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Review

Plant defense using toxic inorganic ions: Conceptual models of the defensive enhancement and joint effects hypotheses

Robert S. Boyd*

Department of Biological Sciences, 101 Rouse Life Sciences Building, Auburn University, Auburn, AL 36849, USA

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ABSTRACT

The concept of plant defense using toxic mineral elements originated as an explanation for extremely elevated concentrations of some elements (termed hyperaccumulation) in some plant tissues. The Defensive Enhancement Hypothesis suggests that hyperaccumulation evolved because, after an initial defensive benefit accrued from a relatively low initial concentration, increased concentration of an element provided increased plant fitness and drove evolution of higher element concentrations until hyperaccumulation was achieved. The Joint Effects Hypothesis postulates that additive or synergistic effects between element-based defenses, or between toxic element and organic chemical defenses, may have contributed to the evolution of hyperaccumulation. By lessening the concentration of an element necessary to provide an initial defensive benefit to a plant, joint effects could decrease the level of an element that provides an initial defensive benefit, allowing additive or synergistic defensive enhancement to take effect. Recent experimental tests have demonstrated defense at relatively low element concentrations, and tests of metal/metal and metal/organic compound combinations have shown joint effects. These hypotheses suggest how hyperaccumulator plants may have evolved in response to plant–herbivore interactions, and suggest that toxic element levels below those used to define hyperaccumulation may be ecologically effective.

Contents

1. Introduction

Hyperaccumulators are plants that typically live on metal-enriched soils, often ultramafic (serpentine) soils, and accumulate unusually high tissue concentrations of an element or its ions [1]. The term refers to extraordinarily high concentrations in the aboveground tissues of at least one specimen growing in its natural habitat [2,3]. There more than 400 known hyperaccumulator species [3,4,5], and this number continues to rise as new hyperaccumulator species are documented [3]. Nickel is the element most often hyperaccumulated: about 75% of hyperaccumulator taxa are Ni hyperaccumulators [3]. There are also substantial numbers of hyperaccumulators of Co, Cu and Se, along with smaller numbers that hyperaccumulate As, Cd, Mn, Tl and Zn [3]. Plants that hyperaccumulate metals are termed “metal hyperaccumulators” but, because some hyperaccumulated elements are not metals

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* Tel.: +1 334 844 1626; fax: +1 334 844 1645.
E-mail address: boydrob@auburn.edu

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(Se, for example), the general term “elemental hyperaccumulator” will be used here. These plants have been comprehensively reviewed, including their physiology and their potential uses in remediation of polluted sites [6].

Some of the pioneering authors studying hyperaccumulators suggested that hyperaccumulated ions protect plants from herbivores (e.g. [7]), in addition to other potential functions such as drought tolerance, elemental allelopathy, etc. (see [8] for an early review). After initial demonstrations of hyperaccumulated Ni as a plant defense, including defense against both herbivores and pathogens, it was suggested that toxic ions hyperaccumulated by plants could be considered as an inorganic defense by plants against herbivores and pathogens [9]. This additional category of plant defense was established to separate it from other plant chemical defenses, which are organic compounds (also called secondary chemicals) manufactured by plants (see review [10]). Inorganic defenses differ from these secondary chemicals in two ways [11]: first, they are absorbed from the soil; and second, they are not subject to chemical degradation by herbivore and pathogen enzymes (because they are inorganic). This latter feature means that this potential detoxification pathway is unavailable to natural enemies as an offensive tactic (sensu [12]), although there are other offensive avenues that might be used to neutralize elemental defenses [13], such as sequestration, re-excretion, etc.

Questions regarding how and why the hyperaccumulation trait evolved have attracted much attention but are only just beginning to be explored. The defensive function of hyperaccumulated inorganic compounds has been referred to as the Elemental Defense Hypothesis (e.g. [6,14–17]), and will herein be referred to more correctly as the Inorganic Defense Hypothesis. Other hypotheses regarding the evolution of hyperaccumulation, including hyperaccumulation as an evolutionary response to toxic minerals in the soil [6], will not be discussed further here. An earlier review [13] introduced two hypotheses regarding inorganic defenses, the Defensive Enhancement and Joint Effects Hypotheses, both of which suggest hyperaccumulation may have evolved in at least some cases because of its role in plant defense. That review introduced each defense hypothesis: the purpose of the present review is to present a conceptual model of each of these hypotheses, including a consideration of some of the assumptions underlying each model. I will then consider how each hypothesis may contribute to our understanding of the evolution of hyperaccumulation by plants, and suggest directions for future research needed to test these conceptual models and their implications.

