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CYANOGENIC GLUCOSIDES IN *LINUM USITATISSIMUM*

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Key Word Index—*Linum usitatissimum*; Linaceae; flax; HCN-potential; cyanogenic glucosides; linamarin; linustatin; lotaustralin; neolinustatin.

Abstract—Cyanogenic glucosides were quantified in different organs of oil flax (*Linum usitatissimum* cv LCSD 200) plants at different stages of development. Monoglucosides (linamarin and lotaustralin) and diglucosides (linustatin and neolinustatin) appeared in developing embryos soon after anthesis, but mature seeds accumulated only diglucosides. Monoglucosides appeared again in germinating seeds and, in young seedlings, they were the only class of cyanogens. High levels of linamarin and lotaustralin were found in leaves throughout the vegetation period, but the highest amounts were in flowers. In contrast, these glucosides occurred in relatively small amounts in roots and in stems. The possible physiological roles of the changes are discussed.

INTRODUCTION

The widespread distribution of cyanogenic glycosides in plant kingdom, as well as some other evidence, indicate that these compounds may play an important role in the primary metabolism of plants [1, 2]. The content of cyanogens in plant tissues varies with age and developmental stage, and can be greatly influenced by seasonal, nutritional and genetic factors [3, 4]. Cyanogenic glycosides could function as nitrogen-containing precursors for amino acid and protein synthesis during seedling development [5] or they could play a protective role against herbivores by liberation of free HCN from injured plant tissues [6, 7]. Cyanide, released from cyanogens, may control seed development and germination in some species [8, 9]. It can also induce the alternative respiratory pathway [10]. Moreover, according to some evidence, HCN controls nitrate reductase activity in *Zea*, *Sorghum* and *Triticum* [11].

Fax plants (*Linum usitatissimum*) contain two major cyanogenic monoglucosides, linamarin and lotaustralin, aglycones of which are derived from L-valine and L-isoleucine, respectively [12, 13]. These two compounds, without apparent exception, always occur together in different species, although not necessarily in the same ratio [14, 15]. The co-occurrence of these two glucosides in linen flax is ascribed to the existence of a single set of biosynthetic enzymes [16, 17]. Moreover, flax seeds contain two cyanogenic diglucosides, linustatin and neolinustatin, the 6′-*O*-glucosides of linamarin and lotaustralin, respectively [18].

Linum usitatissimum is a cyanogenic plant of considerable economic importance. The presence of large amounts of cyanogenic glucosides in seeds severely restricts the amount contained in animal feed. The necessity to improve the commercial value of this species by reducing the content of cyanogenic glucosides is obvious. Knowledge of their qualitative and quantitative changes in different parts of flax plants at different stages of development is a condition for further research leading to modification of cyanogen content.

RESULTS

The concentrations of particular cyanogenic glucosides were determined in seeds of oil flax (*Linum usitatissimum* cv. LCSD 200) during germination, in whole plants during growth and development, and in different separated organs.

In mature dormant seeds and during the first day following the start of imbibition only diglucosides (linustatin and neolinustatin) were detected (Fig. 1A), the amount of linustatin exceeding that of neolinustatin 1.5 times. The content of both diglucosides increased during the first 36 h of germination and then decreased to a nondetectable level on day 3 following germination. Monoglucosides (linamarin and lotaustralin) appeared in the seeds 36 h after germination and on day 2 they reached the same concentration as that of diglucosides (Fig. 1A); subsequently they constituted the only class of cyanogens in growing seedlings and plants (Fig. 1B). The amount of monoglucosides in the 3-day and 30-day old plants was 20 and 100 times

