Cyanogenic Lipids

Utilization during Seedling Development of Ungnadia speciosa

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ABSTRACT

Large amounts of cyanogenic lipids (esters of 1 cyano-2-methylprop-2-ene-1-ol with C:20 fatty acids) are stored in the seeds of Ungnadia speciosa. During seedling development, these lipids are completely consumed without liberation of free HCN to the atmosphere. At the same time, cyanogenic glycosides are synthesized, but the total amount is much lower (about 26%) than the quantity of cyanogenic lipids formerly present in the seeds. This large decrease in the total content of cyanogens (HCN-potential) demonstrates that at least 74% of cyanogenic lipids are converted to noncyanogenic compounds. Whether the newly synthesized cyanogenic glycosides are derived directly from cyanogenic lipids or produced by de novo synthesis is still unknown. Based on the utilization of cyanogenic lipids for the synthesis of noncyanogenic compounds, it is concluded that these cyanogens serve as storage for reduced nitrogen. The ecophysiological significance of cyanolipids based on multifunctional aspects is discussed.

Cyanogenic plants are characterized by liberation of HCN following tissue injuries. This cyanogenesis is caused by hydrolytic cleavage of cyanogenic precursors, such as cyanogenic glycosides or cyanogenic lipids (3). Whereas cyanogenic glycosides are widespread in plant kingdom, cyanogenic lipids are restricted almost totally in their occurrence to the Sapindaceae (9). Thus, in contrast to cyanogenic glycosides and their related hydrolytic enzymes, little is known about the metabolism of cyanogenic lipids. These natural substances are characterized by a hydroxynitrile, which is stabilized by the esterification with a fatty acid. When the fatty acid is removed, e.g. by the action of a lipase, the resulting unstable hydroxynitrile dissociates spontaneously or by catalysis with a hydroxynitrile lyase and HCN is liberated.

In addition to the postmortem breakdown of cyanogenic compounds as described above, cyanogenic glycosides are also metabolized in the living plant: Bough and Gander (2) mentioned a rapid turnover of the cyanogenic glucoside dhurrin in Sorghum leaves, and Lieberei et al. (7) demonstrated that the cyanogenic glucoside linamarin is converted into noncyanogenic compounds during seedling development of Hevea, thereby serving as a storage compound for reduced nitrogen. This mobilization and utilization of linamarin is executed via a special pathway, where the related cyanogenic diglucoside linastatin acts as transport form for the cyanogenic monoglucoside (13). In contrast to the metabolism of cyanogenic glycosides, no data are available for a similar process with cyanogenic lipids which are stored in high concentrations in seeds of several Sapindaceae. It is not known whether these compounds are also metabolized during seedling development and whether they may also be utilized as storage compounds. To answer these questions, we analyzed changes in the content of the cyanogenic lipids and glycosides during development of Ungnadia speciosa (Sapindaceae) which contain large quantities of cyanolipid type 1, esters of 1 cyano-2-methylprop-2-ene-1-ol with the saturated arachidic acid (C:20) or the monounsaturated C:20 fatty acids (9).

Previsouly, NMR spectroscopy was used for quantitative estimation of cyanogenic lipids (11), but this method is not suitable for the screening of large quantities of samples. To analyze changes in cyanogenic lipid content, development of a simple method for their quantitative estimation was necessary.

In this paper, we present a simple method for quantifying cyanogenic lipids, based on alkaline saponification. Using this method, we analyzed changes in the content of cyanogenic lipids and were able to demonstrate that the cyanogenic lipids in ungerminated Ungnadia seeds are metabolized completely during seedling development.

MATERIALS AND METHODS

Plant Materials

Seeds of Ungnadia speciosa used in these experiments were collected at Barton Springs, Austin, TX. Seeds were bubbled with air while soaking in distilled water for 24 h. For germination, they were placed between several layers of moist paper towels and incubated at 25°C. After 3 d, all germinated and healthy seedlings were planted in sterile, moist Vermiculite (Deutsche Vermiculite Dämmstoff GmbH, D-4322 Spröckhövel 2) and cultivated in an incubator at 25°C and 60% RH with a light/dark cycle of 14/10 h. The rate of germination was 80 to 85%. Ungnadia seedlings were harvested at various developmental stages.

Trapping of Liberated HCN

To determine if HCN was liberated during seedling development, several seedlings were grown in Erlenmeyer flasks in

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a gastight system. A stream of moistened air was used to exchange the atmosphere in the experimental system continuously. By bubbling this air through 1 mL of 5 N NaOH, any HCN liberated from the seedlings during the experiment was trapped for quantitative determination.