2. Conceptual model: Defensive Enhancement Hypothesis

The Defensive Enhancement Hypothesis suggests that hyperaccumulation may have evolved in some plant species because the hyperaccumulated elements protected the plants against herbivores and pathogens. For the sake of simplicity, the presentation of these models will refer to defense against herbivores attacking aboveground organs of plants, but similar scenarios can be envisioned regarding defense against pathogen attack and to protection of roots from attack by both herbivores and pathogens. The conceptual model (Fig. 1) plots mean plant tissue element concentration on the *x*-axis. The model assumes that this mean level is initially relatively low and that there is natural variation around that mean level. This variation is dependent upon genetically based differences in the ability of individuals of that species to take up, translocate and store that element in their tissues. The *y*-axis in the model represents the protective benefit of a particular element concentration in plant tissues. “Protective benefit” is a relative concept that can be defined as a reduction in damage inflicted on a plant (for example, a reduction in tissue removed or digested by a herbivore) compared to the damage that would be inflicted if the element were present at a lower level in the plant tissues. One assumption regarding the protective benefit in this model is that element concentrations will vary linearly in influencing the amount of damage a herbivore will inflict upon a plant (the linear assumption is discussed further below). This impact on damage is expressed on the *y*-axis as “protective benefit:” a low protective benefit will result in great damage to the plant and a high benefit little to none.

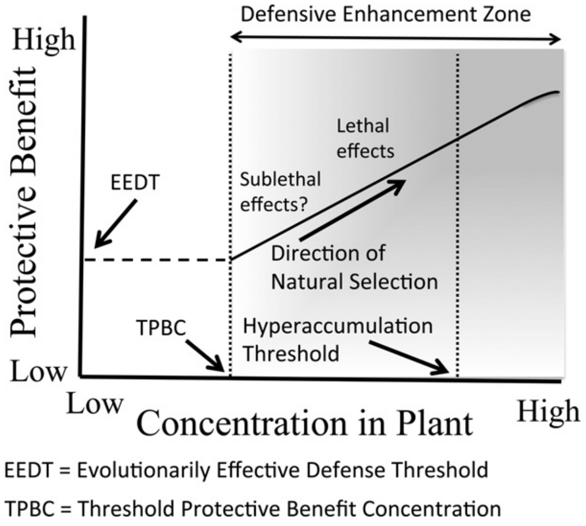

Fig. 1. Conceptual model of the Defensive Enhancement Hypothesis for the evolution of hyperaccumulation [13]. At some threshold value (TPBC: Threshold Protective Benefit Concentration), the inorganic compound concentration in the plant is sufficient to provide protection from herbivore damage great enough so that a selective advantage accrues to the plant. Once this Evolutionarily Effective Defense Threshold (EEDT) is reached, further increase (through natural selection) of plant inorganic compound concentration provides increased defensive benefit. In the model, continual increase in defensive benefit drives an increase in toxic compound concentration to (and beyond) the threshold concentration used to define hyperaccumulation for that element. The net effect is evolution of hyperaccumulation, as natural selection causes evolution of plant toxic compound concentration across the Defensive Enhancement Zone in the model, driven by increased defensive benefits accruing to plants with increased toxic compound concentrations. The question mark for “Sublethal effects” indicates that protective benefits of an element may be non-lethal at lower concentrations: further increases in toxin concentration result in lethal effects that likely provide larger selective benefits than sublethal effects.

The model connects element concentration and the defensive effect that drives evolution of hyperaccumulation as follows: A particular concentration, labeled as the Threshold Protective Benefit Concentration in Fig. 1, represents the point where fitness reductions due to costs of elemental uptake are balanced by fitness gains from reduced herbivore damage. Above this point, a given element concentration in plant tissues will result in increased plant fitness. The element concentration at which natural selection may begin to operate is termed the Evolutionarily Effective Defense Threshold. Below this threshold, no net changes in fitness are expected and variation in element concentration should be hidden from natural selection. In Fig. 1, this point begins the “Defensive Enhancement Zone,” the region of *x*-axis values that provides enough benefit to drive natural selection. Evolution of increased ability to accumulate metal (moving to the right on the *x*-axis) will increase the protective benefit, resulting in movement along the line (and an increase in mean plant tissue element concentration) as shown by the “Direction of Natural Selection” arrow. Presumably this process will continue until costs of hyperaccumulation, and benefits from defense, establish equilibrium: this equilibrium value may be influenced by evolution of herbivore tolerance, as will be explained below.

