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Pattern of the Cyanide-Potential in Developing Fruits[1]

Implications for Plants Accumulating Cyanogenic Monoglucosides (*Phaseolus lunatus*) or Cyanogenic Diglucosides in Their Seeds (*Linum usitatissimum*, *Prunus amygdalus*)

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ABSTRACT

The absolute cyanide content of developing fruits was determined in Costa Rican wild lima beans (*Phaseolus lunatus*), oil flax (*Linum usitatissimum*), and bitter almonds (*Prunus amygdalus*). The cyanide potential (HCN-p) of the lima bean and the almond fruit began to increase shortly after anthesis and then stopped before fruit maturity. In contrast, the flax inflorescence had a higher HCN-p in absolute terms than the mature flax fruit. At all times of its development the bean fruit contained the monoglucosides linamarin and lotaustralin. The almond and the flax fruits contained, at anthesis, the monoglucosides prunasin, and linamarin and lotaustralin, respectively, while, at maturity, only the corresponding diglucosides amygdalin, and linustatin and neolinustatin, respectively, were present.

The occurrence of cyanogenic glycosides in plants is a well-known phenomenon and the combined effort of many investigators has lead to a clear view of their biosynthesis, of the tissue and subcellular organization of these compounds and their catabolic enzymes, and of the related genetics (for review see ref. 3). Cyanogenic glycosides are present in green plant tissue as well as in seeds such as almonds, flax seed, and wild lima beans (see references in Conn [4] for more complete listings). Nonetheless, we still lack knowledge of the more physiological aspects of these compounds and their metabolism. In particular, the processes involved in the accumulation of cyanogenic glycosides in seeds are not well defined. Thus, it is not known whether the glycosides are synthesized *de novo* in the growing fruit, or alternatively, if all, or significant amounts, of the glycosides are translocated from a primary site of biosynthesis in the parent tissue to the storage tissue in the seed. This paper describes the quantitative and the qualitative aspects of the accumulation of cyanogenic glycosides in whole fruits of the Costa Rican wild lima bean (*Phaseolus lunatus*), the oil flax seed (*Linum usitatissimum*), and the bitter almond (*Prunus amygdalus*) from anthesis to the mature fruit.

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MATERIAL AND METHODS

Source of Plant Material

The same collection of wild lima bean seed (*Phaseolus lunatus* L.) described by Frehner and Conn (6) was used. The flax seed (*Linum usitatissimum* L.) used was C. I. 1303. The bitter almond trees (*Prunus amygdalus* L.) were mature trees growing in the Arboretum of the University of California at Davis, The trees used were those having fruits that contained cyanogenic glycoside(s) at the end of the growing season of 1987.

Growth Conditions

Lima bean plants were grown in growth chambers from seeds in plastic pots (3 seeds per pot) using standard soil. Wooden rods were used to support the plants. The growth chambers were set to 10 h light (mixed incandescent and mercury vapor lamps) at 26°C and a RH of about 60%; at night the temperature was 18°C and the humidity about 80%. Flax was grown in growth chambers in groups of three plants per pot containing vermiculite. These chambers were set to 16 h light (mixed incandescent and fluorescent lamps) at 18°C; at night the temperature setting was 18°C. Humidity was not controlled in this experiment. All the plants were watered three times each week and received commercial liquid fertilizer once a week. The almond trees were neither watered nor fertilized.

Sampling

The date of anthesis of individual lima bean and flax flowers was recorded by inspection three times a week during a period of 5 weeks. Each time a small, dated tag was hung around stems bearing newly blossoming flowers; other 1 or 2 d old inflorescences not required for our experiments were removed so that only tagged fruits were allowed to develop. Mature and immature tagged fruits were removed from the parent plant, weighed, frozen in liquid N_2, and lyophilized. Units consisting of whole pots were completely processed on the same day. Sufficiently developed lima bean fruits were divided into seeds and fruit walls prior to weighing (Fig. 1). Several

