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PLANT CYANOGENESIS OF *Phaseolus lunatus* AND ITS RELEVANCE FOR HERBIVORE–PLANT INTERACTION: THE IMPORTANCE OF QUANTITATIVE DATA

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Abstract—Quantitative experimental results on the antiherbivorous effect of cyanogenesis are rare. In our analyses, we distinguished between the total amount of cyanide-containing compounds stored in a given tissue [cyanogenic potential (HCNp)] and the capacity for release of HCN per unit time (HCNc) from these cyanogenic precursors as a reaction to herbivory. We analyzed the impact of these cyanogenic features on herbivorous insects using different accessions of lima beans (*Phaseolus lunatus* L.) with different cyanogenic characteristics in their leaves and fourth instars of the generalist herbivore *Schistocerca gregaria* Forskål (Orthoptera, Acrididae). Young leaves exhibit a higher HCNp and HCNc than mature leaves. This ontogenetic variability of cyanogenesis was valid for all accessions studied. In no-choice bioassays, feeding of *S. gregaria* was reduced on high cyanogenic lima beans compared with low cyanogenic beans. A HCNp of about 15 μmol cyanide/g leaf (fresh weight) with a corresponding HCNc of about 1 μmol HCN released from leaf material within the first 10 min after complete tissue disintegration appears to be a threshold at which the first repellent effects on *S. gregaria* were observed. The repellent effect of cyanogenesis increased above these thresholds of HCNp and HCNc. No repellent action of cyanogenesis was observed on plants with lower HCNp and HCNc. These low cyanogenic accessions of *P. lunatus* were consumed extensively—with dramatic consequences for the herbivore. After consumption, locusts showed severe symptoms of intoxication. Choice assays confirmed the feeding preference of locusts for low over high cyanogenic leaf material of *P. lunatus*. The bioassays revealed total

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losses of HCN between 90 and 99% related to the estimated amount of ingested cyanide-containing compounds by the locusts. This general finding was independent of the cyanogenic status (high or low) of the leaf material.

Key Words—Cyanogenesis, chemical defense, *Phaseolus lunatus*, *Schistocerca gregaria*, herbivory, cyanogenic potential, cyanogenic capacity, plant–herbivore interactions.

INTRODUCTION

Cyanogenesis is widespread in the plant kingdom (Møller and Seigler, 1999). Over 2500 plant species are known to be cyanogenic and have the capacity to release gaseous HCN from endogenous cyanide-containing compounds, generally cyanogenic glycosides (Jones, 1988; Poulton, 1990; Seigler, 1998; Gleadow and Woodrow, 2002). Cyanogenic glycosides are β-glucosides of α-hydroxynitriles derived from protein and nonprotein amino acids (Zagrobelny et al., 2004). Hydrogen cyanide is toxic to the plant itself. Thus, cyanogenic plants must be able to synthesize and accumulate this toxin as inactive precursors to prevent autotoxicity (Vetter, 2000). Cyanogenic glycosides are separated in the intact plant from one or more specific β-glucosidases that are localized in the apoplast. In addition to separation on the cellular level, the cyanogenic component and the enzyme can be located in different tissues (Thayer and Conn, 1981; Frehner and Conn, 1987; Poulton, 1988; Swain et al., 1992). In case of injury, the β-glucosidase is brought into contact with its substrate. By hydrolysis of the cyanogenic glycosides, α-hydroxynitriles are formed that are relatively unstable and dissociate either spontaneously or are enzymatically accelerated by an α-hydroxynitrile lyase into HCN and an aldehyde or a ketone (Conn, 1980; Hösel and Conn, 1982; Poulton, 1990; Poulton and Li, 1994; Swain et al., 1992).