2.1. Implications of the model

An important assumption in the model is the shape of the response curve relating element concentration to protective benefit. The linear relationship used in Fig. 1 is a simplification, as the dose–response curve of a particular chemical administered to a particular herbivore probably is non-linear. For example, there may be a threshold concentration beyond which herbivore response increases greatly or, conversely, reaches an asymptote. It is also likely that different natural enemies will respond differently to the same chemical, or that multiple enemies with different tolerances would result in a sigmoidal curve. While the model simplifies these complex features for the ease of conceptual illustration, it remains valid for a curve with a positive slope and so is relatively robust if the assumption of linearity is violated.

The protective benefit axis (y-axis) in Fig. 1 also simplifies complex events. The y-axis represents the effect of an element on *plant* fitness (reproductive output or competitive ability), so that the effect of a particular element concentration on a herbivore is not directly shown. Instead, the model describes the outcome of that element concentration from the plant perspective, thus integrating the impact of the element on the herbivore into the y-axis measurement. Defensive chemicals can affect herbivores in many ways, but from the plant perspective the important outcome is the net effect upon the plant. The Threshold Protective Benefit Concentration is crucial to the model because that is where natural selection can begin to operate to enhance plant element concentration. Genetically based variation in element concentration is vital to the model, but the relative roles of genetics and environment are not well characterized in determining plant metal concentration of hyperaccumulators under field conditions [18]. Studies of hyperaccumulators show that there is considerable variability of metal concentration among individuals under field conditions, and controlled studies using hydroponic cultures also show significant genetically based differences are present [18]. For the model (Fig. 1), the critical question is how genetically based variability arose in the progenitor of a hyperaccumulator species at the low concentrations in plant tissues needed to provide variance to either side of the Threshold Protective Benefit Concentration. This variability could arise due to an inadvertent effect, where elevated metal concentration occurs due to adaptations that enhance uptake of other plant nutrients [8], or from another non-defensive benefit such as drought tolerance, elemental allelopathy or metal tolerance [8]. Over evolutionary time, this original benefit could be supplemented (or even supplanted) by defense if the defensive benefit of enhanced metal concentration provides a sufficient selective advantage.

The factors that determine the Threshold Protective Benefit Concentration are dependent upon on how a herbivore responds to a particular dose of that inorganic compound. Broadly speaking, and depending on dose, effects of plant defensive chemicals on herbivores can range from severe (lethal toxic effects that result in mortality) to relatively mild (sublethal toxic effects that reduce growth) to deterrence (in which damage is greatly reduced because a herbivore tastes and rejects a plant as a food). The comparative roles of these effects in the model are further explored below.

2.2. The roles of lethal effects, sublethal effects and deterrence

In the Defensive Enhancement Hypothesis model (Fig. 1), the Threshold Protective Benefit Concentration is crucial because that is where natural selection can begin to enhance plant element concentration. In the figure, an element concentration that is toxic to a herbivore (causing "lethal effects") is likely to benefit a plant enough to begin defensive enhancement by resulting in decreased herbivore damage. Protective benefits may also result from sublethal toxic effects (that occur at lower inorganic compound concentrations) because these sublethal effects can cause reduced herbivore growth rate, smaller herbivore size at maturity, reduced fecundity, etc. [19]. What is unclear is whether sublethal effects have strong enough impacts on selection to reach a defensive threshold (as shown by the question mark following sublethal effects in Fig. 1). Sublethal effects may result in less damage to a plant, by stimulating host-switching [20], making a herbivore more susceptible to its own natural enemies [21], etc. On the other hand, sublethal effects may result in more damage to a plant under some scenarios (e.g. [22]). These include prolonged herbivore feeding that causes greater tissue consumption, although this effect may be relatively rare [23]. The increased inorganic compound concentrations in herbivore bodies may negatively impact their natural enemies, providing a beneficial component to the herbivore. This aspect is explored further in Section 2.4.