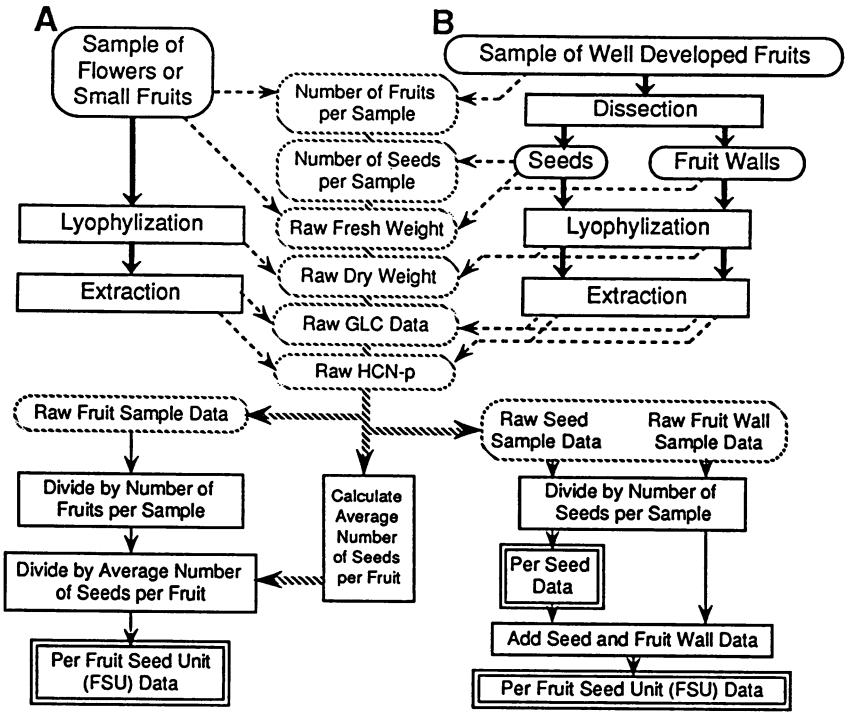

Figure 1. Flow charts of sample and data handling of the lima bean experiment. Fruits younger than 13 DAF were treated according to (A); other fruits, according to (B). Calculation of data based only on seed number or FSU is shown. The average number of seeds per fruit for well developed fruits was 2.08 seeds/fruit ($n = 50$). This value was used to calculate data based on FSU for small fruits. Thick arrows, sample flow; thin arrows and dotted arrows, data flow; simple boxes, actions taken; round boxes, samples; dotted round boxes, data sets; double-framed boxes, data used to generate graphs.

flax inflorescences were combined in one sample and processed as a unit. Almond samples in groups of two to five inflorescences, depending on size, were immediately enclosed in small plastic containers or small plastic bags, were subsequently weighed, frozen in liquid N_2, and lyophilized; bigger fruits were broken into several pieces in a cold mortar with a pestle before lyophylizing. Each data point in a graph corresponds to one sample. Curves were fitted by eye.

The dry weight was taken in all the cases as the weight of the samples after lyophilization.

HCN-p[4] was determined in a sealed vial containing the shredded or crushed sample, sufficient buffer (8) (half-strength, pH 5.5) to cover the sample, 0.5% (w/v) of β-glucosidase (Sigma) dissolved in the buffer, a few drops of toluene to prevent microbial growth, and, in a center well, 0.3 to 1 mL of 1 M NaOH. Incubation was for a minimum of 36 h at 28 to 30°C on a shaker. The NaOH solution of the center well, containing the HCN released from the sample, was diluted to 10 mL in 100 mM NaOH. Duplicate aliquots (5–100 μL) of the diluted NaOH solution were used to determine the HCN-p (6).

GLC

Lima-bean samples for quantitative GLC were crushed and extracted with hot water in a boiling water bath (5 min). To each extract a known amount of lactose (internal standard) was added. Aliquots of these extracts were taken to dryness, dissolved in 50 μL pyridine, and treated with 50 μL *N-N*-bistrimethylsilyltrifluoroacetamide and 20 μL trimethylchlorosilane. One μL of the treated extract was injected into a gas chromatograph (Packard model 417) fitted with a glass capillary column (30 m, 0.75 mm i.d., 1 μm film of SPB-5 by Supelco, Inc., Bellfonte, PA) and a flame ionization detector. Analysis conditions: injector 240°C; flame ionization detector 360°C; carrier gas, helium at 5 mL/min; additional helium at the flame ionization detector 30 mL/min. The temperature program was: 140°C for 1 min; 12°C/min to 326°C; 4 min at 326°C. Quantitative calculations were made using the peak heights of known amounts of standard glycosides.