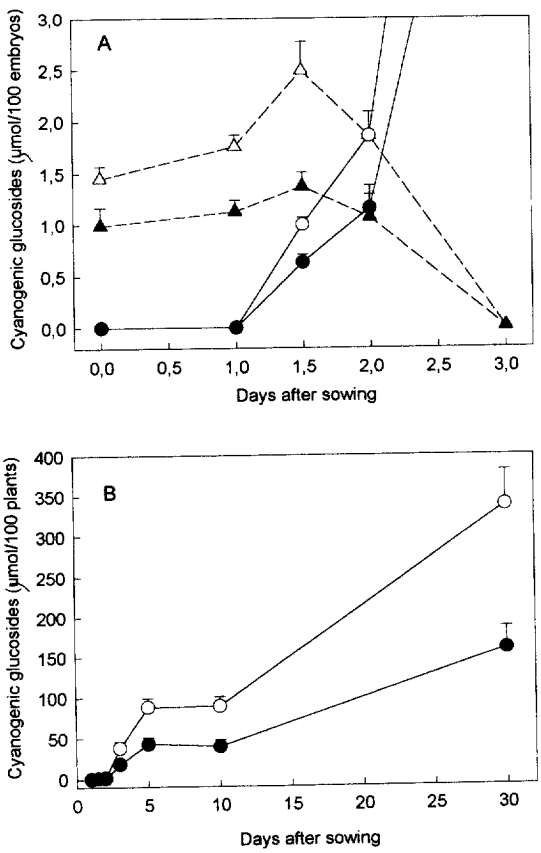

Fig. 1. Cyanogenic glucoside content in flax plants during ontogenesis: A, in germinating embryos; B, at different stages of growth. Monoglucosides: (○ linamarin; ● lotaustralin). Diglucosides: (Δ linustatin; ▲ neolinustatin).

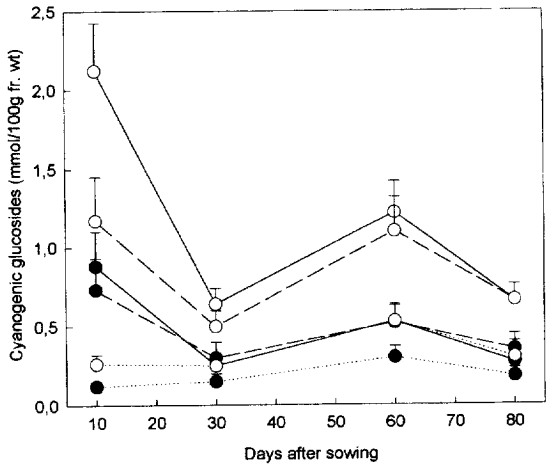

Fig. 2. Concentrations of linamarin (○) and lotaustralin (●) in different organs of flax plants at different stages of development. (——) leaves; (– – –) stems; (. . .) roots.

higher, respectively, than that of monoglucosides (and diglucosides) in 2-day-old plants (Fig. 1). The concentration of linamarin was twice as high as that of lotaustralin in all developmental stages and plant organs studied (Figs 1 and 2).

The main site of accumulation of diglucosides in seeds are cotyledons, in which the content of cyanogens is 4 times greater than that in embryonic axes (Table 1). Nevertheless, their concentration (calculated as the content per 100 g fr. wt) was considerably higher in embryonic axes than in cotyledons. Ratio of neolinustatin to linustatin was somewhat higher in cotyledons (0.6) than in axes (0.5).

Leaves and cotyledons of 10-day-old flax seedlings contained the highest amounts of monoglucosides. Their content in stems of the same seedlings was ca 4 times lower, the lowest amounts being detected in the roots. Similarly, in 10-day-old seedlings, the highest concentrations of cyanogens were found in leaves and stems exceeding several times those in roots (Table 2).

Similar relationships were observed in the organs of mature plants during development (Fig. 2). In leaves, as well as in stems and roots, both linamarin and lotaustralin were present; however, their concentrations were different, depending on plant age and organ. The highest concentration of monoglucosides was found in leaves, slightly lower in stems (especially green ones) and the lowest in roots (Fig. 2). The concentration of cyanogens in young leaves of 10-day-old plants (3.0 mmol/100 g fr. wt) was more than three times higher than that in leaves of 30- and 80-day-old plants (ca 1 mmol/100 g fr. wt), and twice as high as that in leaves of 60-day-old plants. Similarly, stems of 10- and 60-day-old plants contained higher concentrations of glucosides than those in stems of 30- and 60-day-old plants, but the differences were less

Table 1. Cyanogenic glucoside content in different organs of flax embryos 24 h after germination

	Cyanogenic glucoside			
	μmol/100 organs		mmol/100 g fr. wt	
Organ	Linustatin	Neolinustatin	Linustatin	Neolinustatin
Cotlyedons	1.20±0.11	0.73±0.09	0.21±0.02	0.13±0.02
Embryonic axes	0.35±0.08	0.16±0.04	0.47±0.11	0.22±0.06

±s.d. values indicated.

Table 2. Cyanogenic glucoside content in different organs of 10-day-old flax seedlings

	Cyanogenic glucoside			
	μmol/100 organs		mmol/100 g fr. wt	
Organ	Linamarin	Lotaustralin	Linamarin	Lotaustralin
Cotyledons + leaves	64.1 ± 9.6	25.7 ± 6.7	2.12 ± 0.30	0.88 ± 0.22
Stems	12.9 ± 3.0	8.1 ± 0.6	1.17 ± 0.28	0.73 ± 0.05
Roots	5.3 ± 1.3	2.4 ± 0.6	0.26 ± 0.06	0.14 ± 0.03

± s.d. values indicated.

