. H. Orians, pers. comm.).

The following experiment was designed to determine whether particular metabolic compounds varied under environmental stresses so as to suggest that the variation was adaptive and to help generate predictions concerning the concentration of such compounds in nature. A secondary objective was to provide information on the chemical response of plants to NaCl-influence and to combinations of stresses.

Chlorogenic acid (CGA) was selected as a model to study for several reasons. Its chemistry and biosynthesis are reasonably well known. It is extremely widespread among plants (Sondheimer, 1964) and is known to have antibiotic (Farkas and Kiraly, 1962), allelopathic (del Moral and Muller, 1970) and anti-herbivore (Jones, 1971) properties. Several authors (see Wender, 1970) have suggested that it plays an important role in various metabolic functions. Consequently CGA seems to be an allelochemic metabolite. The structure of CGA (3-caffeoylquinic acid) and some related compounds is shown in Fig. 1. Two isomers, band 510 (4-caffeoylquinic acid) and neochlorogenic acid (5-caffeoylquinic acid) occur with CGA and were not separated by the procedures employed. Isochlorogenic acid (ICGA, 3,5-dicaffeoylquinic acid and other minor isomers) was separated and also will be discussed. Corse *et al.* (1965) treat the chemistry of this type of compound.

In Fig. 2, I have summarized possible pathways for CGA formation from Sondheimer (1964), Hanson *et al.* (1967), Steck (1968), and Wender (1970). Note that CGA formation requires energy, that nitrogen availability may influence that pathway, and that deamination of phenylalanine leads directly to cinnamic acid, a CGA precursor. Thus it is reasonable to investigate how changes in these requisites will effect CGA concentration and whether such changes are of adaptive significance.

Helianthus annuus L., var. Russian Mammouth, is an ideal plant for this study. Its grows rapidly controlled conditions, is genetically homogeneous, has been studied in a similar context (Koeppe *et al.*, 1969) and has demonstrable allelopathic (Wilson and Rice, 1969) and phytopathological properties (Johnson and Schaal, 1957). Koeppe *et al.* (1969) showed that CGA concentration in sunflowers increased under moderate UV light, but was suppressed under higher intensities. Nutrient deprivation, particularly of nitrogen and boron, increases CGA concentration in sunflowers and tobacco (Chouteau, 1963; Koeppe and Rohrbaugh, 1968; Watanabe *et al.*, 1964). The NaCl factor has not previously been investi-

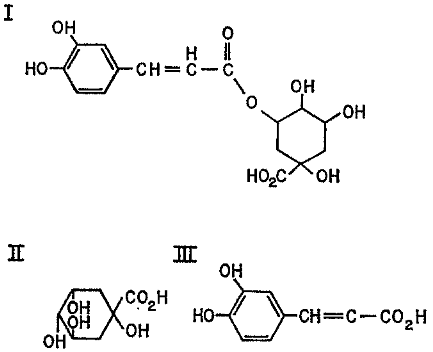

Fig. 1. Chemical structures of : *I* chlorogenic acid; *II* d-quinic acid; *III* t-caffeic acid