Deterrence of herbivore attack occurs when a herbivore reduces or avoids consumption of a plant's tissues [6,13]. In the context of the Inorganic Defense Hypothesis, deterrence of herbivore attack has been demonstrated for all elements tested (As, Cd, Ni, Se and Zn) when damage levels have been compared between high- and low-concentration inorganic compound samples in choice experiments [13]. In terms of the Defensive Enhancement Hypothesis model (Fig. 1), complete deterrence would produce a very strong defensive benefit to a plant since it would not be damaged at all by a herbivore. Complete deterrence seems rare, however: it has been reported only once (for hyperaccumulated Zn) against the grasshopper *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) [24].

2.3. Experimental approaches

Demonstration of the Threshold Protective Benefit Concentration for individual inorganic compounds has been attempted for some herbivore species. A straightforward approach is to use artificial diet amended with different concentrations of a compound, usually to detect a statistically significant effect relative to unamended diet. Elements studied in this way include Ni^{2+} (e.g. [25]), Se as both selenate ($SeO_4{}^{2-}$) and selenite ($SeO_3{}^{2-}$) (e.g. [26]) and Zn^{2+} (e.g. [27]). One drawback to this approach is its limited ability to mimic an actual plant–herbivore relationship: artificial diets are generally optimized to support herbivore growth and have physical characteristics much different from plant tissues, etc. [28]. The advantage is that potentially confounding variables can be controlled or eliminated.

Another approach is to modify the plant material being offered to a herbivore to manipulate the concentration of the toxic material. This can be done in several ways. First, hyperaccumulator species often can be grown on substrates that vary in toxic mineral availability to generate plants with differing toxic concentrations [29,30]. This technique has the advantage of creating a more realistic feeding situation for a herbivore, but its weakness is an inability to control other potentially confounding factors. Changes in growth and chemical makeup of a plant in response to the differing substrates are likely and, in particular, concentrations of organic defensive chemicals may be affected. For example, a "trade-off" hypothesis [29] has been suggested for inorganic defenses, in which one potential benefit of an inorganic defense is allowing a plant defended by toxic inorganic ions to decrease its production of organic defenses. Such trade-offs between inorganic and organic defenses can occur (e.g. [31]), and trade-offs between chemical and other types of plant defenses (e.g. physical defenses) are also possible [32]. Control of confounding variables is a challenge to this approach.

Plant metal concentration may also be modified by infiltrating a plant organ, such as a leaf, with a solution of the toxic compounds, increasing its concentration. Leaf infiltration is used to apply bacteria to plant leaves for varying purposes (see [33]), but could be used to manipulate leaf concentration while not greatly influencing other aspects of leaf chemistry (unless the infiltrated material is toxic to the leaf). Using solutions differing in concentration of an inorganic compound, leaves that vary in dose of that compound could be produced and then fed to a target herbivore. In an example using a similar (but more simple) technique, high and low AsO_2^- lettuce leaves were created by dipping leaves in solutions that varied in AsO_2^- concentrations and offered to nymphs of the grasshopper *Schistocerca americana* Drury (Orthoptera: Acrididae): the nymphs preferred feeding on low AsO_2^- leaves [34]. Potential disadvantages of infiltration (and leaf dipping) include the likelihood that the distribution of the compound within the leaf tissues would differ from that found naturally, as well as the possibility that the chemical form of the element would differ from that in a natural leaf.

Finally, given our constantly improving understanding of the genetic basis of hyperaccumulation [6,35,36,37,38], it is becoming possible to genetically modify plants, changing compound uptake and sequestration pathways to create non-hyperaccumulating individuals of a hyperaccumulator species or to create hyperaccumulating individuals of a non-hyperaccumulator species. This task may be made easier by recent findings that hyperaccumulator and non-hyperaccumulator plants differ primarily in how genes common to both types of plants are expressed and regulated [6,36,37]. The promise of these genetic approaches to studies of inorganic defense is illustrated by use of similar techniques to manipulate plant defense by organic compounds. For example, metabolic engineering of plants can change volatile chemical production and affect responses of both herbivores and predators of herbivores in ways that benefit a plant [39]. While genetic modification provides yet another tool for studying elemental defenses, it does not completely solve the problem of confounded variables. More than one trait of a plant likely will be changed as a result of the genetic modification. Thus, studies of plant defense should integrate multiple approaches, ranging from feeding bioassays to genetic transformation of plants to phylogenetic ecology, to explore both the mechanisms involved and their ecological and evolutionary significance [40].

