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Analysis of the Developmental Regulation of the Cyanogenic Compounds in Seedlings of Two Lines of *Linum usitatissimum* L.

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Analysis of the developmental regulation of the cyanogenic compounds in seedlings of two lines of *Linum usitatissimum* L.

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Abstract: The developmental profiles and tissue distribution of the four cyanogenic compounds in seedlings of two developmentally contrasting inbred lines of flax (*Linum usitatissimum* L.) were examined using HPLC. During germination, the isoleucine-derived compound, neolinustatin, was hydrolysed faster in the more vigorous of the two lines. Furthermore, in this line, the neolinustatin content was higher in seeds and the accumulation of the other isoleucine-derived compound, lotaustralin, was also higher in the cotyledons of seedlings. In contrast, with one exception, the hydrolysis and accumulation of the valine-derived compounds, linustatin and linamarin, was the same in both lines. Differences in the levels of the compounds during germination, and in the hypocotyls, are interpreted as evidence for the involvement of transient levels of hydrogen cyanide in the autocatalytic regulation of ethylene production.

Key words: HPLC, germination, hypocotyl, neolinustatin, lotaustralin, ethylene.

Résumé : Dans les plantules de deux lignées autofécondées à développements contrastés de lin (*Linum usitatissimum* L.) et à l'aide de la chromatographie HPCL, les auteurs ont examiné les profils développementaux et la distribution dans les tissus de quatre composés cyanogènes. Au cours de la germination, la néolinustatine, un composé dérivé de l'isoleucine, s'hydrolyse plus rapidement dans la lignée la plus vigoureuse. De plus, dans cette lignée, la teneur en néolinustatine est plus élevée dans les graines et l'accumulation de l'autre composé dérivé de l'isoleucine, la lotaustraline, est également plus élevée dans les cotylédons des plantules. Au contraire, sauf une exception, l'hydrolyse et l'accumulation des composés dérivés de la valine, la linustatine et la linamarine, sont les mêmes dans les deux lignées. Les auteurs interprètent les différences des teneurs en composés au cours de la germination, et dans les hypocotyles, comme une preuve de l'implication du cyanure d'hydrogène dans la régulation autocatalytique de la production d'éthylène.

Mots clés : HPCL, germination, hypocotyle, néolinostatine, lotaustraline, éthylène.

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Introduction

Flax (*Linum usitatissimum* L.) is one of a group of species, including *Trifolium repens* (white clover), *Manihot esculentum* (cassava), and *Hevea brasiliensis* (rubber tree), that produces two cyanogenic monoglucosides, linamarin and lotaustralin. Most of the species in this group also produce the corresponding diglucosides, linustatin and neolinustatin, respectively. In these and other species with cyanogenic systems, tissue disruption results in the enzymatic hydrolysis of the cyanogenic compounds and the subsequent release of hydrogen cyanide (HCN) that is thought to act as a deterrent against herbivores (Kakes 1990). To protect intact tissues from the potentially detrimental effects of HCN, the cyanogenic compounds and their hydrolytic enzymes are spatially separated in different tissue or cell compartments (Frehner and Conn 1987; Kakes 1990; Conn 1991; Pancoro and Hughes 1992). Nevertheless, at some stages of development, such as germination, the cyanogenic compounds are hydrolysed in vivo, implying a secondary function that may relate to growth and development. One possibility is that the cyanogenic compounds in seeds serve as nitrogen storage compounds, and this nitrogen is released during germination for use in amino acid synthesis (Conn 1981; Selmar et al. 1988).

The cyanogenic monoglucosides, linamarin and lotaustralin, and the corresponding diglucosides, linustatin and neolinustatin, share common biosynthetic and catabolic pathways (Conn 1973; Cutler and Conn 1981; Cutler et al. 1985; Fan and Conn 1985; Hughes 1991; Koch et al. 1992). Although the acetone cyanohydrin in linustatin and linamarin is synthesized from valine and the methylethylketone cyanohydrin in neolinustatin and lotaustralin is synthesized from isoleucine, one set of microsomal enzymes is thought to control the biosynthesis of both cyanohydrins. Furthermore, a single glucosyltransferase mediates the conversion of both cyanohydrins to monoglucosides. The common catabolic pathway involves the sequential hydrolysis of the diglucosides linustatin and neolinustatin through the intermediate

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monoglucosides, linamarin and lotaustralin, respectively, to their cyanohydrins (Fan and Conn 1985). Two β-glucosidases catalyse this sequence. Linustatinase hydrolyses both diglucosides to their corresponding monoglucosides, and linamarase hydrolyses both monoglucosides to their corresponding cyanohydrins. The subsequent release of HCN from both cyanohydrins is also catalysed by a single enzyme. Albeit, the cyanohydrins are relatively unstable and HCN can be released spontaneously, especially at pH 7 or higher (Conn 1980). In flax, immature fruit contain only the monoglucosides, the proportion of diglucoside compounds increases during fruit maturation, and mature seeds contain only the diglucosides (Frehner et al. 1990).