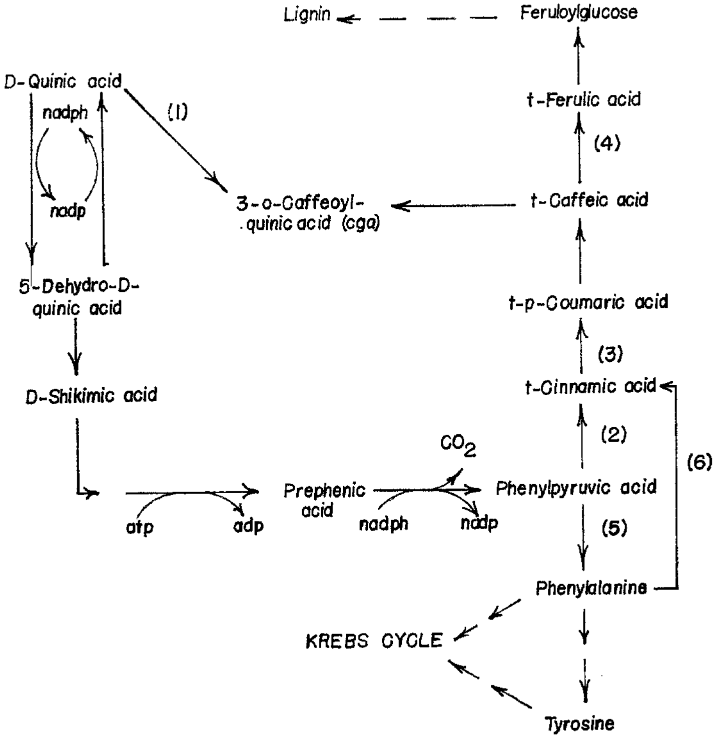

Fig. 2. Possible biosynthetic pathways for chlorogenic acid formation and its relationship to some aromatic amino acids. *1* -OH; *2* ($HONH_3$); *3* -OH; *4* o-methly transferase; *5* transaminase (NH_3); *6* phenylalanine deaminase

gated in this context, though it is an exceedingly important and common variable. The effects of several other variables including temperature and defoliants have been studied in this context (Wender, 1970).

Methods

Sunflower seeds were germinated for seven days in quartz sand culture prior to the imposition of the treatment. Two comparable seedlings were transferred to each opaque water culture vessel fitted with an aerator. Eight vessels were placed in either side of a growth chamber and put under 16 hr light at 28° C and 8 hr dark at 15° C, at 70% relative humidity. At mean plant level after 30 days, illumination was 12900 lux. The chamber was divided into two equal halves with a black cloth, and 4 UV lamps (GE 30T8, germicidal) were placed vertically in the corners of the chamber and shielded with 10 thicknesses of fiberglass screen. An early trial of this experiment resulted in highly abnormal growth and eventual death of all seedlings under UV so that ultimately, high intensity UV was applied for only 1 hr per day. Full strength Hoagland's solution was the normal growth medium. Half the vessels in each side of the growth chamber received a 10% nitrogen Hoagland's solution in which 10% by volume of nitrate solution was added and balanced for the other nutrients. The solutions were changed every three days and brought up to volume daily. A mild stress was induced in half of the vessels with NaCl. Earlier trials with mannitol resulted in massive root infections, while carbowax was not practical in this experiment. During a 24 hr cycle, in which transpiration reduced the culture volume, the osmotic potential of the drought stress vessels varied from —4.0 to —4.3 atm as measured by a Mechrolab osmometer. A specific ion response to either sodium or chloride was, of course, also included in this treatment. For convenience, this treatment will be considered to produce physiological drought stress. The experiment thus resulted in eight factorial treatments each represented by two pots and four plants each. Treatments were randomized within each half chamber (due to the obvious impractically of randomizing the UV treatment) and rerandomized daily to help attain greater uniformity of UV exposure.

After 31 days, the individual plant shoots were harvested and separated according to leaf and stem. Roots in a single pot were not separated into individuals since in may treatments this proved impractical. The dry weight of each segment was obtained. Each portion was extracted for 10 min with 20 volumes of hot isopropanol: water (1:1, v:v) followed by a 10 min extraction with hot isopropanol: benzene:methanol:water (IBMW, 2:1:1:1, v:v). The residue was then extracted for 24 hr with isopropanol. All extracts were combined, evaporated to dryness, and redissolved in IBMW. The entire amount was stripped onto Whatman No. 1 filter paper and chromatographed in one dimension with butanol:acetic acid:water (BAW, 6:1:2, v:v). Identification of the strips was accomplished by determining fluorescence under UV light, relative flow, and comparisons with simultaneously run knowns. (Some chromatograms were initially overloaded and did not effect good separation. These were eluated and rechromatographed after removal of the pigment bands.) The strips were excised and eluted in ethanol and brought to a constant volume.

The solutions were scanned photometrically (Bausch and Lomb Spectronic 505) between 220 and 400 mμ (to assure purity) and peak height recorded at 330 mμ for CGA and 329 mμ ICGA. A calibration curve on reagent CGA and ICGA, run through the same extraction procedures, was used to quantify the extracts. All values were subsequently expressed as μg of phenolic per g dry weight of tissue.