2.4. A potential role for enemy offense

In the presentation of the Defensive Enhancement Hypothesis above, plant defense evolves in response to the plant–enemy interaction but enemy susceptibility to the defense is considered constant. In this scenario, increased levels of an element result in greater protective benefits to the plant. Yet enemy offense also may evolve to counter the plant defense [12]. In the case of metal hyperaccumulator plants, specialist herbivores that are apparently tolerant of elevated plant metal concentration have been discovered. Some of these specialists have relatively high whole-body metal concentrations (e.g. the “high Ni insects” [41]). How these insects tolerate metals is an area needing further investigation. It is likely that a variety of molecular mechanisms [42], including binding of metal ions by metallothioneins, are involved, as well as other tactics such as efficient excretion of metals [43,44], or disposal of metals during transformation between nymphal, pupal and adult stages [43,45]. These tolerant herbivores may play a role in the evolution of hyperaccumulation by providing a selective force for continued increase in plant metal concentration. As an example, assume that an herbivore species contains genetically based variation in sensitivity to metal level, such that the Threshold Protective Benefit Concentration in Fig. 1 causes significant mortality of less tolerant herbivore individuals (resulting in selection for increased metal concentration). Surviving herbivores will be more metal tolerant, so that a greater concentration of metal in plant tissue will be needed to produce an effective defense. In this scenario, evolution of tolerance of the initial Threshold Protective Benefit Concentration by an herbivore will create a new (and greater) threshold by raising the concentration needed to produce a defensive benefit. This suggests that herbivore metal tolerance (and tolerance by other plant enemies such as pathogens) can drive evolution of hyperaccumulation. This role is essentially the classic “Arms Race” escalation scenario [46], in which coevolution between herbivore and plant drive both evolution of escalating herbivore tolerance and increasing levels of plant defense.

As mentioned in the Introduction, prior comparison of inorganic and secondary chemical defenses [11] has pointed out two differences between them: inorganic compounds are absorbed from the soil and are not subject to chemical degradation by herbivore enzymes. The “Arms Race” scenario mentioned above suggests a third difference: secondary chemical defenses have a great potential for evolutionary modification in that they can vary not only quantitatively, but qualitatively through changes in molecular structure. Qualitative variation has been acted upon by natural selection to generate the enormous diversity of these compounds [47]. Inorganic defenses, on the other hand, can vary quantitatively (as reflected by the conceptual model in Fig. 1) but are much more limited in their ability to vary qualitatively. Some elements (such as Se) occur in more than one ionic form in plants [48], and inorganic ions may be bound to differing organic ligands [49] that may influence their defensive effectiveness, but in general this qualitative variation is minor compared to the qualitative diversity found among organic compounds.

Besides contributing to the evolution of hyperaccumulation, evolution of herbivore tolerance may have important evolutionary consequences for the herbivore. One is host specialization of the herbivore on the hyperaccumulator plant. There is little information about this aspect in the literature, but there are some tantalizing suggestions it may occur. For example, the Californian (USA) Ni hyperaccumulator *Strepthanthus polygaloides* Gray (Brassicaceae) hosts a specialist resistant mirid (*Melanotrichus boydi* Schwartz & Wall [50]); the South African Ni hyperaccumulator *Berkheya coddii* Roessl. (Asteraceae) hosts a resistant beetle, *Chrysolina pardalina* Fabricius (Coleoptera: Chrysomelidae) [51], as well as the resistant grasshopper *Stenoscepa* sp. (Orthoptera: Pyrgomorphidae) [52]; and *Stanleya pinnata* (Pursh) Britt. (Brassicaceae), a Se hyperaccumulator from Colorado, USA, is fed upon by a Se-resistant race of the moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) [53]. Careful studies of the herbivores and pathogens of hyperaccumulator plants will likely uncover more such examples, providing opportunities to study mechanisms of metal tolerance in herbivores and how they may have evolved.

