Cyanogenic glycosides and plant-herbivore interactions

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Abstract
Cyanogenic glycosides are nitrogen containing secondary metabolites which offer plant defense mechanism against herbivores while recently various insects have developed ability to detoxify, sequester and synthesize these cyanogenic compounds. Dhurrin was the first identified and isolated CNGs from young leaves of sorghum, *Sorghum vulgare*. The presence of CNGs were confirmed in few species of Chilopods, Diplopods, Heteropterans, Coleopterans and Lepidopterans. Linamarin and lotaustralin were the major CNGs distributed in Lepidopterans. They have developed an ability to de novo synthesize CNGs and detoxify them with the help of β-cyanoalanine synthase and rhodanase. This may be due to the course of insect evolution or the genes responsible for the synthesis of CNGs may get transferred from plants to insects. In future, the responsive genes in these arthropods should be silenced and expansion of transgenic cyanogenic plants may encouraged to ensure plant defense mechanism.

Keywords: CNGs, HCN, plant defense, cyanogenic plant, Zygaenidae and Papilionidae

Introduction
The nitrogen containing secondary metabolites present in plants are CNGs and glucosinolates. The secondary metabolites are not essential for the growth and development of plants instead they play a vital role in plant protection against biotic and abiotic stresses [21]. The CNGs are called as β-glycosides of α-hydroxynitriles (i.e., Cyanohydrins) which is derived from the aliphatic protein amino acids like L-valine, L-isoleucine and L-leucine, the aromatic amino acids like L-phenylalanine and L-tyrosine and from the aliphatic non-protein amino acid like cyclpentenyl-glycine [1]. They are phytoanticipins which is known to be present in more than 2500 plant species and also have been found in few arthropod clades. The CNGs containing major food crops are cassava (*Manihot esculenta*), sorghum (*Sorghum bicolor*), giant taro (*Alocasia macrorrhizos*), bamboo (*Bambusa vulgaris*), apple (*Malus domestica*) and apricot (*Prunus armeniaca*) [20]. They offer plant defense against insects due to its bitter taste and release of toxic HCN upon tissue disruption [27]. They can act as feeding cum oviposition deterrents and also phagostimulant in case of specialist insects on plants containing CNGs. Amygdalin and their synthetic derivative, laetirlate were investigated as potential drugs to treat cancer and were heavily promoted as alternative medicine.

A crucial agent in the co-evolution of plants and insects is that ability of plants to produce and handle bioactive compounds. Plants producing it for defense, but some insects sequester them and opening up new niches with fewer competitors. A few insect species, Zygaenidae and Papilionidae moths, able to carry out both de novo biosynthesis and sequestration of the same CNGs from their feed plants.

Evolution of insects
The insects feeding cyanogenic plants have acquired the ability to metabolize CNGs. They also sequester CNGs from their host plant and use it for predator defense e.g., *Zygaenidae* and *Papilionidae* [27].

Examples
1. *Heliconius* sp: The larvae of *Heliconius* sp. eat passion flower vines (*Passifloraceae*). The bright color wing pattern in adults signal their distastefulness to predators. To be unpalatable and harmful, they are using cyanic characteristics. Amino acids are needed to
make cyanic compounds which they have taken through pollen feeding. When the larvae feeds on the plant with cyanic compounds, they have evolved the ability to neutralize these compounds to protect them from the plant.

2. *Dryas iulia*: The host plants of *Dryas iulia* are lantana, shepherds needle and passion flower.

3. *Parnassius smintheus*: The host plant is *Sedum lanceolatum*.

**CNGs in edible plants**

### Table 1: Presence of CNGs in edible plants

| S. No. | Plants                        | Plant parts | Major CNGs                        | Amount of HCN (mg/kg) |
|--------|-------------------------------|-------------|-----------------------------------|----------------------|
| 1.     | Cassava, *Manihot esculenta* | Roots       | Linmarin, Lotaustalin             | 15-1000              |
| 2.     | Sorghum, *Sorghum vulgare*   | Young leaves| Dhurrin                            | 750-790              |
| 3.     | Flax, *Linum usitatissimum*  | Seed meal   | Linmarin, Linustatin, Neolinustatin| 360-390              |
| 4.     | Lima bean, *Phaseolus lunatus*| Beans       | Linmarin                           | 2000-3000            |
| 5.     | Giant taro, *Alocasia macrorrhizos* | Leaves | Triglochinin                       | 29-32               |
| 6.     | Bamboo, *Bambusa arundinacea*| Young shoot | Taxiphyllin                       | 100-8000             |
| 7.     | Apple, *Malus sp.*            | Seeds       | Amygdalin                          | 690-790              |
| 8.     | Peach, *Prunus persica*       | Kernels     | Amygdalin, Prunasin                | 785-813              |
| 9.     | Apricot, *Prunus armeniaca*  | Kernels     | Amygdalin, Prunasin                | 696-764              |
| 10.    | Nectarine, *P. persica var. nuciopersica* | Kernels | Amygdalin, Prunasin                | 196-209              |
| 11.    | Bitter almond, *Prunus dulcis*| Kernels     | Amygdalin, Prunasin                | 4700                 |