[4] Abbreviations: HCN-p, cyanide-potential; FSU, fruit seed unit.

HPLC

For qualitative analysis of cyanogenic glycosides, samples were extracted in methanol overnight on a shaker at 25 to 28°C. The extract was taken to dryness, resuspended in water, and centrifuged 3 min at 12,000*g* in a Beckman Minifuge. The supernatant was filtered through a syringe microfilter (0.45 μm) and a portion of the filtrate was injected onto a column (RP-18 125 × 4 mm) for analysis. Aliphatic cyanogenic glycosides were eluted with 4% (v/v) acetonitrile in water at 2 mL/min and detected by a refractive index monitor. Aromatic cyanogenic glycosides were eluted with 6% (v/v) acetonitrile in water at 3 mL/min and monitored at 218 nm using an UV detector. Calibration of the chromatography system was done by injecting authentic glycosides.

RESULTS AND DISCUSSION

We present our data in such a way as to describe the growth and development of fruits very closely. For our purposes, expressing absolute data as quantity per fruit or per seed was practical. We preferred a high frequency of sampling with few samples per time point over a low frequency of sampling with

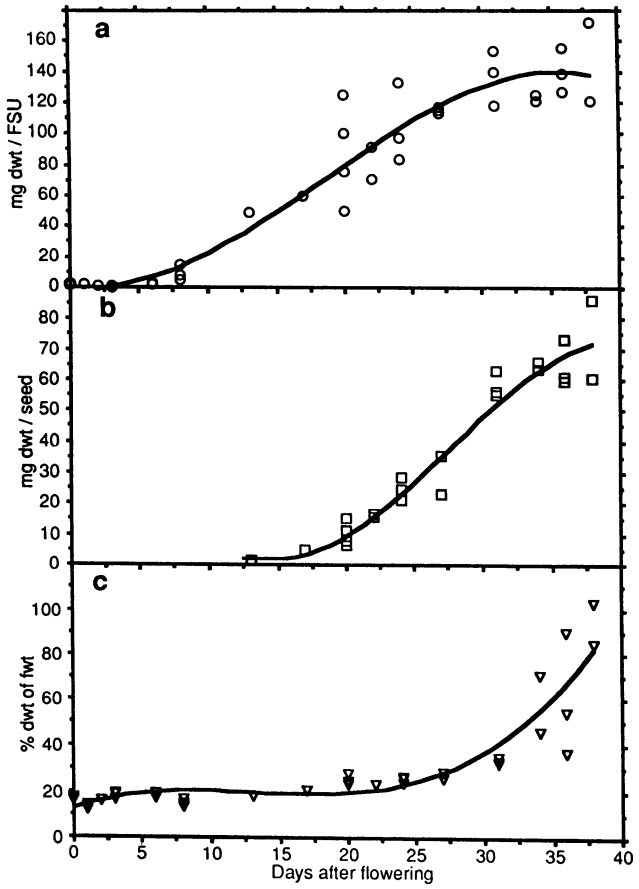

Figure 2. Growth of wild lima bean fruits from anthesis to maturity: weight profiles. a, The dry weight, taken as the weight after lyophilization, is expressed in mg/FSU (n = 42); b, a plotted value corresponds to the average dry weight of a seed of the same sample in mg (n = 24); c, each plotted value corresponds to the ratio of dry to fresh weight of a fruit sample expressed as percentage (n = 42).

many samples per time point. Our sampling procedure describes fruit development almost continuously but does not allow for standard statistical treatment of the data.

Lima Bean

The size of lima bean fruits varied greatly with the number of developing seeds (usually between one and four seeds/fruit, data not shown). Therefore, the data were expressed either as quantity per seed or in the case of whole fruits as quantity of the whole sample divided by the number of seeds contained in it (FSU; Fig. 1).