A second possible consequence of herbivore tolerance is sequestration of elemental defenses into the body of the herbivore, so that they can defend the herbivore against its natural enemies (see review [54] for sequestration of organic defenses). The high Ni tolerating insects [41] seem likely candidates for this, but the defense of high Ni insects only has been explored experimentally for *M. boydi* Schwartz & Wall (Heteroptera: Miridae). In that work, experiments compared survival of several predator species fed either *M. boydi* or low Ni insects of other species [55] or compared survival of *M. boydi* with that of another herbivore, *Lygus hesperus* Knight (Heteroptera: Miridae), when the insects were exposed to an entomopathogenic fungus or two species of entomophagous nematodes [56]. In only one test was there evidence of a potential protective effect of Ni: crab spiders, *Misumena vatia* Clerck (Araneae: Thomisidae), fed *M. boydi* suffered significantly greater mortality than those fed low Ni prey [55]. It should be noted that the experimental design of the *M. boydi* research was potentially confounded because the high and low Ni treatments used insects of different species: a stronger approach would have used *M. boydi* raised on high- or low-Ni foods to create high- and low-Ni individuals.

Experiments with other inorganic ions also suggest potential protective benefits to herbivores. For example, Cd in *Vicia faba* L. (Fabaceae) decreased the growth rate of an aphid, *Acyrthosiphon pisum* (Harris) (Heteroptera: Aphididae), feeding on the plants but resulted in a greater decrease in the growth rate of the aphid's parasitoid, *Aphidius ervi* Halliday (Hymenoptera: Braconidae) [57]. Other experiments suggest that particular (relatively low) doses of some inorganic ions may enhance protection of herbivores from viruses and bacteria, probably by boosting immune responses in the herbivores [58,59,60]. The evolutionary outcome of elemental defenses of herbivores may be evolution of tolerance in their natural enemies, in yet another offensive reaction to an organism's defense. This is illustrated by the Se-resistant parasitoid wasp, *Diadegma insulare* Cresson (Hymenoptera: Ichneumonidae), which attacks a Se-tolerant moth that feeds on the Se hyperaccumulator *S. pinnata* (Pursh) Britton (Brassicaceae) [53].

Finally, this section (and this review in general) has emphasized herbivores as plant enemies, but the models presented are applicable to plant pathogens as well. Protection of hyperaccumulator plants against particular pathogens has been demonstrated in a number of cases [13,15]. But bacteria are able to evolve relatively rapidly, in part due to their short generation times, so evolution of metal tolerance would be expected to have occurred in some cases. Studies of rhizospheric and endophytic bacteria associated with hyperaccumulators show that many bacteria have high tolerances for the hyperaccumulated element [15,61,62,63]. Evolution of metal tolerance in bacteria has resulted from use of metals (e.g. Cu) to protect crops from pathogens [64,65,66], illustrating the potential of inorganic ions to drive evolution of metal tolerance. There is also the potential for metal tolerance to be transferred between bacteria by horizontal gene transfer [64]: this also could contribute to relatively rapid evolution of metal tolerance in pathogens associated with hyperaccumulators. These characteristics may make pathogen metal tolerance more likely than herbivore metal tolerance to serve as a selective force that drives hyperaccumulation.

3. Conceptual model: Joint Effects Hypothesis

The Joint Effects Hypothesis is an extension of the defensive enhancement concept that includes the effects of more than one defensive chemical in the model. As used here, the term "Joint Effects" refers to effects of two defensive chemicals when those chemicals occur in combination (as opposed to their effects when they occur singly). In general, there are three types of joint effects: additivity, synergism (also referred to as potentiation) and antagonism. When two chemicals have a joint effect equal to that of their single effects when combined, the joint effect is additive. If the two chemicals together have an effect greater than an expected additive effect (considering each singly), the joint effect is synergistic, representing a positive interaction when in combination. If the two chemicals together have an effect lesser than an expected additive effect, then the joint effect is antagonistic (representing a negative interaction in combination).

The joint effects concepts noted above can be applied to the conceptual model of the Defensive Enhancement Hypothesis to illustrate how they may: (1) lower the threshold element concentration for which a protective benefit can occur; and (2) affect evolution of elemental accumulation by plants as a consequence. The Joint Effects Hypothesis conceptual model (Fig. 2) assumes that joint effects between two plant defense chemicals will be positive (either additive or synergistic). Using the same concepts and abbreviations as in Fig. 1; and initially considering only a single element in plant tissue; an element at the Threshold Protective Benefit Concentration 1 will produce a protective benefit (at Evolutionary Effective Defense Threshold 1) great enough to begin defensive enhancement of plant element concentration. Now consider the case when a second chemical is present; one that has either an additive or synergistic joint effect with the first chemical. In the presence of the second chemical; a lower concentration of the compound (Threshold Protective Benefit Concentration 2 in Fig. 2) will have an effect on the herbivore that leads to increased plant fitness (Evolutionary Effective Defense Threshold 2 in Fig. 2). This phenomenon will extend the Defensive Enhancement Zone to the left along on the *x*-axis. A synergistic effect would move the Defensive Enhancement Zone farther to the left than would an additive effect: thus synergistic joint effects have the greatest potential to produce defensive enhancement.