Hydrogen cyanide is toxic because of its affinity to the terminal cytochrome oxidase in the mitochondrial respiratory pathway and a number of other reactions (Solomonson, 1981; Brattsten et al., 1983). Despite the obvious toxicity of the products of plant cyanogenesis, its role in the scope of plant–herbivore interaction is very complex (Lieberei, 1988, 1989). A range of aspects determines its effectiveness as a mechanism of plant defense. The potential of cyanogenic glycosides as feeding deterrents depends on their concentration in the host plant. The concentration is often highly variable in natural populations. In addition, its phenotypic appearance depends on environmental conditions, the organ and tissue, and its ontogenetic status (Hughes, 1991; Bokanga et al., 1994; Schappert and Shore, 1999a,b; Gleadow and Woodrow, 2000b). If the concentration of cyanogenic precursors is below threshold toxicity, herbivores might not be affected (Feeny, 1976; Gleadow and Woodrow,

2000a). Herbivores can also avoid threshold toxicity by feeding on cyanogenic plants only as part of a mixed diet (Provenza et al., 1992). Furthermore, different herbivores are influenced in different ways by the presence of cyanogenic precursors because of their mode of feeding. Insect herbivores with sucking mouthparts such as phloem feeders (e.g., aphids) cause minimal tissue disruption during the feeding process and, therefore, avoid the release of toxic HCN or even reach tissues with low concentration of cyanogenic precursors depending on the length of their stylus (McMahon et al., 1995). A further important aspect is the degree of adaptation of the particular herbivore to its cyanogenic host. Specialist herbivores have evolved mechanisms to face cyanogenic glycosides (Provenza et al., 1992). Some use the cyanide as source of nitrogen or for their own defense against predators (Schappert and Shore, 1999b; Engler et al., 2000). For such herbivores, cyanogenic glycosides sometimes act as phagostimulants instead of feeding inhibitors (Calatayud and Le Rü, 1996; Mowat and Clawson, 1996). Thus, cyanogenesis may act as plant defense against generalist herbivores, but the effectiveness depends on many factors on both sides of the plant–animal interaction (reviewed by Gleadow and Woodrow, 2002).

Numerous authors have reported observations for defense functions of plant cyanogenesis against herbivores (e.g., Nahrstedt, 1985, 1988; Hruska, 1988; Jones, 1962, 1988, 1998; Crush and Caradus, 1995; Caradus and Forde, 1996; Schappert and Shore, 1999b,c; Tattersal et al., 2001). However, clear experimental results considering quantitative aspects of the action of HCN as causal factor of plant defense are rare (Feeny, 1976; Patton et al., 1997; Gleadow and Woodrow, 2000a,b). Analyses are complicated by the fact that the concentrations of cyanogenic precursors vary within a single plant and in relation to the ontogenetic status of the organs. For example, the concentration of cyanogenic precursors in leaves of different age differed by up to 90%. Furthermore, quantitative and kinetic analysis of the capacity for release of hydrogen cyanide as reaction to tissue disrupture—the basic measure of plant cyanogenesis—is often neglected. Qualitative statements concerning the release of HCN such as "yes" or "no" or semiquantitative test paper methods are insufficient to describe the complexity of cyanogenesis in interaction with a herbivore.

In view of these gaps, the goal of our study was to investigate quantitatively whether or not and how plant cyanogenesis interacts with the feeding behavior and feeding damage invoked by a generalist herbivorous insect. Furthermore, it has to be considered that deterrent properties of cyanogenic plants could depend on the total amount of bitter-tasting, cyanide-containing compounds stored in a given tissue [cyanogenic potential (HCNp)] or the amount of toxic hydrogen cyanide which can be released per unit time [cyanogenic capacity (HCNc)] or both.

For this, we designed a series of no-choice feeding experiments and additional binary-choice bioassays using lima beans (*Phaseolus lunatus*) as cyanogenic plants and the fourth instars of the African locust *Schistocerca gregaria* as generalist herbivores. Using this model system, we measured the HCNp of every leaf used in the bioassays and, in addition, the release of plant hydrogen cyanide per unit time during the course of feeding as response to damage of the leaf material. In this way, we obtained precise quantitative information on the action of plant hydrogen cyanide.