The development of the lima inflorescence from anthesis to the fully grown fruit took almost 40 d. After a lag period of 6 to 7 DAF, the fruits gained dry weight significantly until 32 to 34 DAF (Fig. 2a). At that time the fruits, which contained dark-colored seeds, were mature and dry. Since lima bean fruits were experimentally separable into fruit walls and seeds at 13 DAF, we observed that the seeds gained dry weight at a linear rate after a lag of 17 DAF until 36 to 38 DAF (Fig. 2b). The relative dry weight (as percentage of the corresponding initial fresh weight) of the whole fruit remained constant at approximately 20 to 25% from anthesis (0 DAF) until about 30 DAF (Fig. 2c); thereafter, the tissue continuously lost water and at 38 DAF, the dry weight almost equalled the fresh weight (Fig. 2c). Qualitatively, the color of the seed coats began to darken at 30 DAF. Seeds were considered physiologically ripe at 38 DAF because thereafter the dry fruits cracked open and expelled the dry seeds.

Cyanide, expressed as HCN-p per FSU, accumulated in fruits after 5 DAF, reached a broad maximum of about 13 μmol/FSU around 25 DAF and thereafter decreased slightly (Fig. 3a); at maturity the HCN-p was about two-thirds of its maximum value during seed development. This final HCN-p value of 6 to 10 μmol/FSU (Fig. 3a) can be taken as a measure of the content of a single seed (see below); this agrees quite well with the mean value of 8.7 μmol per seed of the parent seed (2). It is interesting to note that the tissue-cyanide concentration increased earlier and at a faster rate than did the dry weight of the fruits (*cf.* Fig. 3b with Fig. 2a) and reached

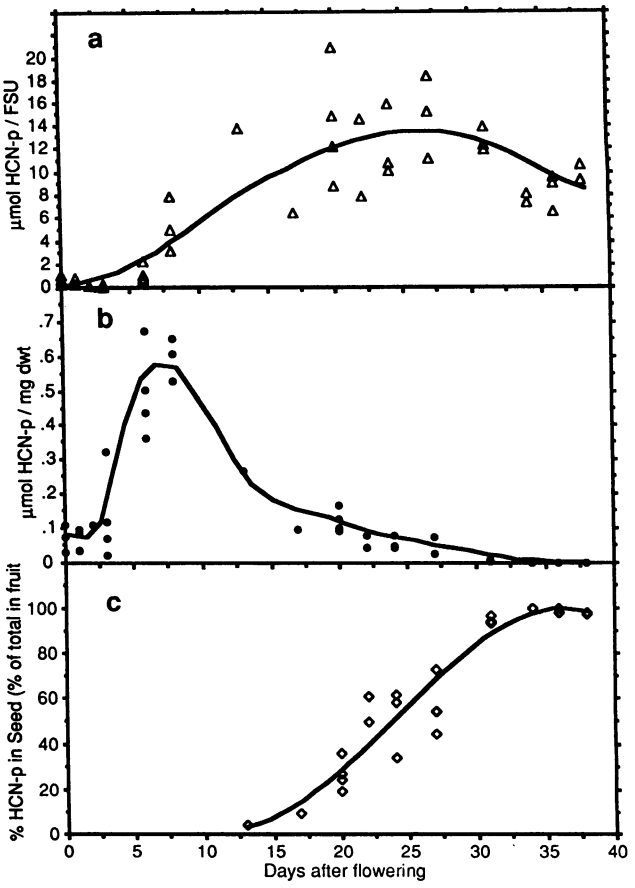

Figure 3. HCN-p profiles of wild lima bean fruits from anthesis to maturity. a, Each plotted value corresponds to the HCN-p of a lima bean fruit sample expressed in μmol HCN-p/FSU (n = 42); b, the HCN-p concentration of lima bean fruit samples is plotted in μmol HCN-p/mg dry weight (n = 42); c, the HCN-p distribution within lima bean fruits is plotted as the ratio of the HCN-p of the seeds of a lima bean fruit sample to the HCN-p of the whole corresponding fruit sample expressed as percentage (n = 24).

a maximum at 7 to 8 DAF. The tissue-cyanide concentration then was diluted by the onset of the rapid gain in dry weight (Fig. 2a), even though the absolute HCN-p per FSU increased further (Fig. 3a).

