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OCCURRENCE OF LOTAUSTRALIN IN THE GENUS *HEVEA* AND CHANGES OF HCN-POTENTIAL IN DEVELOPING ORGANS OF *HEVEA BRASILIENSIS**

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; rubber tree; cyanogenesis; cyanogenic glucosides; linamarin; (*R*)-lotaustralin; HCN-potential; metabolism.

Abstract—The mean HCN-potential (HCN-p) of freshly collected seeds of *Hevea brasiliensis* is 104.8 μmol HCN per g dry weight. More than 90% of the cyanogenic compound is stored in the endosperm. During seedling development under aseptic conditions HCN-p of the entire seedling decreases to 15% within 19 days. The cyanogenic compounds are metabolized during germination to form noncyanogenic substances. Leaves of *H. pauciflora*, *H. benthamiana*, *H. pauciflora* × *H. guianensis* and *H. spruceana* contain both linamarin and (*R*)-lotaustralin, whereas lotaustralin was not detectable in leaves and seeds of *H. brasiliensis*.

INTRODUCTION

Studies of plant cyanogenesis have involved an analysis of the chemical character, distribution and metabolism of cyanogenic precursors in the plant. There exist data supporting the protective role of cyanogenic glycosides, usually against herbivores [1, 2], by liberation of HCN from the injured plant tissue, but primary roles of these secondary plant products in the intact, non-injured or non-infected plant cannot be excluded [3].

There are several observations indicating that cyanogenic glucosides function differently during germination of seeds. Weiss [4] reported liberation of HCN during development of the young apple seedlings suggesting that the cyanogens can act as allelopathic agents [5]; Clegg *et al.* [6] have shown that in germinating lima beans (*Phaseolus lunatus* L.) the cyanogenic glucosides are translocated from the storage tissue into the growing organs of the young plant; there was no loss of cyanogenic potential during seedling development and the content of the cyanogenic precursor linamarin per entire plant remained constant for at least 25 days. Thus, Clegg *et al.* [6] underline the protective character of the cyanogenic glucosides for the lima bean. Further observations show that cyanogenic glycosides are metabolized within the plant [7, 8]. HCN liberated during metabolism may be used for nitrogen supply via β-cyanoalanine [9, 10] and asparagine [11]. Such a process may also occur in germinating tissues but it has never been demonstrated with certainty.

In contrast to lima bean seeds where the reserves are stored in the cotyledons, the storage organ in *Hevea* seeds is not the embryo itself but is a large endosperm tissue which surrounds small, flat cotyledons. During germination the storage products of the *Hevea* seeds have to be translocated from the endosperm tissue into the embryo. As β-glucosidases which split cyanogenic glucosides of *Hevea* and liberate HCN are constituents of the cytoplasm and are found even in the intercellular spaces [12], it seems unlikely that linamarin could be transported without degradation. Therefore, the content of the cyanogenic precursor of *Hevea* seeds and its distribution in different organs of the seedlings during germination were investigated and are presented in this paper.

Seeds of *Hevea brasiliensis* are reported to contain the cyanogenic glucoside linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile [13]; lotaustralin (2*R*-β-D-glucopyranosyloxy-2-methylbutyronitrile) has not been detected. This is exceptional considering the large number of plant species and insects which contain both linamarin and lotaustralin [i.e. 14, 15] and does not fit with the finding that these two glucosides are synthesized by the same set of enzymes, at least in flax [16] and white clover [17]. Therefore, attempts have been made to see whether the various *Hevea* species are able to synthesize lotaustralin as well as linamarin.

RESULTS

HCN-*potential in seeds*

Intact, fresh seeds. The hydrocyanic acid potential (HCN-p) [18] of fresh *Hevea* seeds varied widely by from about 500 μmol HCN per seed to 50 μmol HCN per seed, but no seed was devoid of cyanogenic glycosides (Table 1). Calculated on a dry weight basis of 1.89 ± 0.43 g/seed, the mean content was 104.8 ± 52.7 μmol HCN per g dry weight.