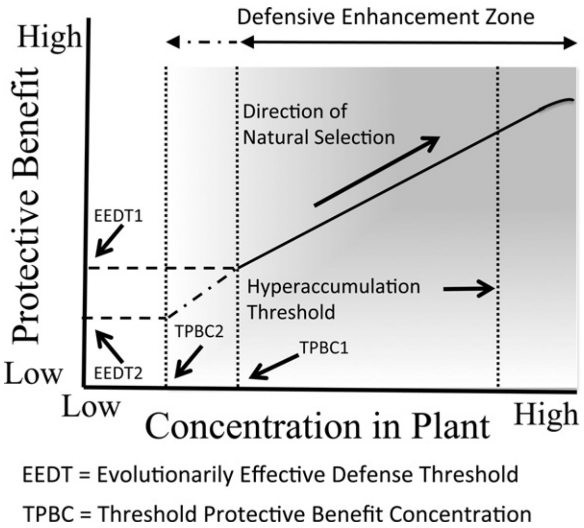

Fig. 2. Conceptual model of the Joint Effects Hypothesis [13], showing how joint effects between an inorganic defense and another defense (either inorganic or organic) can allow a relatively low concentration of an inorganic compound to become defensively effective. This joint effect can initiate evolution of increased inorganic compound concentration and, eventually, hyperaccumulation. In the model, a certain level of an inorganic compound (termed a Threshold Protective Benefit Concentration: TPBC1) is assumed to provide a protective benefit to a plant (labeled the Evolutionarily Effective Defense Threshold: EEDT1 in Fig. 2). According to the Defensive Enhancement Hypothesis for the evolution of hyperaccumulation [5], natural selection can then drive evolution of elevated inorganic compound concentration that may equal or surpass the threshold defining hyperaccumulation of that element in plant tissues. If effects of a second defensive chemical (inorganic or organic) combine with the effects of the original inorganic defense so that an Evolutionarily Effective Defense Threshold for the element is reached at a lower Threshold Protective Benefit Concentration (see EEDT2 and TPBC2 in Fig. 2), the net effect is an extension of the Defensive Enhancement Zone to a lower plant element concentration. This joint effect can allow evolution of hyperaccumulation of an element to begin at an even lower concentration of that inorganic compound than when that component is considered singly.

3.1. Implications of the model

The Joint Effects Hypothesis model has two important implications. First, a protective benefit of a compound may occur at a low concentration of that compound when other chemical defenses are present. Most tests of inorganic defense have used hyperaccumulator plant species or have used single element tests to determine protective benefits in artificial diet experiments [13]. In the former case, the hyperaccumulated compound occurs in plant tissues that also contain other inorganic or organic defense compounds, but the levels of these are not usually determined and their effects not accounted for in the experimental design. Thus, it is difficult to know the concentration of a compound where protective benefits begin. In the latter case, single compound experiments ignore potential joint effects and therefore cannot account for this potentially important factor in determining the effective threshold for a particular single element.

A second important implication of the Joint Enhancement Hypothesis is that evolution of hyperaccumulation can begin with a relatively low concentration of a single element. As discussed above for the Defensive Enhancement Hypothesis, the Threshold Protective Benefit Concentration is a critical feature of the model as that is the point at which natural selection can begin to operate to increase plant element concentration. The lower this threshold, the more likely it is that the stepwise evolution of greater element concentrations can begin.