The fate of cyanide in the developing lima fruit is most interesting. Figure 3c indicates on a relative basis that the developing seeds accumulate HCN-p starting at 15 DAF. This process terminated at 32 to 34 DAF (Fig. 3c), just at the time of rapid water loss from the fruit (Fig. 2c) and therefore at the time when biochemical and physiological processes will be greatly reduced or terminated. These data can be interpreted in terms of translocation of HCN-p from the initially fleshy fruit wall tissue into the developing seed. Alternatively, the HCN-p in the fruit wall could be decreasing due to catabolism and, at the same time and rate, the HCN-p of the developing seeds increasing due to resynthesis in that tissue. Taking into account that (a) at 20 DAF the fruits contained about the same HCN-p as at maturity (Fig. 3a) but only about 20% of it was found in the seed, and (b) that at maturity the HCN-p was found almost exclusively in the seeds (Fig. 3c), we favor the idea of translocation. Since Selmar *et al.* (11), have proposed that cyanogenic diglycosides may act as transport forms of the corresponding monoglycosides, we monitored the pattern of neutral, low-molecular compounds in the developing lima fruits by GLC. At all times during the period of fruit development (between 0 and 38 DAF), only the two cyanogenic monoglucosides linamarin and lotaustralin, known to occur in the lima bean (1), were detected. No peaks coeluting with the corresponding cyanogenic diglucosides linustatin and neolinustatin were detected. The relative amount of lotaustralin averaged 4.5% of the total cyanogenic glycoside content (linamarin plus lotaustralin) and agreed well with the values reported by Butler (1) (4 and 8% for different lima bean samples).

Flax

Most of the data of this experiment were obtained from a large number of whole fruits as separation of different parts of fruits was not feasible. Therefore, the data for flax were calculated on the basis of inflorescence units. (In a subset of maturing inflorescences, the average number of developing seeds per seed capsule was 8.2 ± 1.7 [SE] seeds.) Development of the flax fruit was monitored up to 40 DAF: young inflorescences started to gain dry weight linearly about 5 DAF (Fig. 4a) with no significant gain in dry weight after 28 to 30 DAF (Fig. 4a). At 39 DAF the seed capsule was almost dry and contained dark colored seeds of dry weight similar (approximately 6.9 mg per seed) to that of the parent seed (5.9 ± 0.3 [SE] mg per seed). The relative dry weight of the flax fruit increased linearly with time, starting at 12 DAF (Fig. 4b). This steadily declining water content of the fruit probably reflects oil accumulation in the seeds (5), thus changing the overall ratio of fat to water, the water concentration still being sufficient for biochemical reactions in hydrophilic environments of the cell. Unlike the lima bean (Fig. 2c), the flax fruits did not dry out entirely during the experimental period of 40 d (Fig. 4b); thus, the flax seed might not have reached full physiological maturity at 39 DAF.

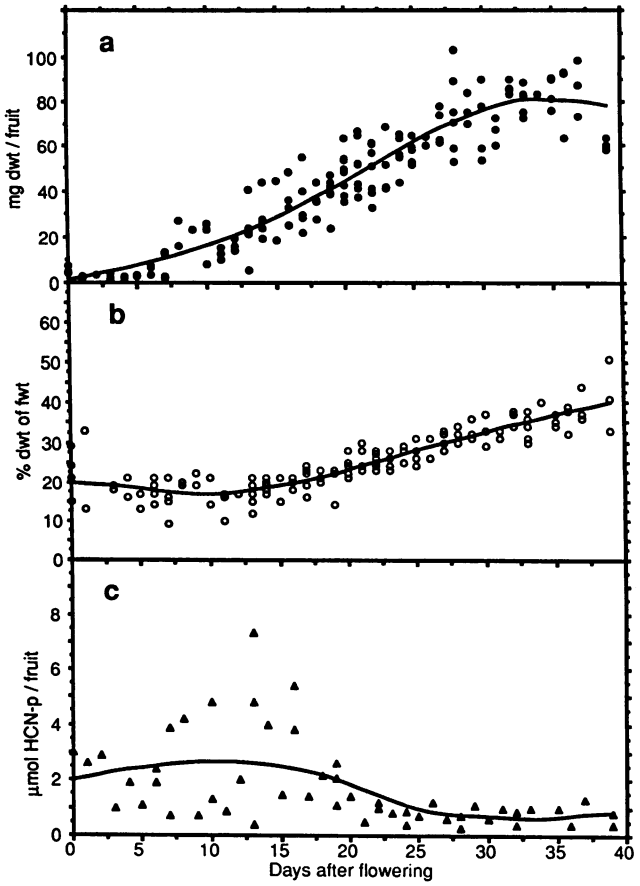

Figure 4. Growth of whole flax fruits from anthesis to maturity. a, The dry weight was plotted using values corresponding to the average weight of a flax fruit of a fruit sample (n = 148); b, the relative dry weight of flax fruits was plotted using the ratio of the dry to fresh weight of fruit samples expressed as percentage (n = 148); c, the HCN-p of flax fruits was plotted using the HCN-p/FSU values of flax fruit samples in µmol HCN-p/FSU (n = 51).