**Source**: Jane Philpott’s Food, Nutrition and Cookery Blog [12-20]

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**Metabolism of CNGs**

The parent amino acid will be subjected to two successive N-hydroxylations followed by decarboxylation and dehydration, as a result aldoxime is formed which is then converted to α-hydroxynitrile (cyanohydrin). These two steps are catalyzed by cytochrome P450 monooxygenase enzyme. The glycosylation of cyanohydrin moiety catalyzed by a UDPG-glycosyltransferase will leads to CNG synthesis [27].

**Fig 1**: Structure of major CNGs in edible plants [20]

**Fig 2**: Bio-synthesis of CNGs in plants, insects and higher animals [10]
Catabolism of CNGs
Catabolism of CNG is initiated through enzymatic hydrolysis by β-glucosidase to afford the α-hydroxynitrile. At pH above 6, α-hydroxynitrile spontaneously dissociates into a sugar, a keto compound and HCN. At lower pH, the dissociation reaction is catalyzed by α-hydroxynitrile lyase [1].

Mechanism of release
In plants, CNGs are stored in the vacuoles. When plant tissue is disrupted by herbivore attack, they are brought into contact with β-glucosidases in the cytoplasm and α-hydroxynitrile lyases that hydrolyze the CNGs and thereby the toxic hydrogen cyanide (HCN) gas is released (Catabolism). Hence the compartmentation will breaks off. The glycosides, cyanohydrins and hydrogen cyanide are collectively known as cyanogens [26, 22].

Cyanogenesis
The ability of living organisms to produce hydrocyanic acid (HCN) is termed cyanogenesis. As such CNGs are not themselves toxic, HCN will be formed when tissues of the plants are crushed or disturbed by herbivores, animal feeding, etc.. Cyanogenesis process will be faster at an alkaline pH and more than 60°C temperature [22].

Detoxification of CNGs
The first step in the detoxification is the formation of β-cyanoalanine from cyssteine by β-cyanoalanine-synthase [14] and β-cyanoalanine is then converted into asparagine. The second step involves the conversion of HCN into thiocyanate by rhodanese [4]. Only the first step of detoxification route occurs in plants and insects while the thiocyanate pathway occurs mainly in vertebrates, plants and insects.

Mode of action of cyanide
The affinity of cyanide towards cytochrome oxidase in the mitochondrial respiratory pathway causes cell death within a short span of time [6].

CNGs and Plant-herbivore interactions
The effectiveness of CNGs in plant defense may vary due to the following factors.
1. The concentration of CNGs in a host plant may be below threshold toxicity.
2. Specialist insects may be evolved to tolerate higher HCN in the diet.
3. Generalist insects may be consumed cyanogenic plant as part of mixed diet, hence the toxicity may be diluted to below threshold value.
4. The mode of herbivore feeding may minimize tissue damage in leaves (e.g. aphids, which are phloem feeders) to limit exposure of CNGs to degradative β-glucosidases [11].
5. Degradation pathway of CNGs may result in accumulation of cyanohydrins, keto compounds, HCN, β-cyanoalanine, thiocyanate and sulfite [6]. All these compounds have defensive properties:
   - CNGs act as feeding deterrent due to its bitter taste.
   - Aldehydes and ketones possess cytotoxic activities.
   - HCN is a respiration inhibitor.
   - β-cyanoalanine is a neurotoxin [17]
   - Thiocyanate and sulfite are enzyme inhibitors.
   - The primary deterrent effect of CNGs is due to their keto compound [13].

Example 1
In Schistocerca americana (Grasshopper, Neoptera) and Hypera brunneipennis (Alfalfa weevil, Coleoptera), the ten natural plant products viz., alkaloids, phenylpropanoids, terpenoids, glucosinolates and CNGs were tested for their deterrence and post-ingestional effects [27]. None of the compounds tested were detrimental, but eight out of ten compounds were deterred feeding [3, 4].