The comparative, developmental study reported here examined the four cyanogenic compounds in seedlings of two inbred lines of flax that differ in growth rate, in plant size at maturity, and in the activity of the catabolic enzyme, linamarase (Fieldes and Gerhardt 2001). Previous research had demonstrated systematic changes in linamarase activity during germination and cotyledon expansion, the retention of a relatively constant level of activity (per hypocotyl) during hypocotyl elongation, and consistently higher linamarase activity during germination in the more vigorous of the two flax lines (Fieldes and Gerhardt 2001). Two aspects of the developmental profiles for linamarase activity in the flax lines delineated predictions about the role of the cyanogenic system in young seedlings. Firstly, linamarase activity increased during the first 12 h after the start of imbibition and then declined over the next 24-h period. It was postulated that this activity peak coincided with the release of nitrogen from the cyanogenic diglucosides stored in the seed. This interpretation predicts that the cyanogenic compounds in the seed are completely hydrolysed during germination, without accumulation of the monoglucosides, and that degradation profiles for the diglucosides may differ in the two lines, which develop at slightly different rates. Secondly, linamarase activity increased basipetally in the hypocotyl from no detectable activity in the top region to high activity at the bottom, suggesting that cyanogenesis in the lower region of the hypocotyl may protect seedlings from soil-associated pathogens. This interpretation of the activity gradient in the hypocotyl predicts that the cyanogenic compounds are also present in the lower region of the hypocotyl, but not in the upper region. To test these predictions, HPLC was used to quantify the cyanogenic compounds in germinating seedlings and seedling tissues of the two flax lines. The quantitative method was a modification of the method reported by Oomah et al. (1992). Preparative procedures were also established to provide purified samples of the three cyanogenic compounds that are not commercially available.

Materials and methods

The two inbred flax lines, L and S, were derived from a fibre cultivar, 'Stormont Cirrus', and are referred to as Durrant's large (L) and small (S) genotrophs. The origins, derivation, and phenotypes of these lines are described elsewhere (Durrant 1971). Two other flax lines, the oilseed cultivar 'Royal' (R) and the fibre cultivar 'Mandarin' (M), were also used in one aspect of the study. Seeds and seedlings were germinated and grown in glass Petri dishes lined with filter paper and supplied with deionized water. The experiments were grown under wide-spectrum growth lamps (model 20713, General Electric Co., Fairfield, Conn.; 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$, at seedling level) using a 16-h photoperiod and a constant temperature of 22–24 °C.

To examine the developmental profiles of the cyanogenic compounds, three samples of each line were prepared every 12 h, from 0 (unimbibed seed) to 84 h, using five seeds or seedlings for each sample. Separate Petri dishes were used for L and S and for each sampling time. Each sample was assayed three times. The data (averages of the three assays) were analysed using one-way analyses of variance and orthogonal comparisons to break down the main component (the 16 combinations of two plant lines and eight sampling times); Fisher's orthogonal polynomials were used to examine the regressions of cyanogenic content on time.

To examine the distribution of the cyanogenic compounds in the tissues of 6-day-old seedlings, L and S were grown together in each of two large Petri dishes (replicates). At day 6, ten seedlings of each line, per replicate, were cut into four sections: the radicle, the bottom and top halves of the hypocotyl, and the cotyledons. The sections from the 10 seedlings were pooled, resulting in two replicates of the eight combinations of plant line and tissue type. Hypocotyl samples from 9-day-old seedlings were obtained in the same way from a separate experiment. The HPLC data (averages of three assays) were analysed using two-way analyses of variance. All coefficients of variation used to assess variability were corrected for small sample size (Sokal and Rohlf 1981). The standard errors for means given in the figure legends were obtained from the error terms of the ANOVAs; the degrees of freedom for the error terms are indicated.

The seed and seedling extracts for HPLC analysis were prepared as follows: In step (*a*), each sample was frozen with liquid nitrogen, ground to powder in a mortar, dispersed in 40 mL of 80% MeOH, and sonicated for 30 min (Branson 1200 sonicator, Branson Ultrasonics Corp., Danbury, Conn.). Following centrifugation at 6000 r/min (1 r = 2π rad; 3000*g*) for 5 min, the supernatant was collected. In step (*b*), the supernatant was evaporated to dryness (using a rotary evaporator) and redissolved in 1mL mobile phase methanol:water : acetic acid (6:93.95:0.05). Internal standard (25 µL of 4 $mg \cdot mL^{-1}$ tertiary butyl alcohol in mobile phase) was added, and the sample was filtered (0.45 µm cellulose acetate Millipore filter, Millipore Corp., Bedford, Mass.) and stored at 4 °C in a sealed vial until analysis.

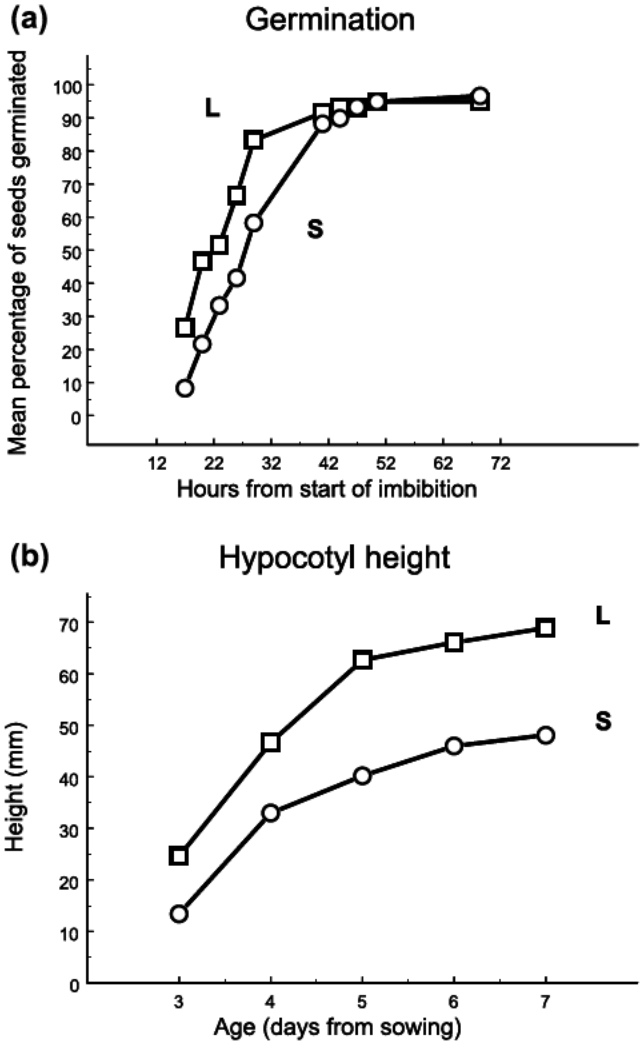
The HPLC system consisted of a Waters 510 pump (Waters Corporation, Milford, Mass.) and a Beckman 156 refractive index (RI) detector (Beckman Instruments Inc., Fullerton, Calif.) (sensitivity of 2×10^7 mv/Δn, where Δn is the differential RI) fitted with a three-way Hamilton valve that allows alternate flushing and filling of the reference and sample sides of the detector. The analytical column (Whatman 4.6 mm × 125 mm, 5 micron C-18, Whatman Inc., Clifton, N.J.) was wrapped with 1/16th inch (1 in = 25.4 mm) copper tubing and cross-connected to the detector heating ports on the RI detector. A thermal water pump (Haake, Thermo Electron Corp., Berlin) in a 20-L water bath maintained the column and detector at 24 °C. A Rheodyne injector (Rheodyne, Rohnert Park, Calif.) and a 20-µL loop were used in the analytical determinations. At a flow rate of