Results

The data are summarized in Table 1. Any stress substantially increased the concentration of both compounds in each tissue and in the plant as a whole. Several facts stand out. Nitrogen deficiency alone

Table 1. Concentration of chlorogenic and isochlorogenic acid in sunflower tissues

Stress applied	µg/g dry weight			
	Chlorogenic acid			
	Leaf[a]	Stem[a]	Root[b]	Average[c]
1. None	71 ± 23	21 ± 9	48 ± 1	43
2. UV	151 ± 53	52 ± 3	120 ± 33	113
3. —H_2O	430 ± 48	78 ± 33	189 ± 10	258
4. UV; —H_2O	640 ± 51	186 ± 25	370 ± 30	455
5. −NO_3	642 ± 60	422 ± 5	239 ± 31	458
6. —NO_3; UV	383 ± 35	218 ± 13	201 ± 25	310
7. —NO_3; —H_2O	1060 ± 143	180 ± 28	234 ± 45	645
8. —NO_3; —H_2O, UV	897 ± 95	156 ± 22	195 ± 31	546

Stress applied	µg/g dry weight			
	Isochlorgenic acid			
	Leaf[a]	Stem[a]	Root[b]	Average[c]
1. None	173 ± 17	45 ± 8	205 ± 70	135
2. UV	220 ± 39	144 ± 26	292 ± 52	203
3. —H_2O	365 ± 48	196 ± 61	444 ± 90	320
4. UV; —H_2O	635 ± 102	275 ± 33	546 ± 139	512
5. —NO_3	1661 ± 210	133 ± 15	1661 ± 223	1065
6. —NO_3; UV	870 ± 125	125 ± 10	1045 ± 140	375
7. —NO_3; —H_2O	2490 ± 308	1170 ± 160	2470 ± 252	2185
8. —NO_3; —H_3O, UV	1235 ± 150	646 ± 95	869 ± 157	979

[a] Mean of four determinations ± standard deviation.

[b] Mean of two determinations ± standard deviation.

[c] Weighted mean of leaf, stem, and root tissues.

causes a great increase in phenolic acid concentration. Ultraviolet light increases phenolic acid concentration when nitrogen is abundant, but inhibits it under low nitrogen. For the treatment levels imposed the three stresses may be ranked in order of decreasing stimulatory effect as nitrogen deficiency, drought, and ultraviolet light.

The manner in which interactions of the stresses affect the concentration of the two chemicals in question is of special interest. The nitrogen status materially influences the interaction between the drought and UV light factors. With normal nitrogen, in each tissue, drought plus UV produces higher concentrations of both CGA and ICGA. However, with nitrogen deficiency, a more complicated pattern results. The stimulatory effects of drought, plus UV are always less than drought alone. In leaf tissue, the addition of drought stress to UV treatments increases both

CGA and ICGA; in root tissue it has little effect; and in stems tissue, it causes an increase in ICGA but a slight decrease in CGA.

In all tissues nitrogen stress alone has a greater effect than either nitrogen plus UV or UV alone. For CGA, the additional presence of drought stress lessens the inhibitory effects of UV in the UV plus nitrogen stress treatment, but this is not so for ICGA.

Drought plus nitrogen deficiency are often synergistic, particularly with ICGA. However, CGA in stems is inhibited by this treatment compared to nitrogen alone, while in roots under UV, drought alone is the most stimulatory. UV inhibits both CGA and ICGA when added to plants stressed by both nitrogen deficiency and drought.

Exceptions to general trends may indicate that in a particular tissue a specific combination of stresses goes beyond stimulation and becomes inhibitory. This may occur as a consequence of interference with general cell biochemistry, in a fashion analogous to the relative inhibition produced by the UV plus nitrogen deficiency which holds for all tissues and both compounds.

The stimulatory effect of the NaCl-drought treatment on CGA concentration is the first reported case where this stress is known to influence CGA or ICGA concentration. The patterns reported for CGA and ICGA are similar suggesting that they are intimately involved in the same of pathways.

The concentration of CGA and ICGA in the entire plant, based on concentrations and the relative weight of the whole plant represented by each tissue, emphasizes that changes in concentration shown in Table 1 do not represent reallocations of the chemicals but real changes in synthetic activity. There are no new patterns evident from this summation, with the pattern of pooled results most closely resembling that of the leaves. On a whole plant basis nitrogen deficiency plus drought produces 15 times as much CGA and 16 times as much ICGA as the non-stress treatment. Nitrogen deficiency alone increase CGA 10-fold and ICGA 8-fold; water stress alone increases CGA 6-fold and ICGA but 2-fold; and UV treatment alone stimulated CGA 2.5-fold and ICGA by 1.5 These summary figures confirm the relationships between the individual stresses detected from the individual tissues.