Example 2
The entire pathway for synthesis of aromatic tyrosine-derived CNG, dhurrin in Sorghum bicolor has been transferred to Arabidopsis thaliana using gene transfer technology to insert the three S. bicolor genes namely CYP79A1, CYP71E1 and UGT85B1. The accumulation of dhurrin prevented the feeding of Phyllotreta nemorum (Coleoptera), thus CNGs can confer resistance to insects [25].

Example 3
In nature, cyanogenic plant, Lotus corniculatus contains two CNGs namely Linamarin and Lotaustralin. The acyanogenic plants either do not synthesize CNGs or lack β-glucosidase for degradation and HCN release. After starvation, insects are generally more willing to feed on cyanogenic L. corniculatus leaves. This indicates that the deterrent capabilities of CNGs is dependent on the demand for food calories. High tolerance to CNGs is a characteristic of many Lepidopteran species. Hence, the role of CNGs in plant protection must be assessed based on species and food demand [27]. The above examples imply that, the primary defensive role of CNGs in plants may be as a feeding deterrent and not as a toxin. It serves as a warning to generalist herbivores that the plant is unpalatable [6]. The CNGs are well suited, cheap type of plant defense [27].

CNGs in Arthropods
Apart from the distribution of CNGs in plants, they are present in a single phylum, Arthropoda. Within arthropods, CNGs are present in Chilopoda (centipedes), Diplopoda (millipedes) and Insecta (Heteroptera, Coleoptera and Lepidoptera) [6].

Chilopoda, Diplopoda and Coleoptera
The defensive secretions of Chilopods, Diplopods and Coleopterans (Paropsis atomaria, Chrysophtharta variicollis and C. amoena) contains aromatic CNGs. These three beetle species synthesize their CNGs as these are not present in their diet. The two species of diplopods namely Oxidus gracilis, Harpaphe haydeniana have evolved biochemical pathways for CNG biosynthesis and degradation similar to higher plants. The H. haydeniana has cyanogenic glands (contain β-glucosidase and α-hydroxynitrilelyase), physically separated from the part containing CNGs. This prevent untimely release of HCN and thereby mimics the phytoanticipin defense effect in plants.

Heteroptera and Lepidoptera
Only a single species of Heteroptera, has been proposed to sequester CNGs from its host plant. In contrast to other arthropods, Lepidopterans are able to synthesize CNGs as well as sequester CNGs from their host plants. They contain mainly aliphatic CNGs as contrast to other arthropods.
Foresters and burnets (Zygaenidae: Lepidoptera)
The resistance of Zygaena to HCN has been reported in the beginning of the 20th century. The Zygaena species can tolerate HCN atmosphere for an hour and revive quickly when transferred to normal atmosphere. The HCN was released from crushed tissues of Zygaena species which reared on acyanogenic plants. This proves the ability of synthesis of CNGs.

The presence of β-cyanoalanine synthase in Zygaena larvae proves that that can effectively produce and detoxify HCN. This may have enabled them to commence feeding on cyanogenic plants. This would be in agreement with the shift of host plant specificity from Celastraceae to Fabaceae within the Zygaenidae.

Linamarin and lotaustralin distribution in Zygaenidae
The CNGs have been found in three subfamilies of Zygaenidae namely Zygaeninae, Procridinae and Chalcosiinae and in addition, two groups viz., Charideinae and Anomoeotinae, that were formerly placed in the Zygaenidae. A total of 45 species from these five groups contains linamarin and lotaustralin. They are sequestered from host plants (Fabaceae, e.g. L. corniculatus). The presence of linamarin and lotaustralin in several life stages of Zygaena transalpina were proved by LC-MS profiling.

In Zygaena filipendulae larvae, the amount of lotaustralin was greater than the amount of linamarin. After pupation, linamarin was the dominant CNG in Z. filipendulae. The equal distribution of linamarin and lotaustralin was observed in both larvae and imagines of Z. transalpina (Host: Hippocrepis comosa), although eggs contained more linamarin and empty pupae contained more lotaustralin. The ratio of linamarin and lotaustralin accumulated in Zygaenidae may vary due to the amount present in their diet and because of the ratio generated in the insect during synthesis.

In Zygaena trifolii (Host: Lotus), only small amount of CNGs (<1%) were found in the gut and the fat body while the majority was present in haemolymph and integument. A large proportion of the accumulated toxic secondary plant products may be excreted or lost as exuviae. As opposed to this, Zygaena larvae are able to retrieve CNGs from the old cuticle, since exuviae contain only minute amount of CNGs.