Table 1. Amounts (µg/5 seeds) of the two cyanogenic diglucosides in three samples of seed (1, 2, 3) from each of the two inbred lines (L and S) in *Linum usitatissimum* (flax).

	Linustatin			Neolinustatin		
Seed sample	Amount	SE	CV	Amount	SE	CV
L1	72.5			87.9		
L2	65.7			78.2		
L3	73.1			88.0		
Mean for L	70.4 (356.2)	2.37	5.8	84.7 (428.2)	3.25	6.7
S1	38.2			33.8		
S2	41.1			35.4		
S3	41.9			44.0		
Mean for S	40.4 (221.0)	1.12	4.8	37.8 (206.5)	3.17	14.5

Note: Samples were prepared from five seeds and assayed three times. Data are averages of the three assays; the means are averages of the three samples. Standard errors (SE) for means (n = 3) and coefficients of variation (CV) illustrate the variability among samples. Data in parentheses are conversions for mean content (µg/5 seeds converted to mg/100 g seed).

Fig. 1. Differences in germination and hypocotyl height between the L and S lines of *Linum usitatissimum* (flax). (*a*) Mean (n = 2) percentage of seeds germinated (two samples of 40 seeds per line); at 20 h, SE = 3.5, df = 2. (*b*) Mean (n = 4) hypocotyl height (four samples of five seedlings per line) at day 5, SE = 2.54, df = 3.



0.6 mL·min^{-1} of MeOH–H_2O–HOAc (6:93.95:0.05), the retention times were 5.4, 7.4, 9.5, 12.9, and 17.8 min for linamarin, linustatin, *t*-butyl alcohol, lotaustralin, and neolinustatin, respectively. A Hewlett Packard 3395 integrator (Hewlett-Packard Canada Ltd., Mississauga, Ont.) provided areas for elution peaks of the cyanogenic compounds in each sample. The amounts of the compounds (expressed as µg/5 seeds or seedlings) were determined using calibration curves obtained from purified samples of the four cyanogenic compounds. The calibration curves were all linear regressions (significant at $P < 0.01$). Calibration curves were obtained using commercially available linamarin (Sigma Chemical Co., St. Louis, Mo.) and purified samples (standards) of the other three cyanogenic compounds from the preparative method described below.

The HPLC elution peaks for linustatin and neolinustatin in seed extracts were initially identified using purified standards kindly provided by Dr. Palmer, South Dakota State University. Since no source of lotaustralin was available, the putative elution peak for lotaustralin was purified and its identity was confirmed using ^{1}H and ^{13}C nuclear magnetic resonance (NMR). NMR also demonstrated that our standards of linustatin, neolinustatin, and lotaustralin were more than 90% pure (Smith et al. 1980). None of the standards displayed any impurity peaks using the analytical method.

The extract used to purify linustatin and neolinustatin was obtained from six batches of 30 seeds. The initial extraction followed steps *a* and *b* described above, except that a second extraction was done on the residue remaining at the end of step *a*. The six batches of seeds were processed separately until the end of step *a*, when all solutions from the primary and secondary extractions were pooled. At the end of step *b*, the dried extract was redissolved in 5 mL of 10% MeOH. The extract used to purify lotaustralin was obtained in the same way using a single batch of 90 seedlings that had been germinated and grown in water for 48 h.

The preparative method used the HPLC system described above, an Altex 15 mm × 25 cm, 10 micron C-18 preparatory column (Beckman/Altex, Fullerton, Calif.), a Waters U6K variable volume injector (Waters Corporation), and, typically, 100-µL injections. The initial purifications were done using 10% MeOH as the mobile phase and a flow rate

Table 2. Differences between the L and S lines of *Linum usitatissimum* (flax) (*i*) during germination and early seedling growth, (*ii*) in the contents of the four cyanogenic compounds, and (*iii*) in the rates of changes in content with age.

Parameter measured	L	S	*F* value
% Germination at 20 h and hypocotyl elongation at day 5			
Germination (%) (n = 2)	46.7	21.7	$F_{[1,2]}$ = 25.00*
Hypocotyl height (mm) (n = 4)	59.6	43.3	$F_{[1,6]}$ = 14.02**
Regression mean (μg/5 seedlings) and regression coefficient (slope, μg·h^{-1})			
Linustatin (36–72 h)			
Mean (n = 4)	24.6±4.65	33.0±3.69	$F_{[1,32]}$ = 1.60ns
Slope	–1.6±0.35	–1.5±0.28	$F_{[1,32]}$ < 1.0ns
Neolinustatin (0–84 h)			
Mean (n = 8)	46.0±4.04	28.5±3.02	$F_{[1,32]}$ = 28.04**
Slope	–1.1±0.15	–0.6±0.11	$F_{[1,32]}$ = 18.81**
Linamarin (48–84 h)			
Mean (n = 4)	265±38.5	294±36.9	$F_{[1,16]}$ < 1.0ns
Slope	13±2.9	17±2.8	$F_{[1,16]}$ = 1.40ns
Lotaustralin (36–84 h)			
Mean (n = 5)	378±47.5	166±19.8	$F_{[1,20]}$ = 22.88**
Slope	21±3.1	10±1.4	$F_{[1,20]}$ = 13.85**
Regression means (μg/5 seedlings) and slopes (μg·h^{-1}) for exponential increases (36–84 h)			
Linamarin			
Mean (n = 5)	2.00±0.129	2.06±0.033	$F_{[1,20]}$ < 1.0
Slope	0.038±0.008	0.034±0.002	$F_{[1,20]}$ < 1.0
Lotaustralin			
Mean (n = 5)	2.26±0.138	1.94±0.044	$F_{[1,20]}$ = 9.59**
Slope	0.037±0.008	0.032±0.003	$F_{[1,20]}$ < 1.0