**Extraction of Cyanogenic Lipids and Cyanogenic Glycosides**

Immediately after harvesting and careful removal of the hard seedcoat and adhering vermiculite, each seedling was divided into two fractions consisting of the cotyledons only and the remainder of the seedling composed of leaves, stem, hypocotyl, and roots. These were frozen and crushed in liquid nitrogen with mortar and pestle and subsequently freeze dried.

To extract the lipids, the freeze-dried powder was suspended with 10 mL of CHCl₃ in an Ultra Turrax homogenizer (3 × 60 s). The homogenate was filtered and the insoluble residue was removed from the filter, resuspended in 5 mL of CHCl₃, homogenized, and refiltered. This washing procedure was repeated two additional times with only 4 mL of CHCl₃. The filtrates (about 23 mL) were combined and made to an exact final volume of 25 mL with CHCl₃.

The cyanogenic glycosides in the insoluble residue remaining after chloroform extraction were then extracted with MeOH. For this, after evaporation of CHCl₃, the residue was suspended in 4 mL of MeOH and homogenized with an Ultra Turrax homogenizer (3 × 60 s). The homogenate was filtered and the insoluble residue was removed from the filter, resuspended in 4 mL of MeOH, homogenized, and filtered again. This washing procedure was repeated two more times with 3 mL of MeOH. The corresponding filtrates (about 14 mL) were combined, and the exact final volume was adjusted with MeOH to 15 mL. This extract contained all of the cyanogenic glycosides, since analysis of the MeOH-insoluble residue revealed that it contained no cyanogenic material.

**Saponification of Cyanogenic Lipids**

For quantitative determination, the cyanogenic lipids in the chloroform extracts were cleaved by saponification. This

![Graph](https://via.placeholder.com/150)

**Figure 1.** Alkaline hydrolysis of cyanogenic lipids. Saponification of cyanogenic lipids was performed as described in "Material and Methods." 100% corresponds to a cyanogenic lipid content of 85 μmol.
a steam of air. After addition of enzyme solution (0.5 mL),
the reaction vial was closed with a cap of aluminum foil, and
the assay was incubated for 60 min at 30°C. After incubation,
NaOH (500 μL, 1 N) was added to guarantee complete disso-
ciation of all cyanohydrins produced. Aliquots (100 μL)
were transferred to phosphate buffer (4.8 mL, 5 mM [pH 6.0]);
in samples with small amounts of cyanogenic glycosides,
this volume was increased to as much as 1 mL. Immediately
before the cyanide test, HCl (100 μL, 0.5 N) was added, and
this nearly neutral to slightly acidic solution was used for
cyanide determination. Control assays with different incuba-
tion times indicated that the enzymatic cleavage of cyanogenic
glycosides was complete after 60 min.

Cyanide Determination

Cyanide was estimated with the Merck Spectroquant kit for
cyanide (data sheet 130 259 8 DO dt/5, Fa. Merck). This
assay is based on the method of Aldridge (1). Absorbance
was measured at 585 nm, ε_{585} = 131,600 1 × mol⁻¹ × cm⁻¹.

Protein Determination

Protein concentrations were determined by the Biuret
method with bovine serum albumin as standard.

RESULTS

Saponification of Cyanogenic Lipids

The alkaline hydrolysis of cyanogenic lipids strongly de-
dpends on the incubation conditions (Fig. 1). With chloroform
extracts from Ungnadia seeds, it could be shown that com-
plete hydrolysis of cyanogenic lipids was achieved in less than
90 min when saponification was performed at 70°C, whether
1 or 5 N NaOH was used. In contrast, the reaction was much
slower at 30°C, and nearly complete hydrolysis was achieved
after 6 h with 5 N NaOH only.

The cyanide produced was determined after saponification.
As the cyanide assay requires samples adjusted to pH 6 to 7,
the aliquots used for cyanide estimation must be neutralized
carefully. No difficulties were observed with the 1 N NaOH
saponification assay, but some problems in reproducibility
arose when aliquots of the 5 N NaOH assay were neutralized.
Quantification of cyanogenic lipids by complete saponifica-
tion is best accomplished by an incubation assay with 1 NaOH
at 70°C for 2 h.

Utilization of Cyanogenic Lipids during Seedling
Development

Ungerminated Ungnadia seeds contain about 380 μmol
cyanogenic lipids, whereas older Ungnadia plants lack cyan-
genic lipids (Fig. 2). To determine whether the decrease in
the amount of cyanogenic lipids is caused by cyanogenesis or
is due to catabolic reactions without liberation of HCN, some
seedlings were grown in Erlenmeyer flasks. Analysis of air
blown through the flasks revealed that no or only traces (less
than 1 μmol) of HCN was liberated from intact seedlings
during seedling development. Only seeds and seedlings which
were infected with pathogens (mostly fungi) released HCN
via wound-induced cyanogenesis to a higher extent. During
germination and development of healthy seedlings, no HCN
was liberated, although the cyanogenic lipids initially present
in the seeds completely disappeared within a period of 3 weeks
(Fig. 2). Therefore, this consumption of cyanogenic lipids
must be due to metabolic reactions which either are different
from wound-induced cyanogenesis or which ensure that the
HCN produced via cyanogenesis is refixed completely by the
plant. In the course of this process, either the cyano-nitrogen
from the cyanogenic lipids is transformed to noncyanogenic
compounds, or the corresponding cyanohydrin is used directly
to synthesize cyanogenic glycosides. For this reason, it was
necessary to analyze the increase and changes in the content
of cyanogenic glycosides during seedling development.