Infected seeds. More than half of the *Hevea* seeds tested failed to germinate; such seeds were infected by different fungi. The fungal development in the seeds followed a typical colonization pattern. The first mycelial development was observed in the embryonic axis and in the cotyledons, followed by colonization of the endosperm within 3 days. All seeds showed the same degree of damage

* This paper contains parts of the "Habilitationsschrift" of R.L.

Table 1. HCN-potential of *Hevea brasiliensis*

Plant material	μmol HCN / organ	dry matter / organ	μmol HCN / g dry weight
Entire seeds	40 to 487 $\bar{x} = 187 \pm 112$	1.35 to 2.62 $\bar{x} = 1.9 \pm 0.4$	22.6 to 188.7 $\bar{x} = 104.8 \pm 52.7$
Infected seeds No. 1	38.8	1.85	20.9
2	0	1.85	0
3	0	1.63	0
4	0	1.23	0
5	0	0.86	0
6	0	0.71	0
		$\bar{x} = 1.4 \pm 0.5$	
Embryonic axis	0.53 ± 0.15	0.007 ± 0.001	76.8 ± 24.4
Cotyledons	21.9 ± 9.9	0.22 ± 0.01	100.2 ± 46.3
Endosperm	285.7 ± 131.4	1.31 ± 0.15	205.7 ± 105.8

The seeds were controlled for intactness before use. Intact seeds reveal a whitish, turgid endosperm and flat white to yellow-coloured cotyledons, whereas infected seeds contain dark yellow to brown endosperm tissue. Changes in colour are visible before any fungal mycelia is detectable. The variability of HCN-p in *Hevea* seeds is large. Seeds: $n = 25$; embryonic axis, cotyledons, endosperm $n = 6$.

in that the cotyledons had been infected profusely but the endosperm was almost free of mycelia. Six of the infected seeds were tested for their HCN-p. Though the infection had not spread throughout the tissue, only one of the six seeds revealed a residual HCN-p (Table 1). All others were devoid of cyanogens.

Distribution of HCN-*p within the seed.* The endosperm represents almost 85% of the seed dry matter and generally contains more than 90% of the cyanogenic precursors. On the basis of dry weight the endosperm tissue contains two to three times the HCN-p of the cotyledons (Table 1). However the HCN-p per g dry weight of the embryonic axis is quite similar to that of the cotyledons.

HCN-*potential in seedlings*

Hevea seedlings were analysed at four different growth stages for their HCN-p (Fig. 1). During the first three days of germination the HCN-p of the entire seedlings remains unchanged but is distributed throughout the whole plantlet. There is a loss of cyanogenic precursor content in the seed tissue itself and the yellowish rootlets and the red to green coloured stem contain about 16% of the total cyanogen (Fig. 2a). On a dry weight basis, the HCN-p in roots and in the stem is equal to that of the seed. A significant loss of total HCN-p per plant is seen after 14 days of germination and seedling development. Only a third of the seeds' content of cyanogenic glycosides remains in the entire plant (Figs 1 and 2).

After 19 days of germination the primary leaves of the seedlings are completely unfolded and the new flush of secondary leaves is produced. During this stage there is only a small residual HCN-p in the plant (Fig. 2a). The relative content of HCN precursor per dry weight is highest in the roots and almost equally low in the stem and in the leaves (Fig. 2b). Within 19 days of germination the HCN-p of developing seedlings of *H. brasiliensis* declines to values less than 15% of the seed.