Note: *F* values from the analyses examine the difference between L and S. **, P < 0.01; *, P < 0.05; ns, not significant at P = 0.05.

of 2 mL·min^{-1}. For the seed extracts, retention times were 12–14 min for linustatin and 18–20 min for neolinustatin. Individual fractions for these times were collected from multiple injections, pooled, evaporated to dryness, redissolved in 5 mL MeOH–H_2O–HOAc (6:93.95:0.05), filtered through a 0.45-μm filter, and stored. Using the same column and procedure, the retention time for lotaustralin in the seedling extract was 16–18 min. This fraction was also collected from multiple injections, pooled, evaporated to dryness, redissolved in 5 mL, filtered, and stored.

Each compound was repurified using the same preparative column and MeOH–H_2O–HOAc as the mobile phase. A flow rate of 2 mL·min^{-1} MeOH–H_2O–HOAc (6:93.95:0.05) was used for linustatin (retention time 5–6 min) and for lotaustralin (retention time 22–24 min), and a flow rate of 3 mL·min^{-1} MeOH–H_2O–HOAc (7:92.95:0.05) was used for neolinustatin (retention time 15–16 min). For each compound, fractions collected from multiple injections were pooled, evaporated to dryness, further dried for 2 h using a vacuum pump, weighed, redissolved, and used for the calibration curves.

Results

Cyanogenic glucoside content in seed

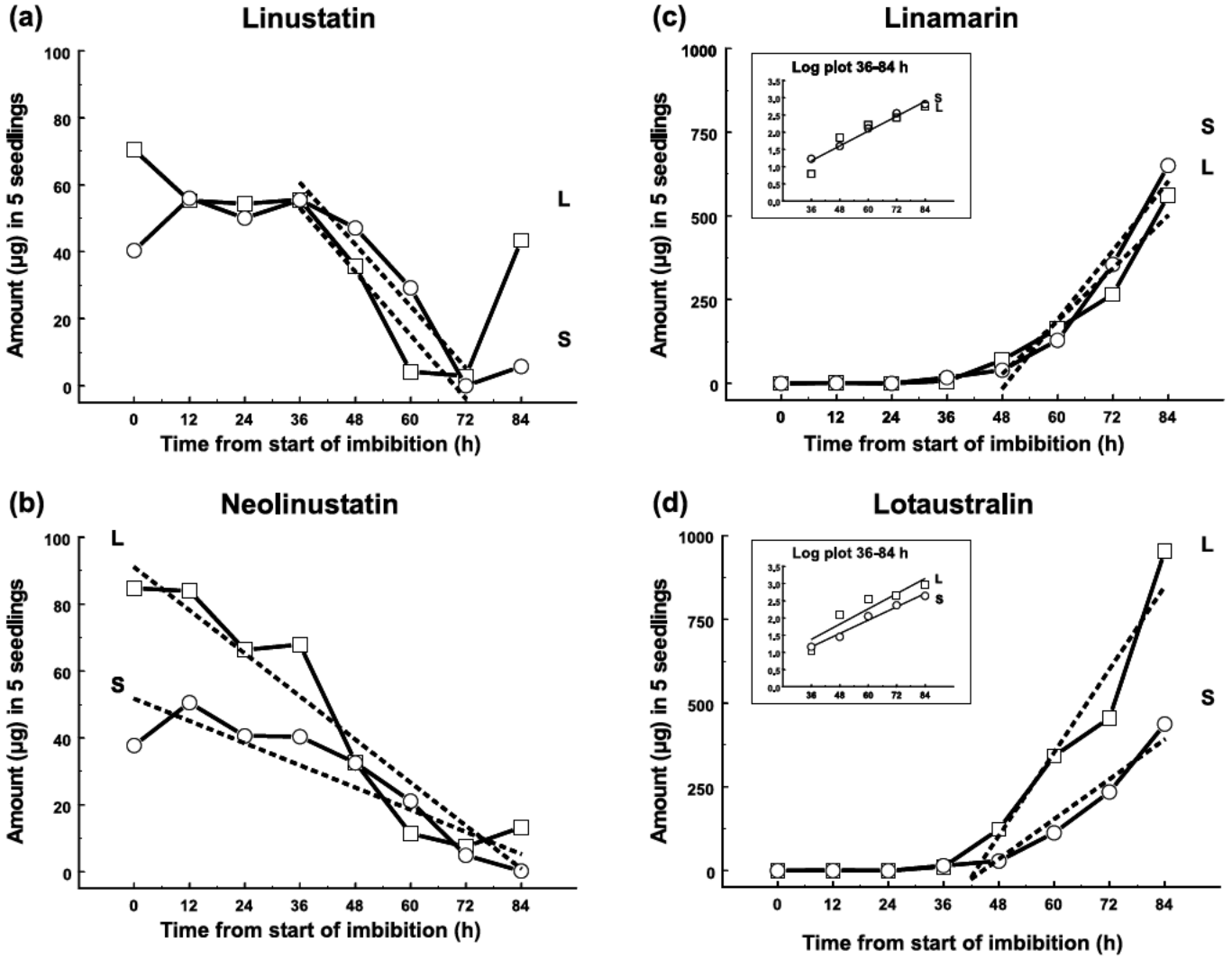
Seed samples contained only the diglucosides linustatin and neolinustatin, with no indication of either of the corresponding monoglucosides. For seed samples, the variability among the three samples for each line averaged 6.5%, and the total amount of the cyanogenic compounds in five seeds was approximately 155 μg for L and 78 μg for S (Table 1). Based on an average number of seeds per gram (253 for L and 273 for S), the estimated amount of the cyanogenic compounds in 100 g seed was 784 mg for L and 428 mg for S.