Table 3. Concentration of cyanogenic glucosides in flowers and immature seeds of flax plants

	Cyanogenic glucoside mmol/100 g fr. wt			
Organ	Linamarin	Lotaustralin	Linustatin	Neolinustatin
Flowers	10.20 ± 1.41	4.24 ± 0.78	0	0
Immature seeds	1.90 ± 0.55	0.73 ± 0.19	0.61 ± 0.14	0.33 ± 0.09

± s.d. values indicated.

significant than for the leaves. The concentration of linamarin and lotaustralin in roots of 60-day-old plants (0.83 mmol/100 g fr. wt) was twice that of younger and older plants (Fig. 2).

Among the flax organs studied, the highest concentration of cyanogenic glucosides was found in the flowers (14.4 mmol/100 g fr. wt) (Table 3). This corresponds to a concentration of 360 μmol/100 flowers. Already formed, but still immature seeds, extracted from fruits on day 14 following anthesis, stored not only monoglucosides, but also both diglucosides, in lower amounts. However, the concentration of cyanogens in such seeds was much less than that in flowers (Table 3).

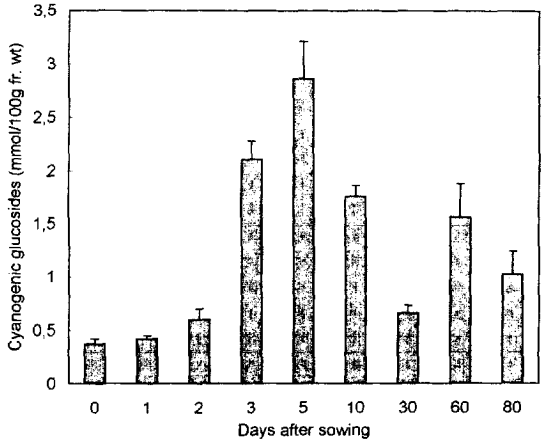

Fig. 3. Cyanogenic potential (sum of cyanogenic glucosides—mmol/100 g fr. wt) of whole flax plants at different stages of growth and development.

DISCUSSION

The cyanogenic potential (capacity to produce HCN [19]) changed during germination, seedling development and subsequent growth of flax plants (Fig. 3). The total amount of cyanogens in germinating seeds did not change significantly but it increased markedly during early seedling growth, especially at day 5, when the young leaves began to expand. After this stage, the HCN-potential in flax tissues decreased, and then it increased again before the onset of flowering (Fig. 3). These observations could indicate the involvement of cyanoglucosides in the control of some organogenetic processes. The highest concentration of cyanogens at these extremely important stages of development may also indicate that these very sensitive stages are specially protected against pathogens and herbivores. On the other hand, according to some opinions, cyanogenesis may be harmful to the plant, impairing the action of other defence mechanisms [20].

Changes in cyanoglycoside content during germination and seedling development of cyanogenic plants have also been reported earlier [21, 22]. During germination of highly cyanogenic seeds, the cyanogenic potential of seedlings may decrease rapidly, as shown in *Hevea* [2, 19], or remain constant, as demonstrated for lima beans (*Phaseolus lunatus*) [21]. On the other hand, acyanogenic or weakly cyanogenic seeds, like *Sorghum* [23] or *Lotus corniculatus* [24],

rapidly synthetise cyanoglucosides during germination. Similar changes in cyanogen content during development have been observed in several varieties of white clover plants; being highest at the start and end of the vegetative period [25].

As in cassava [22, 26], linamarin is the major monoglucoside in *Linum usitatissimum*. Lotaustralin, which is the main glucoside in *Trifolium repens* [15], occurs in flax in smaller amounts. Although the amount of monoglucosides in flax tissues fluctuates at different stages of development (Figs 1 and 2), the ratio of linamarin to lotaustralin is fairly constant. It may indicate, that both monoglucosides play a similar role during growth and development of a flax plant.

The suggestion of Selmar *et al.*, that diglucosides play the role of transport form of cyanogens [5, 27, 28] does not appear to be confirmed by the flax results. Diglucosides are found only in flax seeds (Fig. 1 and Table 3). This observation is in full agreement with the data of Smith *et al.* [18], who detected diglucosides only in flax seeds. Similarly, Oomah *et al.* [29] found diglucosides as the dominant cyanogens in seeds of 10 flax varieties and, in some varieties low amounts of the monoglucoside, linamarin. The concentration of cyanogens in flax seeds of variety LCSD 200 in the present study is twice as low as that observed by Oomah *et al.* The apparent absence of diglucosides in the vegetative parts of the plant, and the observation that the concentration of cyanogens in seeds was much lower than that in other tissues (Figs 1 and 2) does not support the hypothesis that diglucosides are involved in the transport of cyanogens in flax seed. Accumulation of diglucosides during flax seed maturation, observed by Frehner *et al.* [30] and also in this paper (Table 3), concerns another developmental phase and is not involved in the long-distance transport of cyanogens, postulated by Selmar *et al.* [27, 31].