In the consequences discussed above, the type of joint effect that may occur is of great importance. The Joint Effects Hypothesis requires a positive effect: antagonism would reduce the effect of an element, resulting in a decreased defensive benefit. Additive joint effects contribute to the Joint Effects Hypothesis model, but synergistic effects are the strongest positive effect and therefore would contribute most to the model. Relatively few studies have explored joint effects: it has been pointed out a number of times (e.g. [40,67,68,69]) that most studies of toxicological properties of chemicals (including plant defensive chemicals) do not investigate effects in combination. A study using snails, *Helix aspersa* Müller (Eupulmonata: Helicidae), and the Zn hyperaccumulator *Noccaea caerulescens* (J. Presl & C. Presl) F.K. Mey (Brassicaceae) documented herbivory levels to leaves and examined several factors (dry matter content and nitrogen, Zn and glucosinolate levels) to determine if they could explain the herbivory patterns observed [70]. Results were complex, but glucosinolate concentration was more important than Zn concentration in explaining herbivore preference. The only experimental study involving inorganic defenses and organic defense chemicals to date [71] reported results from experiments in which artificial diet was amended with defensive chemicals and fed to larvae of the crucifer specialist moth, *P. xylostella* (L.) (Lepidoptera: Plutellidae). They found additive joint effects between some pairs of metals (Zn + Ni, Zn + Pb, Zn + Cd) and between Ni when combined in certain concentrations with some example organic defense chemicals (atropine, nicotine, and tannic acid).

Finally, it was mentioned earlier that a “trade-off” hypothesis [29] has been suggested for inorganic defenses, in which one potential benefit of an inorganic defense was allowing a plant defended by toxic inorganic ions to decrease its production of organic defenses. For example, glucosinolate production by the Zn hyperaccumulator *N. caerulescens* fell when Zn concentrations in plants were relatively high [31]. The nature of the joint effect probably will determine if trade-offs will occur, as well as their extent. In the model (Fig. 2), the magnitude of the shift to the left of the Defensive Enhancement Zone depends on the magnitude of the joint effect. If the joint effect is additive, then the shift to the left will be relatively small. Synergistic effects, especially strong synergistic effects, will produce a large leftward shift. The ability of a plant to trade off an organic defense would therefore be greater if a strong synergistic effect occurred between an inorganic ion and an organic compound. This would allow a plant to reduce its organic compound concentration yet still extend the Defensive Enhancement Zone relatively far to the left in the model. Trade-offs therefore would be more likely to occur for synergistic rather than additive joint effects.

3.2. Future exploration

Exploration of the Joint Enhancement Hypothesis would be assisted by knowing what combinations of inorganic and organic compounds actually occur in hyperaccumulator plants. There seems to be no unified source of this information: a preliminary analysis found that Ni hyperaccumulation did not correlate with plant taxa that produce aromatic substances and volatile oils [72], but a more comprehensive and detailed analysis has not been done. Plant families or genera that contain hyperaccumulator species contain certain types of organic defense compounds. As examples, the Brassicaceae contains many hyperaccumulator species of Ni and Zn [3] and many members of the family contain glucosinolates [73]. The genus *Psychotria* (Rubiaceae) has Ni hyperaccumulator species [5] and members may contain alkaloids as well as other types of defensive organic compounds [74]. The Euphorbiaceae contains hyperaccumulators of Ni and Cu and the family is known for its latex that contains a suite of toxic organic chemicals [75], etc. Often what is not known is whether particular hyperaccumulator species contain particular types of organic compounds: in part this lack of knowledge stems from an incomplete inventory of hyperaccumulator species, as some have been discovered relatively recently (e.g. [76]). There is a great need for studies specifically targeting hyperaccumulator species that will investigate both inorganic and organic chemical defenses. Using phylogenetically controlled contrasts (sensu [77]), these studies could explore patterns of inorganic and organic defenses among carefully selected groups of hyperaccumulator and non-hyperaccumulator species. These types of investigations could shed light on the ways chemical defenses may have evolved in concert and influence plant defense. Critical to these studies is a more complete phylogenetic understanding of hyperaccumulation, which despite initial attempts (e.g. [78,79,80,81]) is still woefully incomplete.

4. Conclusions

Hyperaccumulation is a fascinating plant physiological phenomenon. Its evolutionary origins are still unclear, but inorganic defenses may be an explanation for how hyperaccumulation evolved in at least some of the many known cases. The conceptual models developed here allow researchers to examine the assumptions that underlie new hypotheses dealing with plant defense and element hyperaccumulation (Defensive Enhancement Hypothesis and Joint Effects Hypothesis), along with their predictions and ecological implications. Continued experimental explorations, using multiple approaches and experimental systems and bringing to bear new molecular tools, will increase our understanding of these unusual plants and their evolutionary history.

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