Developmental profiles for the cyanogenic compounds

The developmental profiles for the four cyanogenic compounds were obtained for the period from 0 to 84 h after the start of imbibition. This period is characterized by germination (16–40 h), which is faster in L than in S (Fig. 1*a*, Table 2), and by hypocotyl and radicle elongation, both also faster in L than in S (data not shown). At 72 h, the cotyledons are not fully expanded, but the seed coats have usually been discarded, and the hypocotyl, which is longer in L than in S (Fig. 1*b*) with lower fresh mass in L (Fieldes and Gerhardt 2001), is still elongating. In flax seedlings, the rate of elongation of the hypocotyl increases acropetally; upper regions of the hypocotyl elongate faster than lower regions (M.A. Fieldes, data not shown). Cotyledon fresh mass is 6%–7% lower in S than in L throughout development (Fieldes and Gerhardt 2001).

During the period from 0 to 84 h, the amount of cyanogenic diglucosides decreased (Figs. 2*a* and 2*b*) and the amount of cyanogenic monoglucosides increased (Figs. 2*c*

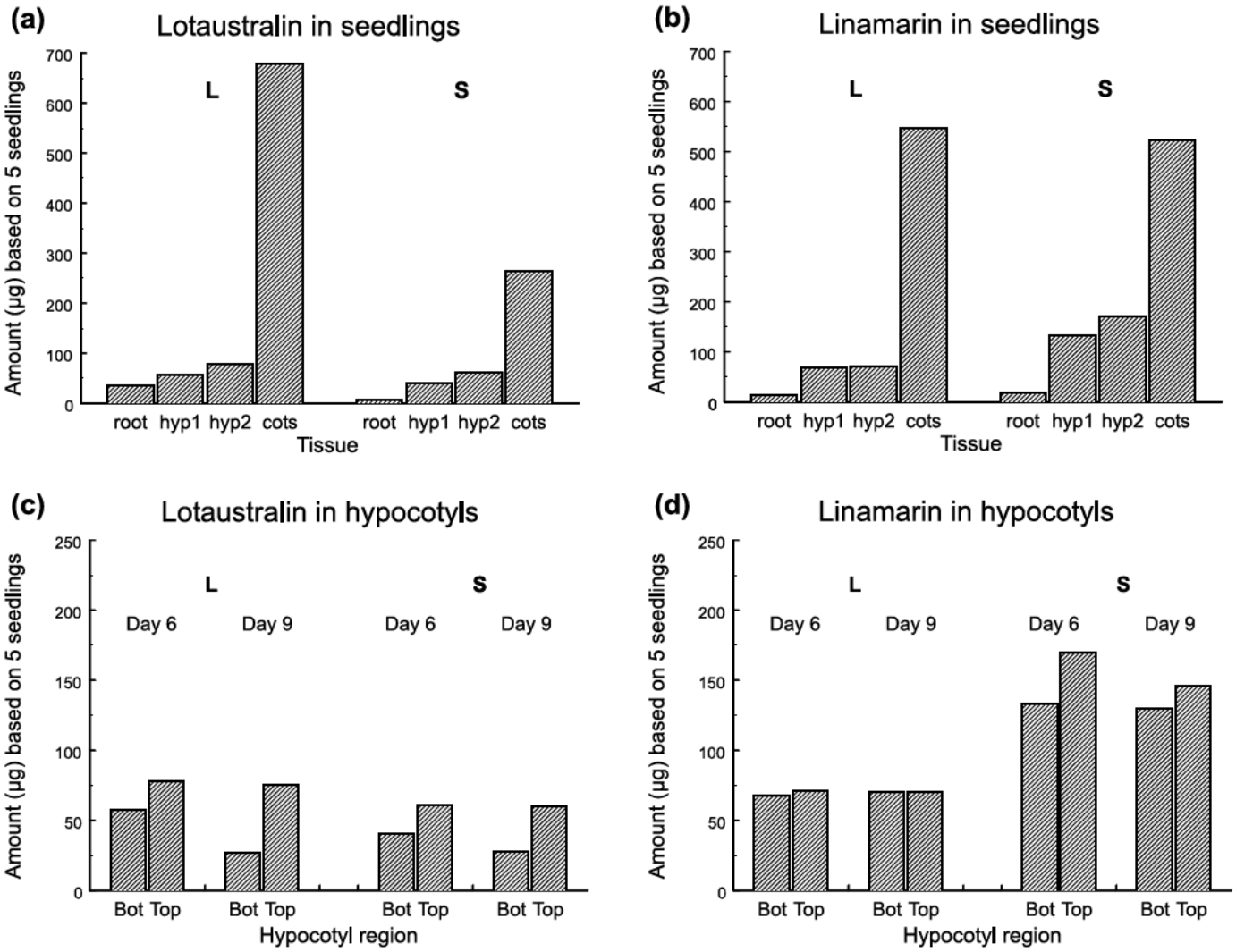
Fig. 2. Developmental profiles for the amounts (µg/5 seedlings) of the four cyanogenic compounds in seeds and seedlings of the L and S lines of *Linum usitatissimum* (flax). Line plots for the mean (n = 3) amount of (*a*) linustatin (SE = 6.7, df = 32), (*b*) neolinustatin (SE = 6.6, df = 32), (*c*) linamarin (SE = 64, df = 16), and (*d*) lotaustralin (SE = 70, df = 20) in five seeds or seedlings of L (squares) and S (circles) during the first 84 h of imbibition and regressions (dashed lines) fitted to the linear region of each plot.