On the other hand, it has been demonstrated earlier that cotyledons are the only site of cyanogen biosynthesis in flax [13]. Similarly, in emerging cassava seedlings, cyanogen biosynthetic activity is also confined to the cotyledons [22]. Therefore, one can assume that cyanogenic diglucosides are transported throughout the plant, and the absence of detectable amounts of diglucosides in parts of the flax plant, other than seeds, could be due to the activity of corresponding glucosidases in these organs.

EXPERIMENTAL

Plant material

Seeds of oil flax (*L. usitatissimum* L.) cv. LCSD 200 harvested in 1992 were received from S.H.R. Sobótka (Poland) and stored dry at 4° in darkness. Seeds were sown in lots of 50 in 9 cm diameter Petri dishes lines with 2 discs of filter paper moistened with 6 ml of H_2O and then incubated for 5 days under the following conditions: 16 h photoperiod, light/dark temp. 25°/16°, light intensity 150 μmol m^{-2} s^{-1}. Seven-day-old plants were grown in pots (14 cm diameter, 2–3 seedlings per pot) on garden soil. Plants were given H_2O twice a week and, alternatively, with modified Hoagland nutrient solution (pH 6.5).

Extraction and TLC of cyanogenic glucosides

Fresh plant material (0.2–0.1 g) was frozen in liquid N_2, ground to a fine powder in a mortal and pestle and extracted with 100 vols of boiling 80% aq. EtOH for 5 min. The slurry was filtered under vacuum through Whatman No. 1 paper (two layers, glass Buchner funnel) and the solvent evapd in a rotary evaporator at 40° to yield a syrup. The residue was redissolved in H_2O and extracted with $CHCl_3$ to remove lipids. The aq. phase was evapd *in vacuo* at 40° and the residue extracted with 2 ml of 10% aq. *iso*-PrOH. The extract containing cyanogenic glucosides was clarified by centrifugation, if necessary, and applied (10–60 μl) to TLC plates (cellulose MN300). Chromatograms were developed in H_2O satd *n*-BuOH [32]. R_f values of linustatin and neolinustatin were 0.12 and 0.21, respectively; linamarin and lotaustralin were 0.59 and 0.64, respectively. Cyanogenic glucosides were detected on chromatograms by a sandwich technique with Feigl-Anger testpaper [33], after spraying with soln of β-glucosidases in NaOAc buffer (0.1 M, pH 5.6). The minimum amount of HCN detectable was 10 nmol, 4 times higher than that of the method of Ref. [33].

Extraction of crude β-glucoside

Intact, dry seeds were ground with 5 vols of dry ice–Me_2CO, in a Waring blender for 1 min. The slurry was filtered under vacuum through Whatman No. 1 paper and the residue was re-extracted × 3 with cold Me_2CO. The resulting Me_2CO powder was left to air-dry for 1–2 h and then placed in a desiccator under vacuum to remove traces of Me_2CO. The Me_2CO powder was resuspended in NaOAc buffer (0.1 M, pH 5.6) (1:10 w/v) and stirred in an ice bath for 1 h. The slurry was spun off at 10,000 *g* for 20 min and the supernatant dialysed overnight against NaOAc buffer (0.1 M, pH 5.6). The resulting prepn (a crude source of β-glucosidase) was used for spraying chromatograms when analysing cyanogens [33] and for hydrolysis for cyanogenic glucosides eluted from chromatograms.

Quantitative analysis of cyanogenic glucosides

Cyanogenic glucosides were first separated by TLC and the zones of chromatograms corresponding with individual cyanogens were then removed and eluted with NaOAc buffer (0.1 M, pH 5.6). Glucosides were then hydrolysed with the β-glucosidase prepn. Incubation was carried out at 37° for 3 h with 0.2 ml enzyme (equivalent 6 seeds)—for monoglucosides; and for 21 h with 0.3 ml enzyme (equivalent 9 seeds)—for diglucosides. The amount of glc liberated was

determined in incubation mixts using the glucose oxidase (GOD) and peroxidase (POD) method [34], against a non-incubated blank. Each expt was performed using 2 to 3 replicates, except for immature seeds (Table 3), where only one replicate was used. Each value is a mean of at least 8 determinations. Standard deviation (s.d.) was calculated and its range is shown in tables and figures.