Discussion

Various forms of physiological stress will materially change the concentrations of phenolic acids (Wender, 1970). To this list I can now add physiological drought as induced by sodium chloride. Isochlorogenic acid is produced from CGA by the addition of a second caffeoyl group, probably as a consequence of the lignification process (Wender, 1970). I will therefore discuss these compounds together.

Hanson *et al.* (1967) and Sondheimer (1964) demonstrated that CGA is a growth regulator. It inhibits IAA oxidase (Gortner Kent, 1958) and Taylor (1968) suggests that it is also indirectly involved in lignin synthesis. Chlorogenic acid is also an important anti-pathogen (Farkas and Kiraly, 1962). It is found in low concentrations until invasion or injury stimulates its production. This suggests that antibiosis is a principal function, while the metabolic functions are per force primary ones.

Wilson and Rice (1969) and del Moral and Muller (1970) showed that CGA and ICGA are effective allelopathic compounds. Natural leachates of sunflower leaves and *Eucalyptus* litter contain quantities of CGA which inhibits seedlings growth both under natural and controlled conditions. In low concentrations they may act in concert with related phenolic inhibitors. Effective allelopathy occurs only when fairly high concentrations of phytotoxins are produced and released into the environment by leaching or decomposition. A mechanism whereby allelopathic toxins occur *de novo* in high concentrations without concommitant self-poisoning is difficult to immagine. I thus consider the alleopathic function to be derived. However, the ability of these compounds to inhibit competitors is of distinct selective advantage. Selection for increased concentration and retention of the compounds results even if their metabolic functions become absolete. Thus they may become principally allelochemics.

Under many conditions the expected phenolic acid concentrations would be high in response not only to stress but also in response to strong allelochemic factors. Thus pattern prediction with respect to phenolic acid concentration based on one hypothesis may be confirmed erroneously. However, certain conditions can be imagined which would favor high concentrations if physiological stress were a major selective agent but favour lower concentrations if herbivores or competitors were major selective agents. These will be discussed subsequently.

The question arises whether plastic CGA response to physiological stress is a positive adaptive response or whether it represents a passive metabolic comprise to a metabolically difficult situation. Let us consider the response to several stresses.

Nitrogen deficiency may lead to the formation of CGA in one of two ways. Inhibited amino acid production creates a temporary surplus of aromatic precursors which shunt into the CGA pathway while nitrogen is utilized in more essential amino acid pathways. Alternatively the deamination of phenylalanine releases CGA precursors (Fig. 2). The increase in CGA, ICGA, and their phenolic acid precursors is favored if resulting concentrations inhibit IAA oxidase.

The values obtained here with nitrogen deficiency are within a range stimulatory to auxin production (Sondheimer, 1964). Such auxin stimulation would be adaptive if a more favorable reallocation of the available growth resources results. McKee (1962) reports an example in which boron deficiency led to an increase in the proportion of meristematic tissue and a greater surface to volume ratio. Thus under nutrient deficiency the stimulation of auxin by means of enhanced CGA concentration may lead to a plant response appropriate to the nature of the environmental stimulus. This speculation is easily amenable to direct test under rigorously controlled conditions.

Drought stress acts to slow growth and as a consequence lignification may be inhibited. Lignin precursors such as ferulic acid, caffeic acid, and ultimately CGA, accumulate. As with nitrogen deficiency, the accumulation of CGA under drought stress may result in an increased absorbing surface relative to that produced in the absence of the CGA mechanism. The metabolic responses to this stress need a more careful elucidation.

The influence of UV light on CGA concentration has been investigated by several workers (Frey-Wyssling and Bäbler, 1957; Lott, 1960). UV radiation disrupts cellular functions and plants grown in the absence of UV develop normally except that they contain low concentrations of phenolic acids. Lott (1960) suggests that the increase in CGA production results from enzyme activation by UV energy. This suggests that the process has adaptive significance. Koeppe *et al.* (1969) suggested that UV stimulation of phenolic compounds in alpine plants "could affect the ability of plants to survive in these regions through either changes as metabolic regulators or as phytotoxins". However, they did not elaborate or suggest mechanism or important restrictions on the importance of either role.

High concentrations of phenolic compounds may be a form of negative feedback. Parsons (1968) summarized evidence that slow vegetative growth is adaptive under stringent environmental conditions. In harsh environments a considerable proportion of the limited available energy is partitioned into maintenance functions, water and minerals may be in critically limited supply, and competition for light may be minimal. Under these conditions more conservative individuals are more likely to survive and reproduce. While some species occur with "slow growth" ecotypes in response to temperature or mineral conditions, other alpine species may find that UV light provides an adequate cue to severe conditions and that response by virtue of self-inhibition with phenolic acids is also an effective strategy. This would be a flexible mechanism of adaptive growth retardation. Further, it would minimize mistakes based on more variable cues such as temperature.