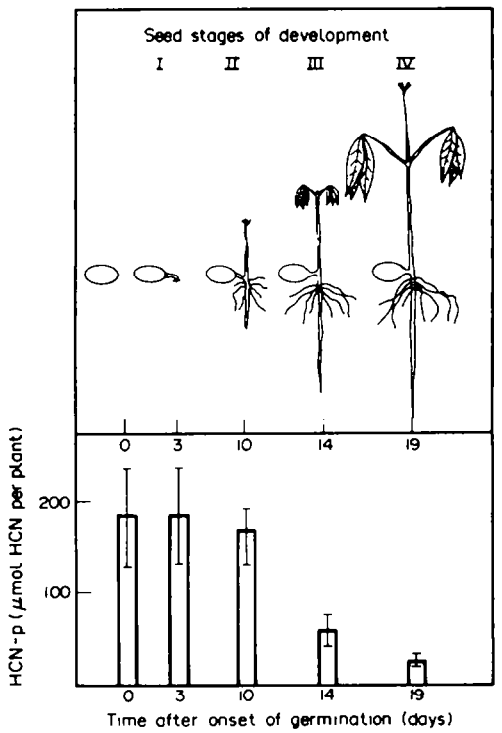

Fig. 1. Decline of HCN-potential during seedling development of *H. brasiliensis*. Seedling stages are: I, Hypocotyl and seedling root are visible. The hypocotyl is white to reddish. II, The main root and the first secondary roots are developed. The sprout emerged from the seed, length about 4 cm, colour reddish to green. The leaves are still folded. III, Primary leaves are unfolded, soft, reddish to green in colour, the bud for the next leaf flush is visible. IV, Primary leaves are hardened and turning to green. The leaves of the second flush are beginning to unfold. $n = 5$ for each stage.

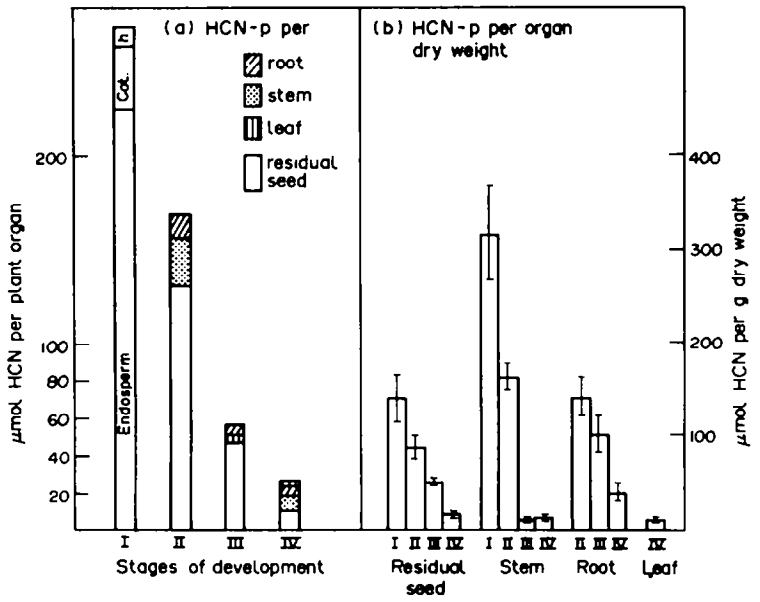

Fig. 2. HCN-potential of seedling organs of *H. brasiliensis*. For stages of development see Fig. 1. The initial value of HCN-p in stage I is higher than the mean value of HCN-p in seeds given in Table 1 because another seed population was used in this experiment. The data given represent the mean values for five individual plantlets tested per developmental stage. The variation between the different individuals are shown in Fig. 2b. The mean HCN-p of young leaf stages from grown up *Hevea* plants is about 288 µmol HCN/g leaf dry weight [25]. h = hypocotyl; cot = cotyledon.

HCN-*liberation in the course of germination*

Although there is a dramatic decrease in HCN-p during seedling development only a small amount of HCN was liberated during germination. This occurs first when adventitious rootlets begin to develop and again when the sprout extends from the seed. During this phase a slight injury of the petiole tissue of the cotyledons occurs, as indicated by the formation of small latex droplets in this area. HCN liberation was correlated with the occurrence of tiny wounds in the outer tissue layers of the seedling. However, the total amount of HCN liberated during germination and seedling development was at most only 18.1 nmol per seed, corresponding to only 0.0097% of the mean of 187.3 µmol HCN per fresh seed (Table 1). Therefore the drastic diminution of HCN-p which takes place during seedling development cannot be explained by the liberation of HCN from the germinating seed. In contrast to the pattern of HCN liberation just described for germinating *Hevea* seeds, HCN-liberation from fungally-infected seeds was strikingly different, starting with a loss of HCN directly at the beginning of the incubation which decreases drastically to zero within seven days (Fig. 3).