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REFERENCES

1. Eyolfsson R., *Fortschritte der Chemie Organisher Naturstoffe*, 1970, **28**, 74.
2. Lieberei R., Selmar D. and Biehl B., *Plant Systematics and Evolution*, 1985, **150**, 49.
3. Conn, E. E., *Encyclopedia of Plant Physiology*, 1979, **8**, 461.
4. Seigler, D. S., Conn, E. E., Dunn, J. E. and Janzen, D. H., *Phytochemistry*, 1979, **18**, 1389.
5. Selmar, D., Lieberei, R., Biehl, B., Nahrstedt, A., Schmidtmann, V. and Wray, V., *Phytochemisty*, 1987, **26**, 2400.
6. Jones, D. A., In *Cyanide in Biology*, ed. B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing. Academic Press, London, 1981, pp. 509–516.
7. Nahrstedt, A., *Plant Systematic and Evolution*, 1985, **150**, 35.
8. Taylorson, R. B. and Hendricks, S. B., *Plant Physiology*, 1973, **52**, 23.
9. Dziewanowska, K., Niedźwiedź, I., Chodelska, I. and Lewak, S., *Physiologie Vegetale*, 1979, **17**, 297.
10. Esashi, Y., Sakai, Y. and Ushizawa, R., *Plant Physiology*, 1981, **67**, 503.
11. Eck, H. V. and Hageman, R. H., *Crop Science*, 1974, **14**, 283.
12. Conn, E. E., *Naturwissenchaften*, 1979, **66**, 28.
13. Cutler, A. J. and Conn, E. E., *Archives of Biochemistry and Biophysics*, 1981, **212**, 468.
14. Butler, G. W., *Phytochemistry*, 1965, **4**, 127.
15. Collinge, D. B. and Hughes, M. A., *Journal of Experimental Botany*, 1982, **33**, 154.
16. Hahlbrock, K. and Conn, E. E., *Phytochemistry*, 1971, **10**, 1019.
17. Cutler, A. J., Sternberg, M. and Conn, E. E., *Archives of Biochemistry and Biophysics*, 1985, **238**, 272.
18. Smith, C. R., Weisleder, D. and Miller, R. W., *Journal of Organic Chemistry*, 1980, **45**, 507.
19. Lieberei, R., Nahrstedt, A., Selmar D. and Gasparotto, L., *Phytochemistry*, 1986, **25**, 1573.
20. Lieberei, R., Biehl, B., Giesemann, A. and Junqueira, N. T. V., *Plant Physiology*, 1989, **90**, 33.
21. Clegg, D. O., Conn, E. E. and Janzen, D. J., *Nature*, 1979, **278**, 343.
22. Koch, B., Nielsen, V. S., Halkier, B. A., Olsen, C. E. and Moller, B. L., *Archives of Biochemistry and Biophysics*, 1992, **292**, 141.
23. Bough, W. A. and Gander, J. E., *Phytochemistry*, 1971, **10**, 67.
24. Blain, H., *Pamiętnik Puławski*, 1975, **62**, 131.
25. Stochmal, A. and Oleszek, W. In *Szata roślinna Polski w procesie przemian*, ed. Z. Mirek and J. J. Wojcicki. TEXT, Kraków, 1995, p. 380.
26. McMahon, J. M., White, W. L. B. and Sayre, R. T., *Journal of Experimental Botany*, 1995, **46**, 733.
27. Selmar, D., Lieberei, R. and Biehl, B., *Plant Physiology*, 1988, **86**, 711.
28. Selmar, D., Irandoost, Z. and Wray, V., *Phytochemistry*, 1996, **43**, 569.
29. Oomah, B. D., Mazza, G. and Kenaschuk, E. O., *Journal of Agricultural and Food Chemistry*, 1992, **40**, 1346.
30. Frehner, M., Scalet, M. and Conn, E. E., *Plant Physiology*, 1990, **94**, 28.
31. Selmar, D., *Planta*, 1993, **191**, 191.
32. Nahrstedt, A., *Phytochemistry*, 1970, **9**, 2085.
33. Kakes, P., *Biochemical Systematics and Ecology*, 1991, **19**, 519.
34. Bergmeyer, H. U. and Bernt, E., in *Methods of Enzymatic Analysis*, 2nd edn ed. H. U. Bergmayer. Academic Press Inc., London, 1974, **3**, 1205.