The suggestion that an increasing concentration of phenolics would be selected for by means of a mechanism of increasing allelopathic potential bears close scrutiny. This selection pressure would be operative if potential competitors displayed differential susceptibility to these toxins, if the plants are sufficiently "leaky", if the stimulated compounds were produced in sufficiently high concentration, and if competition for resources is sufficiently severe (i.e. selective). However, the stimulatory effect of UV on phenolic acid concentration is very common. It would therefore seem more likely that this response is to the presence of UV itself, rather than to any correlated process. Phenolic compounds absorb strongly in the UV range. High intensity ultraviolet light has an extremely damaging effect on cellular organization. Consequently the enhanced production of phenolic compounds confers a degree of protection to those cells able to store them in high concentration. The ability to store potentially toxic substances exacts a cost on the system. If such substances were maintained even when UV conditions were alleviated, the efficiency of the organism would suffer and it would eventually be replaced by more efficient, plastic biotypes. This later response to ultraviolet may be much more common than one involving growth regulation.

The synergistic effects of nitrogen deficiency and drought stress may occur because nitrogen deficiency curtails phenylalanine production while drought slows lignification. One effect increases the supply of CGA precursor while the other curtails the drain on these precursors. The mechanism by which UV acts is not clear though it probably is a generally disruptive element, but the addition of the third stress must thoroughly overburden the abilities of the plants to respond at all, placing the plant near death. Under such conditions, there are many biosynthetically earlier demands on potential CGA precursors, so the pool is not filled.

Study of the CGA production systems has shown that CGA and related compounds are increased under environmental stress. This is explicable by the hypothesis that these accumulations alleviate environmental stress and that this function is their most important one. The primary function of CGA may have involved more normal metabolic control. Defense against herbivores and competitors may be viewed as important but derived or even tertiary functions for these compounds.

Many phenolic compounds, including CGA, are allelopathic, but the argument presented here suggests that this condition is derived and results from the happy coincidence that high stress environments lead to higher concentrations of phenolics which, if subsequently released, may confer additional advantage by detering potential competiters. The interactions of physiological stress, metabolic regulation, pathogens, herbivores, and competitors with phenolic acid production is of interest. Physiological stress selects for increased CGA by means previously discussed because CGA can act to ameliorate the effects of this stress. Consideration of the energy demands of the plant suggests that a plastic response is more adaptive than an ecotypic, fixed response, and therefore ability to produce large concentrations of CGA rapidly is incorporated into the genotype. Higher concentrations and the ability to produce high concentrations rapidly lead to greater defense capacities against herbivores and pathogens; hence a positive feedback loop is established where pathogens select for disease resistance and unpalatability and higher CGA concentration which in turn selects for CGA resistant parasites.

Still higher levels of CGA result in some release into the soil by leaching or decomposition of senescent material. Leachability of plant material may be adaptive if cellular concentrations begin to approach the level of self-intoxication. Once out of the plant, CGA can, under the appropriate circumstances inhibit potential competitors. The effectiveness of CGA as an allelopathic agent depends upon its becoming concentrated to inhibitory proportions in the environment or its action in concert with other growth inhibitors. If fitness is increased by this inhibition the ability to produce and tolerate still greater CGA concentrations is selected for. The limit to this cycle of ever higher CGA concentrations is reached when the ability of the cell to cope with sustained high concentrations of phenolic toxins is overwhelmed.

The study of inhibitory chemical substances produced by plants permits the study of evolutionary processes and demonstrates that many such substances are the products of several selective forces. It also indicates the subtlety of many adaptions of plants to their environments. In view of the remarkable plasticity exhibited by sunflower in this regard, one can only wonder whether chemotaxonomists should investigate qualitative responses to stress in more detail. Ultimately studies of chemical plasticity will lead to a more complete understanding of how multifactorial selection operates on particular traits. I suggest that all adaptions to harsh environments do not involve alteration in metabolic efficiencies or those subjects commonly pursued by physiological ecologists. Accommodation to adverse environmental conditions by mechanisms such as those suggested here may well prove to be of much greater significance than heretofore realized.

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