and 2*d*). The linustatin profile was linear for L but quadratic for S (Fig. 2*a*), the neolinustatin profiles were linear for both L and S (Fig. 2*b*), and the profiles for linamarin and lotaustralin were all third-order regressions (Figs. 2*c* and 2*d*). In each profile, there was a period when the change in cyanogenic content was more or less linear. The data for these periods were reanalysed and the slopes and means of the regressions for L and S were compared. The linear regressions for the valine-derived compounds, linustatin and linamarin, were the same for L and S (Figs. 2*a* and 2*c*; Table 2). In contrast, the amounts of both isoleucine-derived compounds were higher in L than in S and the linear changes (decreases in neolinustatin and increases in lotaustralin) were faster in L than in S (Figs. 2*b* and 2*d*, Table 2). Semilogarithmic plots were examined for both linamarin and lotaustralin using the data for the period from 36 to 84 h (inserts in Figs. 2*c* and 2*d*, Table 2). Significant linear regressions of log(amount) against time demonstrated that the changes during this period were exponential and confirmed that the profiles for linamarin were identical in L and S. Nevertheless, although the rate of exponential increase in lotaustralin was the same in L and S, a difference in the regression means (Table 2) indicated that the increase in lotaustralin content began earlier in L than in S.

No monoglucoside content was detected in the seed samples. Trace amounts of these compounds were detected at 12 h (linamarin: L = 2.2 µg, S = 0; lotaustralin: L = 1.2 µg, S = 0.6 µg), but not at 24 h. By 48 h, the amounts of linamarin (L = 70 µg; S = 39 µg) and lotaustralin (L = 123 µg; S = 28 µg) were comparable to the amounts of linustatin and neolinustatin in the seed, even though the levels of the diglucosides had not decreased to zero. At 48 h, the total amount of the diglucosides had decreased by approximately 50% in L and 10% in S, relative to the initial amounts in seed. Nevertheless, 24 h later at 72 h, only trace amounts of the diglucosides remained (linustatin: L = 2.9 µg, S = 0 µg; neolinustatin: L = 7.6 µg, S = 4.9 µg). At 84 h, linustatin was detected in two samples of L (Fig. 2*a*)

Fig. 3. Distribution of (*a*) lotaustralin (SE = 45, df = 7) and (*b*) linamarin (SE = 86, df = 7) in the root, lower (hyp1) and upper (hyp2) hypocotyl, and cotyledons (cots) of 6-day-old seedlings of the L and S lines of *Linum usitatissimum* (flax) and comparisons of (*c*) lotaustralin (SE = 7.0, df = 7) and (*d*) linamarin (SE = 11.0, df = 7) in the bottom (Bot) and top (Top) halves of hypocotyls from 6- and 9-day-old seedlings.



and in one sample of S (17.2 µg); this observation cannot be explained without information on the period from 84 h to day 6.

Distribution of the cyanogenic compounds in seedling tissues

Linustatin was detected in one of the cotyledon samples of S (190 µg, 14% of the total content), but otherwise there were no indications of the cyanogenic diglucoside in any of the samples from 6-day-old seedlings or in the hypocotyl samples from 9-day-old seedlings. In 6-day-old seedlings, the majority of the total cyanogenic content, 79% for L and 64% for S, was located in the cotyledons, and 2% of the total content was found in the roots (Figs. 3*a* and 3*b*). In the cotyledons, the linamarin content was the same in L and S and the lotaustralin content was higher in L than in S (Table 3*a*). In 6-day-old seedlings, there were no significant differences in the cyanogenic content of the hypocotyl between L and S or between the top and bottom regions. Nevertheless, two trends in the data were unexpected. Firstly, contrary to our initial premise, there were indications of higher amounts of the cyanogenic compounds in the top, rather than in the bottom, of the hypocotyl. Secondly, in contrast to the situation in all other tissues where the linamarin content was the same in L and S, the linamarin content in the hypocotyl was higher in S than in L. To further examine these points, hypocotyl sections from 9-day-old seedlings were examined, and the hypocotyl data for both ages were compared (Figs. 3*c* and 3*d*, Tables 3*a* and 3*b*). There were no significant differences between the two ages, and analysis confirmed the significance of the trends seen in the data for 6-day-old seedlings. Except for the linamarin content in L, which was the same in both regions of the hypocotyl, the amounts of the cyanogenic compounds were

Table 3. Amount (μg/5 seedlings) of cyanogenic compound in tissues of 6- and 9-day-old seedlings in the L and S lines of *Linum usitatissimum* (flax).

(*a*) Tissue distribution and hypocotyl differences.

	L	S	*F* value[a]
Tissue distribution in 6-day-old seedlings			
Linamarin			
Cotyledons (*n*=2)	546	524	$F_{[1,7]} < 1.0$ns
Other tissues (*n*=6)	152	321	$F_{[1,7]} < 1.0$ns
Lotaustralin			
Cotyledons (*n*=2)	679	265	$F_{[1,7]} = 41.66$**
Other tissues (*n*=6)	171	109	$F_{[1,7]} < 1.0$ns
Hypocotyls of 6- and 9-day-old seedlings			
Linamarin (*n*=8)	70.1	141.4	$F_{[1,6]} = 83.38$**
Lotaustralin (*n*=8)	59.9	47.6	$F_{[1,6]} = 6.13$*

(*b*) Bottom vs. top of hypocotyl in 6- and 9-day-old seedlings.