Synthesis of Cyanogenic Glycosides during Seedling
Development

Although ungerminated Ungnadia seeds do not contain
cyanogenic glycosides, the strongly cyanogenic nature of adult
genic lipids completely decrease cyanogenic, umol are cosides in Changes and S4.
Changes in the development of U. speciosa seeds decreases cyanogenic lipids, the difference between cyanogenic lipids and cyanogenic glycosides (280 μmol of cyanogens or HCN, respectively) corresponds to the amount of nitrogen which is utilized from the cyanogens to produce noncyanogenic nitrogen-containing compounds. Assuming that the total amount of cyanogenic glycosides is produced completely via de novo synthesis and no direct transformation of cyanogenic lipids to related cyanogenic glycosides takes place, an even greater amount of cyano-nitrogen is converted in noncyanogenic compounds. In this case, the whole amount of cyanogenic lipids would serve as nitrogen source, and 380 μmol of cyano-nitrogen would be utilized for the synthesis of noncyanogenic nitrogen-containing compounds.

Ungnadia seeds contain about 170 ± 43 mg protein in each seed (n = 10). Assuming an average value of about 14 mg nitrogen per 100 mg protein, about 1700 × 10⁻⁶ g atoms of nitrogen are present in the total proteins of each Ungnadia seed. Comparison with the nitrogen stored in cyanogenic

![Image of Figure 3](image-url)

**Figure 3.** Synthesis of cyanogenic glycosides during seedling development of U. speciosa. Content of cyanogenic glycosides was determined by estimation of cyanide after enzymatic cleavage of the glycosides in the MeOH extracts of seeds and seedlings. The total content (upper curve) represents the amount of cyanogenic glycosides present in the cotyledons (dotted area) and in the rest of the seedling, consisting of leaves, stem, hypocotyl, and roots (hatched area). The stages and time scale are equivalent to those in Figure 2.

Ungnadia plants is due exclusively to these compounds (Fig. 3). Substantial synthesis of cyanogenic glycosides must occur during the development and the growth of Ungnadia plants. Significant quantities of cyanogenic glycosides are present first in stage S1. The highest rates of synthesis (see the slope of the upper curve in Fig. 3) are detectable between stage S2 and S4.

During seedling development, most of the cyanogenic glycosides are found in the large cotyledons, which constitute nearly the whole seedling. Later, the cyanogenic glycoside content of the shrinking cotyledons decreases drastically, and at the same time it increases in the young seedlings (Fig. 3).

**Changes in the HCN-Potential during Seedling Development**

The HCN-potential in Ungnadia seedlings consists of the content of cyanogenic glycosides and cyanogenic lipids. Whereas during seedling development the cyanogenic lipids disappear completely (Fig. 2), synthesis of cyanogenic glycosides occurs (Fig. 3). Comparison of the amounts of cyanogenic lipids metabolized and of cyanogenic glycosides synthesized revealed that the high HCN-potential (380 μmol cyanogenic lipids) in seeds decreases to a much lower level (100 μmol cyanogenic glycosides) in the young plant (Fig. 4 which is a combination of those data in Figs. 2 and 3). During this decrease of cyanogens, no significant amount of HCN is liberated to the atmosphere. Therefore, large quantities of cyano-nitrogen must be converted to yield noncyanogenic compounds. Assuming that the newly formed cyanogenic glycosides are produced by direct transformation of cyanogenic lipids, the difference between cyanogenic lipids and cyanogenic glycosides (280 μmol of cyanogens or HCN, respectively) corresponds to the amount of nitrogen which is utilized from the cyanogens to produce noncyanogenic nitrogen-containing compounds. Assuming that the total amount of cyanogenic glycosides is produced completely via de novo synthesis and no direct transformation of cyanogenic lipids to related cyanogenic glycosides takes place, an even greater amount of cyano-nitrogen is converted in noncyanogenic compounds. In this case, the whole amount of cyanogenic lipids would serve as nitrogen source, and 380 μmol of cyano-nitrogen would be utilized for the synthesis of noncyanogenic nitrogen-containing compounds.