The pattern of cyanide accumulation in flax fruits (Fig. 4c) showed remarkable differences compared with that of lima beans (Fig. 3a). At anthesis (0 DAF; Fig. 4c) flax inflorescences contained 2 to 3 µmol HCN, which was considerably more than in the almost mature fruits (0.4–1.3 µmol at 35–40 DAF, Fig. 4c). These final values agree well with the HCN-p of the fruit of the parent seed; a calculation using the above mentioned 8.2 seeds per fruit capsule, the capsule tissue with an HCN-p of approximately zero (7), and the HCN-p of the parent seed of 0.05 nmol HCN per seed yields a result of about 0.4 µmol HCN per parent fruit. A broad maximum of HCN-p per flax fruit appeared between 7 and 18 DAF (Fig. 4c) which was characterized by a high variability among the particular samples taken. Lüdtke (7) mentioned a similarly broad maximum of the HCN-p in flax fruits in the field.

In a few samples the cyanogenic glycosides were determined qualitatively by HPLC. All glycosides known to exist in flax seeds (linamarin, lotaustralin, linustatin, and neolinustatin [12]) were detected, the biochemically-related valine series (linamarin and linustatin [4]) predominating over the isoleu-

cine series (lotaustralin and neolinustatin [4]) This analysis showed that the ratio of monoglycosides to total cyanogenic glycosides shifted from 100% (*i.e.* only linamarin and lotaustralin) at anthesis to 0% (*i.e.* only the diglucosides linustatin and neolinustatin) at maturity: Young flax fruits (0–18 DAF) contained mainly the monoglucosides linamarin and lotaustralin (>90% of total cyanogenic glycosides), whereas about 30% of the cyanogens in older fruits (25–40 DAF) were the diglucosides linustatin and neolinustatin. The parent seed, in contrast, contained exclusively the diglycosides, about two-thirds being linustatin and one-third neolinustatin. It is very likely that the fruits at the end of the experimental period (39 DAF) were not quite physiologically mature because they would be expected to contain qualitatively the same glycosides as the parent seed. This apparent glucosylation of the monoglycosides could be the result of translocation of cyanogenic glycosides as proposed by Selmar *et al.* (11): that is, the monoglucosides linamarin and lotaustralin produced in parental tissues of the flax plant would be translocated into the growing seeds as the corresponding diglucosides linustatin and neolinustatin, respectively. The diglucosides would persist in the seed as such until the seed germinated, at which time, degradation and metabolism of the diglucosides would occur.

Almond

The experimental data obtained for the almond has been calculated on a per fruit basis. All the flowers and young fruits sampled were composed of one carpel only; thus each fruit represented one seed (except for the closed flower buds, which were not opened to check the number of carpels). On d 52 of the year 1988 (*i.e.* February 21, 1988) most inflorescences of the trees under investigation were blossoming; therefore, that day was used to determine the age of the fruits on a DAF basis. As shown in Figure 5a, significant increase in dry weight started at 10 DAF and was probably not finished at 140 DAF (d 190, Fig. 5a). At this time the almond fruits were still green but had just about reached their final size; the endocarp was quite hard and the embryos were well developed. Similar to the flax fruit (Fig. 4b), the relative dry weight of the almond fruits increased slowly after a lag of about 50 DAF (Fig. 5b). This apparent loss of water was probably due to the onset of oil synthesis and accumulation. As shown in Figure 5b, the observation period did not last until the fruits were physiologically mature; by d 190 of 1988 the fruits were still moist with a relative dry weight less than 40%.