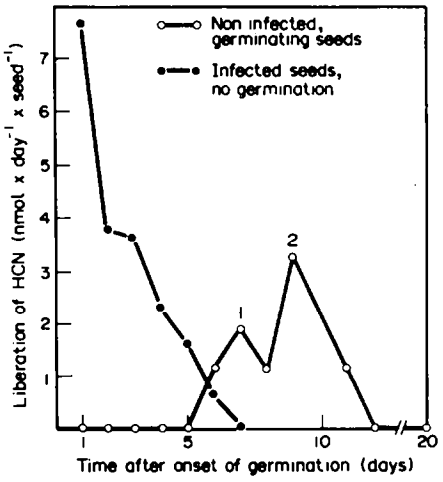

Fig. 3. HCN-liberation from seeds of *Hevea brasiliensis*. The data given here are from one representative experiment of a number of 12 experiments. Variation about a mean value from all twelve experiments are not shown as these would cover the two peaks of HCN liberation which coincide with the emergence of side roots and stem respectively, but which have been clearly visible for each single experiment. Depending on the start of germination the maximum of HCN liberation caused by emergence of side roots appeared between the 4th and 9th day, that caused by the stems between the 9th and 12th day. All infected seeds showed a remarkable decrease of HCN from the beginning of the experiment, which indicates that HCN was already lost before the onset of the experiment. 1, Side roots appear; 2, stem appears.

Cooccurrence of linamarin and (R)-*lotaustralin*

Samples of seeds and leaves of *Hevea* species were analysed by GC for linamarin and lotaustralin. Linamarin was present in both seed and leaves of *Hevea brasiliensis*, in leaves of *H. pauciflora*, *H. benthamiana*, *H. viridis*, *H. guianensis*, *H. pauciflora* × *H. guianensis*, *H. camargoana*, and *H. spruceana*, whereas lotaustralin was detected only in leaves of *H. benthamiana*, *H. pauciflora*, *H. pauciflora*

× *H. guianensis* and *H. spruceana* (Table 2). Moreover, the lotaustralin in these species was indeed low in that it did not exceed more than 3% of the total cyanogenic glucoside. Lotaustralin could not be detected either in the leaves or in the seeds of *H. brasiliensis* (limit of detection 0.5% of linamarin content). In order to confirm the presence of lotaustralin indicated by GC, leaf material of the four positive species was combined and a pure lotaustralin fraction was isolated. The ^{1}H NMR spectrum of the compound was identical to published data of lotaustralin [19]; (*R*) configuration at C-2 of lotaustralin was established by means of GC of the peracetates [20].

DISCUSSION

Hydrocyanic acid potential (HCN-*p*)

All seeds of *Hevea* examined in this study contained cyanogenic compounds. The amounts of cyanogenic material per seed varied greatly but were much larger than those reported by Butler [13] who observed 91 μg HCN per g fresh weight. This concentration corresponding to about 4–5 μmol HCN per g dry weight, is less than 5% of those encountered in this investigation. It is unlikely that the differences are due simply to individual variability. The seeds analysed by Butler [13] were obtained from the Madrid Botanical Garden, whereas the material used in the present study was freshly collected from Brazilian rubber plantations and germinated well.

Low content of cyanogenic glycosides in *Hevea* seeds may be due to fungal infection or due to prolonged storage of seeds. It is well known that *Hevea* seeds lose their capacity for germination rapidly when they are stored for several weeks [e.g. 21]. The loss of ability to germinate is a consequence of physiological and/or structural changes during storage. The seeds lose about 68% of water and about 90% of their cyanogenic potential during 4 weeks of storage under 'normal ambient conditions' (Ivory coast) [22]. Unfortunately Butler [13] gives no data about germination capacity or storage time of the plant material used in his study.