Cuticular cavities in Zygaenidae
The larvae in two subfamilies, Chalcosiinae and Zygaeninae, have cavities on their dorsal side in which they store the cyanide and can excrete it as defensive droplets against predators. The fluid would be highly viscous and colorless in nature. The defensive fluid from Z. trifolii is composed of linamarin and lotaustralin (7% CNGs), β-cyanoalanine (0.3%), proteins (8% including β-glucosidase) and water. A 1:1 (linamarin: lotaustralin) ratio was measured in the defensive secretion of Zygaena larvae whereas 19:1 was measured in their haemolymph. This indicates that lotaustralin is transported more effectively than linamarin because of their increased lipid solubility.

The larger cavities release their droplets by slight irritation whereas smaller cavities release much smaller droplets on severe irritation. This may be reabsorbed in few seconds after irritation has stopped. In contrast, diplopods and chilopods have specialized cyanogenic glands.
isoceicline, respectively because they are not present in their host plants (Passifloraceae). The amount of linamarin is higher than lotaustralain. The CNGs have the same bodily distribution as observed in Z. trifolii [9].

Toxicity of CNGs to humans

The CNGs containing edible plants should not be eaten as raw; processing techniques will reduce the toxicity. In case of insufficient processing, HCN may be released in the body, until the low pH of the stomach deactivates the β-glucosidase enzyme. The chronic sub-lethal dose of cyanide will cause lower birth rates, neonatal deaths, impaired thyroid function and slower response time. The acute symptoms include rapid respiration, drop in blood pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhoea, mental confusion, twitching and convulsions. The death can occur when the limit of cyanide exceeds that an individual is able to detoxify [8].

Lethal dose

The lethal dose of HCN in animals is 0.66 to 15 mg/kg body weight whereas the acute lethal dose of human is 0.5–3.5 mg/kg. The lethal dose of free cyanide for an adult man in cassava and its processed products constitutes 50–60 mg [8].

Case study

Table 2: Fumigation LC50 values (g/ml) of volatile natural and synthetic cyanohydrins

|                  | M. domestica | 95% fiducial limits | R. dominica | 95% fiducial limits |
|------------------|--------------|---------------------|-------------|---------------------|
| Naturally occurring cyanohydrin (DMK) | 0.07         | 0.06, 0.09          | 0.40        | 0.35, 0.46          |
| Synthetic cyanohydrin (CHP)         | 0.056        | 0.049, 0.063        | 0.37        | 0.14, 0.42          |
| Cyanohydrin ether CHP-me         | 0.41         | 0.34, 0.49          | 0.88        | 0.75, 1.04          |
| Cyanohydrin ester CHP-ace | 0.26         | 0.23, 0.30          | 0.37        | 0.32, 0.45          |

As a result, Park and Coats (2002) [22] found that natural and synthetic cyanohydrins are effective against stored product pests. They had not been conducted any experiments for acute and chronic toxicity to mammals, birds, movement to ground water and persistency in soil. Also, testing should be done for carcinogenicity, mutagenicity or teratogenicity in mammals.

Conclusion

The CNGs are not effective against all insects and not all cyanogenic plants release enough cyanide to be toxic. Maintaining some acyanogenic genotypes is advantageous because it will not be preferred by the specialized insects. The ability of synthesizing CNGs is a basic trait in some insects.

CNGs in Papilionoidea (Brush-footed butterflies)

The super family papilionoidea also contains linamarin and lotaustralain. The imagines of sub families namely Heliconiinae, Acraeinae, Nymphalinae and Polyommatinae [18, 15, 16] synthesize linamarin and lotaustralain from valine and

Fig 4: Zygaena filipendulae larva feeding on its cyanogenic host plant Lotus corniculatus exuding stored CNGs defense compounds through internal cuticular cavities when caterpillar is disturbed [7]

Fig 5: Structure of type 2 defensive cyanogenic gland in spirostreptid millipede [24]
This may be due to insect evolution (or) the genes encoding the enzymes involved in biosynthesis, degradation and detoxification of CNGs been horizontally transferred to the insects from host plants (or) the result of convergent evolution. Hence, an insects only need cyanogenic host plants to minimize their own biosynthesis of CNGs. Transferring genes across plants enables them with an altered qualitative and quantitative content of natural products thereby bypassing millions of years of co-evolution of plants and their pests. E.g., Transgenic A. thaliana plants accumulating the tyrosine-derived CNG dhurrin act as strong feeding deterrent against flea beetle Phyllotreta nemorum.

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