The HCN-p of flower buds (d 40) was slightly higher than the HCN-p of the youngest fruits (d 70, Fig. 5c) and increased slightly starting at d 90. After d 140, the HCN-p of the fruits rose sharply to reach about 20 times the level of the flower buds (*cf.* values of d 41 and 187, Fig. 5c). Up to the onset of significant cyanide accumulation (around d 100, Fig. 5c), all the glycoside present in the fruits consisted of prunasin (Fig. 5d), the cyanogenic monoglucoside of several species of the *Rosaceae* (4). Thereafter the relative abundance of the corresponding diglucoside amygdalin increased significantly (Fig. 5d) and after d 160 amygdalin composed more than 90% of the total cyanogenic glycoside content. This pattern of accumulation is consistent with the fact that bitter almond seeds

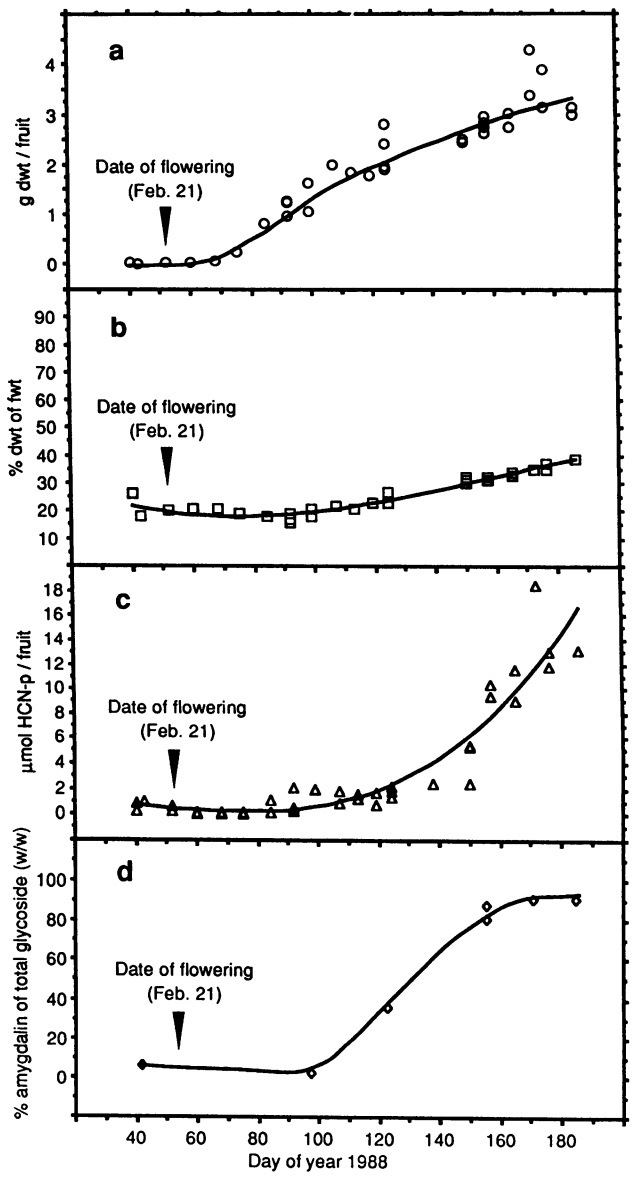

Figure 5. Developing almond fruits. a, The dry weight of almond fruits was plotted using the average fruit weights of the samples after lyophilization ($n = 35$); b, the relative dry weight of almond fruits was plotted as the ratio of dry to fresh weight in % ($n = 35$); c, the HCN-p of almond fruits was plotted using the HCN-p/fruit of the samples of almond fruits in µmol HCN-p/fruit ($n = 39$); d, the relative abundance of amygdalin in almond fruits was calculated as the percent ratio of the amount of amygdalin divided by the total amount of cyanogenic glycosides in the samples ($n = 7$).

contain mainly amygdalin as their cyanogenic glycoside (9). A similar pattern of accumulation and relative abundance of prunasin and amygdalin has been observed in *Prunus avium* (9) and in *Cotoneaster bullata* (10).

Storage and Transport of Cyanogenic Glycosides

The wild lima bean plant contains the cyanogenic glucosides linamarin (2) and to a lesser extent lotaustralin (1, and this paper), both being monoglucosides. During the period of fruit and seed development the plant accumulates the same glucosides first in the whole fruit (Fig. 3, a and b) and the same kind of glucosides accumulate later in the developing seeds (Fig. 3c). There the linamarin concentration increases to about 3.5% (fresh weight) in mature seeds (2). Similarly, *Hevea brasiliensis* contains the monoglucoside linamarin both in vegetative tissues and in the seeds (11).