	Bottom	Top	*F* value[b]
Linamarin (*n* = 4)			
L	69.0	70.1	$F_{[1,6]} < 1.0$ns
S	125.0	157.8	$F_{[1,6]} = 8.79$*
Lotaustralin (*n* = 8)			
L and S	38.8	68.8	$F_{[1,6]} = 36.68$**

Note: **, $P < 0.01$; *, $P < 0.05$; ns, not significant at $P = 0.05$.
[a]*F* values from the analyses examine the difference between L and S.
[b]*F* values from the analysis examine the difference between the bottom and top hypocotyl regions.

higher in the top than in the bottom of the hypocotyl, and the linamarin content was higher in S than in L.

The relationship between the valine-derived and the isoleucine-derived compounds

The detection of differences between L and S in isoleucine-derived compounds but not in the valine-derived compounds was unexpected because the two types of compound are thought to share common metabolic pathways. To further examine the relationship between the two types of compounds, the relative contributions of the isoleucine-derived compounds were examined (Table 4). Although the percentages of the isoleucine-derived compounds were usually higher in L than in S, the relationship between L and S changed during development. For example, the percentage of neolinustatin decreased during germination (0–48 h) and, because the decrease was faster in L than in S, the difference between L and S, which was significant at 12 h, was not significant at 48 h (Table 4*a*). When the percentage of neolinustatin or lotaustralin differed in the two lines, it usually reflected an increase in the amount of the isoleucine-derived compound in L. An exception was seen in the top of the hypocotyl, where the percentage of lotaustralin was higher in L than in S because of the combined effects of higher lotaustralin content and also lower linamarin content. The percentage of lotaustralin in the seed of L was higher than the percentage in those of S and higher than the percentages seen in the seed of two other flax lines, 'Royal' (R) and 'Mandarin' (M) (Table 4*b*). In this comparison of L, S, R, and M, the seed stocks used had been collected from four experiments grown in different years, whereas the seed stocks of L and S used in the other studies had come from a single experiment. The variability for samples from different seed stocks (Table 4*b*) was higher than the variability for different samples from the same seed stock (Table 1). Sampling variability was also higher for seedlings sampled at 48–84 h than for those sampled at other times (Table 4*a*). Data for the percentages of isoleucine-derived compounds were less variable than data for the cyanogenic compounds individually (Table 4).

Discussion

Modifications to the extraction and HPLC procedures

The method used by Oomah et al. (1992) was modified so that small samples could be used to quantify the amounts of the cyanogenic compounds in seeds and was extended to include analysis of the cyanogenic compounds in seedlings. The recovery of the cyanogenic content from five seeds was proportionally comparable to that obtained previously using 1 g of seeds (Oomah et al. 1992), and there did not appear to be a cost in terms of sample variability in using the smaller sample size. Increasing the ratio of the extraction volume to tissue mass and replacing a filtration step with centrifugation probably helped maintain the recovery levels. An excess of liquid N_2 was used in the first step of the extraction to dry the seedling tissues and ensure that all of the samples were extracted at the same methanol concentration. The combination of liquid N_2 and extraction in 80% methanol minimized the possibility of enzymatic degradation of the cyanogenic compounds during sample preparation. In the HPLC method, the mobile phase was acidified using acetic acid rather than phosphoric acid. Acetic acid improved pH control, sharpened the chromatographic peaks, and was important for the purity of the standards (preparative method); phosphoric acid leaves a residue, whereas acetic acid is readily volatilised. Tertiary-butyl alcohol was chosen as the internal standard because it is not a normal component of the plant material and has a retention time in the middle of the range of the HPLC elution peaks for the cyanogenic compounds.

Differential responses in the valine-derived and isoleucine-derived compounds

Three aspects of the developmental profiles demonstrated differences in cyanogenic content between L and S that occurred in the isoleucine derivatives but not in the valine derivatives. Seeds of L contained more neolinustatin than seeds of S, neolinustatin was hydrolysed faster in L than in S during early germination, and the biosynthesis of lotaustralin in seedlings began earlier in L than in S. These differential responses in isoleucine compared with valine derivatives were unexpected because, except for the initial precursors, the two types of derivative are thought to be synthesized in a common pathway (Cutler et al. 1985; Hughes 1991) and degraded by the same hydrolytic enzymes (Fan and Conn 1985). Furthermore, because the substrate specificity of purified flax linustatinase is higher for linustatin than for neolinustatin (Fan and Conn 1985), the differential hydrolysis of linustatin rather than neolinustatin might have been expected. Although the differential hydrolysis of neo-

Table 4. Difference between the L and S lines of *Linum usitatissimum* (flax) in the relative content of the isoleucine-derived cyanogenic compounds, neolinustatin and lotaustralin, at different developmental ages and in different tissues.

(*a*) Cyanogenic compound in tissue and stage.

Compound	L	S	*F* value[a]
% Neolinustatin in total cyanogenic diglucoside content (n = 3)			
Seed	54.7	48.2	$F_{[1,12]}$ = 3.77ns
Seedling (12 h)	60.3	47.3	$F_{[1,12]}$ = 14.84**
Seedling (48 h)	48.3	41.7	$F_{[1,12]}$ = 3.81ns
CV: linustatin = 8.2; neolinustation = 4.2; % neolinustatin = 6.2			
% Lotaustralin in total cyanogenic monoglucoside content (n = 3)			
Seedling (48 h)	56.9	42.3	$F_{[1,12]}$ = 33.00**
Seedling (60 h)	68.1	47.0	$F_{[1,12]}$ = 47.45**
Seedling (72 h)	64.6	39.2	$F_{[1,12]}$ = 68.90**
CV: linamarin = 40.0; lotaustralin = 39.6; % lotaustralin = 11.4			
% Lotaustralin in total cyanogenic monoglucoside content (n = 2)			
Cotyledons (day 6)	55.4	33.6	$F_{[1,9]}$ = 59.16**
Hypocotyl top (day 6)	52.3	26.5	$F_{[1,9]}$ = 82.42**
Hypocotyl top (day 9)	51.4	29.3	$F_{[1,9]}$ = 60.27**
CV: linamarin = 10.0; lotaustralin = 12.4; % lotaustralin = 3.2			

(*b*) % Neolinustatin in four sets of seed samples collected in different years (n = 4).