Cyanogenic glucosides

Our investigations support Butler [13] who detected only linamarin in seeds of *H. brasiliensis*. Furthermore, lotaustralin was not detectable in leaves of *H. brasiliensis*. Some other species, however, contained measurable amounts of lotaustralin in their leaves (Table 2); thus, the genus *Hevea* does not represent a biochemically unique group of plants which synthesize linamarin exclusively as suggested by Butler [13]. The lotaustralin of *Hevea* posesses the same configuration at C-2 as do all the 'lotaustralins' detected in nature and for which the (*R*)-configuration has been established; one exception is the mixture of (*R*)-lotaustralin and (*S*)-epi-lotaustralin isolated from seedlings of *Triticum monococcum* [23]. Other plants and Lepidoptera containing these two cyanogenic glucosides show a large variability in their linamarin:lotaustralin ratio [13–15]. There are indications that the ratio is influenced by the supply of the biogenetic precursors and/or that the precursors are utilized with different effectiveness by the synthesizing system [24]. Thus, the low content of lotaustralin in the four *Hevea* species investigated and its absence in *H. brasiliensis* seed may indicate an inadequate supply of isoleucine for the synthesizing enzyme system.

Metabolism of cyanogenic glycosides

During germination and plantlet development a drastic decline in HCN-p occurs in seedlings, which is not compensated by an increase in the HCN-p of roots, stem and leaves. On the other hand no significant amount of HCN was released to the surrounding atmosphere. Only during appearance of the side roots and during development of the stem was a small amount of HCN liberated (less than 0.01% of the total HCN-p). At this time tiny injuries of the epidermal and subepidermal tissues were observed which may be responsible for HCN liberation. As the germinating seeds were grown aseptically, any diminution of HCN by microbial activities can be excluded.

Physiological significance

The results discussed above indicate that in the case of *Hevea* the cyanogenic substances do not function primarily as protective substances against herbivores or microbial attack at least during the germination phase. More than 85% of the cyanogenic glycosides disappear presumably in order to recycle the nitrogen in the growing plant. Furthermore the newly formed, thin-walled primary leaves, which offer no structural barrier against attacking organisms, reveal only a very low HCN-p, i.e. less than 4% of that of the young leaf stages of adult *Hevea* plants [25]. It is unlikely that the concentration of a protective substance would be reduced so drastically at this stage in leaf development. Therefore these results indicate that cyanogenic substances may function in *Hevea* as storage compounds for protein synthesis [26].

EXPERIMENTAL

Freshly collected *Hevea* seeds were received from the National Brasilian Rubber and Oilpalm Research Center in Manaus, Amazonas. Immediately after arrival the seeds were washed, the hard seed cover was removed and the seeds were placed into moistened peat culture substrate or in Vermiculite (Deutsche Vermiculite Dämmstoff GmbH, 4322 Sprockhövel 2) for germination. Two weeks after germination the seedlings were fertilized with Hoagland nutrition soln twice a week [27]. The plants were grown at 90% r.h. in a glasshouse at 24°. For the analysis of HCN liberated during germination the seeds were surface sterilized directly after arrival. The hard seed cover was removed under sterile conditions and the seeds were placed into sterile 500 ml flasks containing 80 ml 0.8% agar (Oxoid No. 3) adjusted to pH 5.0 with HCl. A constant stream of prewashed sterile air (80 ml/min) was led through the flasks into 1 ml of 0.1 M NaOH for trapping the HCN. The NaOH was changed at 24 hr intervals. HCN was determined using the Merck spectroquant kit for cyanide, according to the Merck data sheet 'cyanide 130 259 8 Do dt/5r' (Merck, Darmstadt, FRG). This method is based on the formation of König's salt [28].