Seed of *Linum usitatissimum* and *Prunus amygdalus* differed qualitatively from the lima bean in their pattern of accumulation of cyanogenic glycosides during fruit development. Although the inflorescences and young fruits contained their species-characteristic cyanogenic monoglucosides (linamarin and lotaustralin in the flax; prunasin in the almond), the temporal pattern of HCN-p buildup in the fruits differed considerably between the flax (Fig. 4c) and the almond (Fig. 5c), and the well developed and nearly ripe fruit of both species contained mainly their characteristic diglucosides (linustatin and neolinustatin in flax, amygdalin in the almond).

It would be interesting to identify the physiological and biochemical factors that determine the two qualitatively different patterns of storage of cyanogenic glucosides observed in this study. The dissection and analysis of developing lima fruits allowed us to calculate the relative distribution of HCN-p between the fruit wall and the seeds (Fig. 3c). Figure 3c shows a shift of HCN-p within the developing fruit. This shift can be interpreted as translocation of the glucosides from the fleshy fruit walls into the growing seeds. Based on their data, Lüdtke (7), Nahrstedt (9), Clegg *et al.* (2), and Selmar *et al.* (11) also suggested the possibility of translocation of cyanogenic glucosides. Selmar *et al.* (11) presented experimental evidence for translocation in *Hevea brasiliensis* in a model involving the diglucoside linustatin as a transport form of the monoglucoside linamarin. This model could also explain the translocation of linamarin from the fruit walls of the lima bean into its growing seeds. It has been shown earlier (6) that the β-glucosidase (linamarase) which acts on linamarin is located in the extracellular space (water accessible space of tissue, excluding protoplasts and their contents) of lima bean plants, space which a linamarin molecule would have to cross on its way to the seed. Such translocation would seem unlikely unless the linamarin was protected from hydrolysis by linamarase in the extracellular space. On the other hand glucosylated linamarin (linustatin) is not hydrolyzed by the extracellular lima bean linamarase (6). Assuming that the translocation model involving diglucosides (11) holds for seeds that accumulate the monoglucoside (*e.g. Phaseolus* and *Hevea*) as well as for seeds accumulating the diglucoside (*e.g.* flax and almond), the difference between the two accumulators would be in their posttranslocational metabolism. In the first case the diglucoside could simply be hydrolyzed back to the monoglucoside, and in the second case the translocated molecule would be stored unaltered as a diglucoside.

Based on the results presented in this paper, it is possible to describe the physiological state during which translocation most likely would occur: In the lima bean fruits the period of fast translocation appeared to occur during seed growth between DAF 20 and 30 (Fig. 2b) because the HCN-p at that time was already high (Fig. 3a) and the relative amount of the total HCN-p of the fruit increased dramatically in the developing seeds (Fig. 3c). During this period we would expect to be able to find a transport form of linamarin if translocation really occurs and if linamarin is modified (*e.g.* glucosylated) for translocation. The almond fruit shows a window of time during which to search for a UDPG:prunasin glucosyltransferase, the one enzyme still undescribed in the biosynthesis of amygdalin (or any other cyanogenic diglycoside). Between DAF 80 and 130 (about day 130–180, Fig. 5c) the HCN-p of the almond fruit increased rapidly and the prominent glucoside shifted from the monoglucoside prunasin to the diglucoside amygdalin (Fig. 5d). During this period the fruit was still relatively soft and contained only a few woody elements so that it would be experimentally useful. The flax fruit does not show such clearly defined periods of glycoside accumulation. The HCN-p was high in the young fruit (DAF 10–18, FIg. 4c) but was lower in the almost mature fruit (DAF 35–40, Fig. 4c), so that no period of rapid synthesis of cyanogenic glucosides was observed. The ratio of mono- to diglucoside changed only slowly during fruit development, thus diminishing any hope for detecting an active UDPG: linamarin glucosyltransferase activity in crude extracts. In this case weak glucosyltransferase activities could be sufficient to account for the observed minimal rate of diglucoside appearance.

It is likely that some of the missing information in the biochemistry and the physiology of cyanogenic glycosides in plants could be obtained by analyzing chosen plants (or parts thereof) at a specific physiological state.

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