METHODS AND MATERIALS

Plants. P. lunatus seems to be an obligate cyanogenic plant. Extensive screenings of wild and weedy forms and cultivated genotypes revealed no acyanogenic forms (Baudoin et al., 1991). We tested seven defined *P. lunatus* accessions for resistance: L 2357 [origin: Spain; seed color: white/brown/violet, speckled; 100 grain weight (gw): 149.99 g], L 2233, wild type (origin: Cuba; seed color: gray/brown/black; 100 gw: 8.91 g), L 1315 (origin: Peru; seed color: white; 100 gw: 267.73 g), L 1259 (origin: unknown; seed color: brown/red/black, speckled; 100 gw: 82.39 g), L 2441 (origin: Bulgaria; seed color: white; 100 gw: 134.21 g), L 8079 (origin: unknown; seed color: white; 100 gw: 42.65 g), and L 8071 (origin: unknown; seed color: auburn; 100 gw: 45.23 g). In the following, only the numbers are used for specification of the accessions (2357, 2233, 1315, 1259, 2441, 8079, and 8071). Seed material was provided by the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben, Germany.

In addition to these plants, the almost noncyanogenic *Phaseolus vulgaris* L. cultivar "Saxa" was used as control. This cultivar with the stock number 40.176 was purchased from Carl Sperling & Co., Lüneburg, Germany.

Growing and Treatment of Plants. To work with homogenous, biochemically well-defined plant material, single plants of the respective accessions were cultivated and vegetatively propagated as clones. The clones were cut as one node cutting from the mother plant, rooted in water, and, after a 2 wk rooting phase, transferred into standard substrate (TKS®-1-Instant, Floragard®), which was mixed with one third sand of a grain size of 0.3–0.7 mm and one third sand with a grain size of 1–2 mm. Plant containers with a diameter of 18 cm were used.

Between July and August, clonal collections of at least 30 plants were prepared per accession. Plant material was cultivated under greenhouse conditions at a light regime of 16:8-hr light/dark by a photon flux density of 400 μmol s^{-1} m^{-2} at the plant container and 900 μmol s^{-1} m^{-2} on the top of the plants, respectively. Accessional light was provided by 400-W high-pressure

sodium lamps with plant-grow broad-spectrum fluorescent bulbs (Son-Targo 400, Philips®). Temperature in the chamber was 30/20°C in a 16:8-hr light/dark period and ambient relative air humidity (range, 60–70%). Climatic conditions and irrigation of the plants were computer-based and controlled by INTEGRO® software. Plants were fertilized with a nitrogen-phosphate fertilizer (Blaukorn®-Nitrophoska®-Perfekt, Compo GmbH) twice a week.

Leaf material was classified by morphological parameters of development into the three classes "young," "intermediate," and "mature." In addition, the insertion position of the leaves at the stem was documented. Leaves at the apex or a side stem that were unfolded for at least 3 days were classified as "young." These leaves did not exceed one third to one half of the final leaf size. Leaves that were located one to two leaf insertion positions below the category of "young" leaves were defined as "medium" leaves. Leaves assigned to this category were not completely expanded and still showed thin and delicate leaf tissue. "Mature" leaves were located at the stem at least two insertion positions below the leaves that were classified as "medium." These leaves were characterized by a dark green color and a hardened midrib, and were always completely expanded.

The occurrence of pests and diseases was controlled, and the harvest of leaf material of a particular plant was followed by at least 4 wk until the next harvest of leaves to minimize possible effects of various induced defense mechanisms known from the Fabaceae (Fischer et al., 1990; Liu et al., 1992; Underwood, 1999; Underwood et al., 2000; Heil, 2004).

Insects. S. gregaria is a polyphagous insect and an economic pest throughout many regions of Africa (Mainguet et al., 2000). Locusts were provided by the Biocenter Grindel and Zoological Museum (University of Hamburg) and were held under the same conditions as the plants. They were offered a diet consisting of leaf material of different Poaceae (*Poa annua* and *Agropyron repens*) together with fruits (apple and banana). The fourth instars were used as herbivores.