Estimation of the HCN potential was performed as follows: The plant samples were homogenized with prechilled Na_2HPO_4 (0.067 M), using 4 ml soln per 1 g of plant material. Under these conditions no loss of HCN from the samples was observed during homogenization, as was confirmed by comparing this method with the H_3PO_4-homogenization method used by Cooke [29]. In order to hydrolyse the cyanogenic glucosides, aliquots of the

Table 2. Content of HCN-p and linamarin:lotaustralin ratio of leaves of four *Hevea* species in which lotaustralin was detected. Leaves of *H. spruceana* have been obtained air dried; the linamarin:lotaustralin ratio was not measured exactly.

Species	Clone	μmol CN^-/g d.m.	% ratio linamarin:lotaustralin	
H. benthamiana	F 4512	113	97.2	2.8
H. pauciflora	PA 31	71	97.8	2.2
	P 10	89	99	1
	PUA 5	102	99	1
H. pauciflora × *guianensis*	PUA 7	334	98.6	1.4
H. spruceana (air dried)		~10	++++	+

d.m. = dry matter.

homogenates were incubated in Thunberg vessels. The incubation mixtures consisted of 0.05 ml plant homogenate, 0.45 ml of NaH_2PO_4 (0.067 M), 0.1 ml *Hevea*-β-glucosidase in McIlvaine phosphate–citrate buffer pH 5.5 (activity of about 0.22 μmol *p*-nitrophenol liberated per min in the assay described in ref. [30]). The incubation for enzymatic cleavage of the cyanogenic precursor was run at 30° for 20 min. The reaction was stopped by adding 0.6 ml 0.2 M NaOH which had been placed in the side tube of the Thunberg vessel before the assay was started. Aliquots of the stopped incubation mixture were used for HCN estimation after neutralization as described above. Dry wt was estimated after continuous drying of plant samples at 85° to constant wt.

Isolation and identification of the cyanogenic glucosides. The plant material (leaves, seeds) was freshly collected, powdered in liquid N_2 and subsequently freeze dried. The material was defatted with petrol and extracted with an azeotropic mixture of MeOH and EtOAc. This extract was taken to dryness and the residue chromatographed on silica gel (2.5 × 40 cm) using MeOH–CH_2Cl_2 (3:17) as an eluant. Cyanogenic fractions were monitored using β-glucosidase from *Hevea* leaves [31] and Feigl–Anger paper test strips [32]. The cyanogenic fractions were combined, taken to dryness and chromatographed by HPLC on RP-18 (1.6 × 25 cm) with H_2O–MeCN (19:1) (flow rate 5 ml/min) using an RI-detector. Linamarin (R_t *ca* 18 min) and lotaustralin (R_t *ca* 42 min) were collected separately and taken to dryness. The lotaustralin fraction was chromatographically pure and used to obtain a ^1H NMR spectrum in Me_2CO-d_6 (400 MHz, Bruker-WM-400). (*R*)-Configuration at C-2 was established by GC of the peracetate [20] on silar-10C (12% on gaschrom Q 100–120 mesh, 1 m × 2 mm, glass; N_2 as carrier gas, 25 ml/min; 240° isothermally, FID), R_t of (*S*)-epi-lotaustralin peracetate: 45 min, R_t of (*R*)-lotaustralin peracetate: 50.7 min.

Estimation of linamarin:lotaustralin ratio. The lyophilized plant material was defatted with petrol and extracted with an azeotropic mixture of MeOH and EtOAc. An aliquot of the extract was taken to dryness, dissolved in 0.1 ml of dry pyridine and silylated with 0.1 ml *N,N*-bis-trimethylsilyltrifluoroacetamide and 0.05 ml trimethylchlorosilane. Several μl of the soln were injected into a GC system: OV-101 3% on chromosorb AW-DMCS 80–100 mesh, 180 × 0.2 cm, glass; N_2 as carrier: 25 ml/min; 155–230°, 2°/min; R_t of TMS-linamarin 21.9 min, R_t of TMS-(epi)lotaustralin 24.3 min. The ratios of the two compounds was calculated from the area of their signals.

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