![Image of Figure 4](image-url)

**Figure 4.** Changes in the HCN-potential during seedling development of U. speciosa. This curve contains data from Figure 2 and Figure 3. The total HCN-potential (upper curve) consists of the amount of cyanogenic glycosides present in the entire seedling (shown as dotted area) and the corresponding amount of cyanogenic lipids (white area).
lipids gives a ratio of 1 to 4.6 (380 × 10⁻⁶ g atoms nitrogen present in 380 µmol cyanogenic lipids to 1700 × 10⁻⁶ g atoms nitrogen present in proteins). This demonstrates convincingly that, in Ungnadia, cyanogenic lipids function as major storage compounds for reduced nitrogen.

**DISCUSSION**

**Synthesis of Cyanogenic Glycosides**

Cyanogenic lipids of *Ungnadia speciosa* seeds are completely metabolized during seedling development. At least three-fourths of the corresponding nitrogen is utilized for the synthesis of noncyanogenic compounds, but cyanogenic lipids (Formula 1) also may serve as precursors (not more than 26%) for the cyanogenic glycosides produced during this developmental phase. In this case, cyanogenic glycosides synthesized in young seedlings must reveal an aglycone-structure similar to that of the hydroxynitrile moiety found in the cyanogenic lipids of *Ungnadia*. The corresponding glucoside based on this cyanohydrin (1 cyano-2-methylprop-2-ene-1-ol) is proacacipetalin (Formula 1). Our recent analyses revealed that, during early development stages of *Ungnadia* seedlings, proacacipetalin does occur (14), whereas in older *Ungnadia* plants the spectrum of cyanogenic glycosides is completely different and no proacacipetalin could be detected. Further and detailed analyses of the glycosidic fractions of developing *Ungnadia* seedlings are necessary to document the conversion of cyanogenic lipids into cyanogenic glycosides. An understanding of such an interrelationship between cyanogenic lipids and glycosides as discussed by Hübel (5) will provide important steps for understanding the metabolism of cyanogenic compounds.

**Mobilization and Utilization of Cyanogenic Lipids**

Mobilization and utilization of cyanogenic lipids during seedling development of *Ungnadia* strongly suggest that cyanogenic lipids function as storage compounds for reduced nitrogen like the cyanogenic glycosides in *Hevea* (13). Although it is not clear whether the cyanogenic lipids are exclusively transferred to noncyanogenic compounds, or if the nitrogen of about one-fourth of them is used for the synthesis of cyanogenic glycosides, cyanogenic lipids must be regarded as a major nitrogen source for the developing seedling.

As shown for cyanogenic glycosides (13), cyanogenic lipids can be decomposed by two different processes: (a) wound-induced cyanogenesis and (b) mobilization and utilization. By decompartmentation due to tissue injuries, cyanogenic lipids come into contact with the related hydrolytic enzymes. Lipases hydrolyze the cyanogenic lipids, the resulting hydroxynitrile dissociates, and HCN is liberated. In contrast to this wound-induced cyanogenesis, no free HCN is liberated to the atmosphere during mobilization of cyanogenic lipids and the conversion to noncyanogenic compounds. The reactions involved in these two different processes are still unknown, but the fact that both of them occur in the same plant indicates that cyanogenic lipids perform more than one function within one plant. Due to its different effect on potential herbivores, the HCN which is liberated during cyanogenesis and the cyanogenic compounds, respectively, are believed to provide a protective function for the plant (for review see Ref 6). Recently, however, Hruska (4) pointed out that the repellent effect of cyanogens is weak or even negligible. In other studies, Lieberei et al. (8) demonstrated that cyanogenesis inhibits active defense reactions of plants against fungi. Although the function of cyanogenic compounds in the interaction of cyanogenic plants and other organisms is unknown, it is well accepted that such synchronological significance depends on the process of cyanogenesis. Thus, it is easy to distinguish these functions from those established by processes without any liberation of free HCN, such as mobilization and utilization. Because cyanogenic lipids are involved in cyanogenesis and yet are stored, mobilized, and utilized in the intact tissue without HCN-liberation, their significance must be multifunctional.

In addition to functions as storage compounds or as repellents, cyanogenic compounds may yet be responsible for other effects, such as the regulatory responses, such as flower induction by HCN in *Lemna* (15). The multifunctionality of secondary plant products, here demonstrated for cyanogenic compounds, also occurs with other N-containing secondary plant products and is probably a general phenomenon. Other examples include storage and consumption of toxic pyrrolizidine alkaloids (16) and nonprotein amino acids (10). The fact that different metabolic reactions are involved in the varying functions of cyanogenic compounds makes them suitable for further analysis of multifunctionality of secondary products in plants.

**LITERATURE CITED**

4. Hruska AJ (1988) Cyanogenic glucosides as defense com-