L	S	*F* value[a]	Royal	Mandarin	*F* value[b]
52.9	39.2	$F_{[1,9]}$ = 87.64**	35.1	44.7	$F_{[1,9]}$ = 42.61**
CV: linustatin = 36.1; neolinustation = 36.7; % neolinustatin = 5.5					

Note: Coefficients of variation (CV) illustrate the average variability among the means for monoglucoside content, diglucoside content, and percentage of isoleucine-derived compounds for the ages shown. **, $P < 0.01$; ns, not significant at $P = 0.05$.

[a] *F* values from the analyses examine the difference between L and S.

[b] *F* values from the analyses examine the difference between Royal and Mandarin.

linustatin during the initial 36 h of imbibition is as yet unexplained, differences between L and S in neolinustatin content and in the rate of neolinustatin degradation suggest that the activity of linustatinase is higher in L than in S. Thus, the peak of linamarase activity during this period and the higher linamarase activity in L compared with S (Fieldes and Gerhardt 2001) may be substrate concentration responses, dependent on the lotaustralin being produced from the neolinustatin.

Potential role for cyanogenic compounds in seeds during germination

The developmental profiles suggest that two distinct processes occur during germination and early seedling growth: the hydrolysis of the diglucosides and, after about 36 h, the de novo biosynthesis of the monoglucosides. The early degradation of the neolinustatin without accumulation of lotaustralin is consistent with the idea that the cyanogenic compounds in the seed have a secondary function during germination. Both the higher neolinustatin content in seeds and the faster rate of neolinustatin degradation in L compared with S indicate that this compound could participate in regulating the rate of germination and may mediate the earlier germination seen in L.

The role of the cyanogenic compounds in the hypocotyl

The distribution of cyanogenic compounds did not support the prediction that the cyanogenic system in the hypocotyl protects seedlings from soilborne pathogens. Nevertheless, the inverse relationship between linamarase activity (Fieldes and Gerhardt 2001) and cyanogenic content in the top relative to the bottom of the hypocotyl (Fig. 3) continues to suggest that the cyanogenic compounds have a secondary role in the hypocotyl. In flax seedlings, the cyanogenic compounds are only synthesized in the cotyledons (Cutler and Conn 1981). Thus, one possibility is that the inverse relationship occurs because these compounds are translocated to the hypocotyl, where they are hydrolysed by linamarase. In cassava, cyanogenic compounds produced in the cotyledons are translocated to the roots, where high levels of the monoglucosides accumulate (Selmar 1994). In cassava, however, it is the diglucosides that are translocated; unlike the monoglucosides, the diglucosides are not hydrolysed by the linamarase in the hypocotyl (Selmar 1994). In contrast, in flax seedlings, only relatively low levels of monoglucosides were detected in the root, and no diglucosides were detected in the hypocotyl (Fig. 3). Furthermore, the cyanogenic monoglucosides were present in the top of the hypocotyl (Fig. 3) even though they would be unable to serve a protective role

in this region, because it contains no detectable levels of linamarase activity (Fieldes and Gerhardt 2001).

The secondary roles of the cyanogenic system may relate to HCN production

It is difficult to reconcile the idea that the linustatin and neolinustatin contents of flax seeds are hydrolysed to provide nitrogen during the earliest stages of germination, given the large amounts of linamarin and lotaustralin that are synthesized shortly thereafter (Fig. 2). It is also difficult to imagine that the cyanogenic system serves two different secondary roles: one during germination and another in the hypocotyl. Therefore, the recent study of *Arabidopsis* is of particular interest (McMahon Smith and Arteca 2000). This study examined the effects of HCN treatments on the two enzymes (1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase) that catalyse the penultimate and ultimate steps of ethylene biosynthesis, respectively, and regulate the rate of ethylene production. HCN treatments increased transcription of an ACC synthase gene (*AC6*) and also of an ACC oxidase gene (*ACO1*) (McMahon Smith and Arteca 2000). The response to HCN was sensitive and rapid and, although the HCN produced during ethylene production is regulated by β-cyanoalanine synthase, it suggests that transient levels of in vivo HCN may mediate the autocatalytic biosynthesis of ethylene (McMahon Smith and Arteca 2000).

The nitrogen component of the HCN produced during hydrolysis of the cyanogenic compounds in flax and other species is recycled to asparagine (Conn 1991) by β-cyanoalanine synthase (Miller and Conn 1980; Selmar et al. 1988). Nevertheless, transient levels of this HCN may also upregulate ethylene biosynthesis. Ethylene is known to break seed dormancy and promote embryonic growth (Abeles et al. 1992) and to play a direct role in regulating germination in, for example, pea (Petruzzelli et al. 2000) and chickpea (Gómez-Jiménez et al. 2001). Furthermore, ethylene inhibits hypocotyl elongation in seedlings (Reid 1995). Thus, if HCN from the hydrolysis of cyanogenic diglucosides in flax seed mediates germination by mediating ethylene production, then the differences in neolinustatin content and hydrolysis between L and S could account for their different growth rates during germination. Furthermore, in seedlings, HCN-mediated ethylene production would be expected in the lower regions of the hypocotyl, rather than the upper regions, where it could inhibit elongation. In flax and in other cyanogenic plants, transient levels of HCN produced during the hydrolysis of cyanogenic compounds may be more important, in terms of regulating germination and hypocotyl elongation, than any potential use of these compounds as a nitrogen source for amino acid biosynthesis.

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