Cyanogenic Features of Plants. Plant cyanogenesis requires at least two basic physiological properties: the ability to synthesize and accumulate cyanide-containing compounds [the cyanogenic potential (HCNp)] and the capacity to release hydrogen cyanide from these endogenous compounds [the cyanogenic capacity (HCNc)]. Therefore, release of HCN is described by two cyanogenic features. The HCNp is defined as the total amount of HCN that can be released from a specific tissue (Loyd and Gray, 1970). HCNc describes the release of HCN per unit time (Lieberei, 1988).

Analysis of Cyanogenic Potential. HCNp was measured by extraction of the cyanogenic precursors from leaf material. To avoid a premature release of HCN by degradation, the complete procedure from harvest of the leaves to extraction of the cyanogenic precursors was conducted with cooled solutions and devices (4°C). In addition, all steps of processing were conducted on ice.

Any injury of leaf material during harvest and transport was avoided, and the harvested leaves were immediately put into water-filled Eppendorf® tubes to minimize effects of wilting. Only the laminae of the leaves were used for analysis. Leaf material was ground with a mortar and pestle for extraction by adding 0.067 mol/l disodium hydrogen phosphate (2 ml/g fresh leaf material). The homogenized sample was filtered and centrifuged for 20 min at $13000 \times g$ and 4°C. The supernatant was used for further analysis.

For enzymatic degradation of the cyanogenic precursors, exogenous β-glucosidase was added to the respective sample to cleave the precursor glycosides and to detect all HCN that can be released. Different cyanogenic substrates require different β-glucosidases. Therefore, the cyanogenic substrate must be matched with appropriate and possibly specific β-glucosidases. The rubber tree *Hevea brasiliensis* (Willd.) Muell. Arg. (1865) contains the cyanogenic glycosides linamarin and lotaustralin, as does *P. lunatus* (Lieberei et al., 1986). Thus, the β-glucosidase of *H. brasiliensis* was assumed to show specific activity for hydrolysis of the cyanogenic precursors found in the lima bean. Fresh leaf material of *H. brasiliensis* was frozen in liquid nitrogen, ground with mortar and pestle by adding cooled phosphate buffer (4°C), and adjusted to pH 6.8 [1 g leaf fw/4 ml buffer (fw = fresh weight)]. The homogenized sample was filtered and centrifuged for 20 min at $13000 \times g$ and 4°C. The supernatant was filtered through membrane caps with a pore size <10,000 kD (Schleicher & Schuell Bioscience GmbH, Dassel, Germany) and frozen at −20°C for further analysis. Generally, β-glucosidase activity was determined by using *p*-nitrophenyl-β-D-glucopyranoside (Merck) as artificial chromogenic substrate. The amount of β-glucosidase added per sample corresponded to 20 nkat. Thunberg vessels were used as reaction flasks for the determination of the HCNp. These were stoppered by a glass stopper with a side bulb of about 1-ml volume. Thus, the Thunberg vessel contained a closed headspace, and the released HCN could not leak from the preparation. The mixture for incubation consisted of 0.05 ml supernatant of the centrifuged sample, 0.45 ml 0.067 mol/l aqueous sodium dihydrogen phosphate solution, 0.10 ml β-glucosidase solution in McIlvaine buffer, pH 5.6 (20 nkat), and 0.60 ml 0.2 mol/l NaOH in the side bulb of the stopper. This mixture was incubated in a water bath for 20 min at a temperature of 30°C. The enzymatic reaction was stopped by the NaOH solution, which was added from the side bulb of the stopper to the incubation mixture. Sodium salt of HCN was formed, and the concentration of cyanide was measured spectrophotometrically by use of the Spectroquant® cyanide test (Merck). This test is based on the formation of cyanogen chloride. This compound reacts with pyridine forming glutacone dialdehyde, which condenses with 1,3-dimethyl barbituric acid to a violet polymethine dye with a maximum absorbance at a wavelength of 585 nm. One mol of polymethine dye corresponds to 1 mol cyanide.

The standard preparation for spectrophotometric measurement of cyanide consisted of one aliquot (0.1 ml sample) that was taken from the stopped incubation mixture. The sample was neutralized by adding an aliquot of 0.1 mol/l HCl (0.1 ml) and made up to 5 ml with 4.8 ml aqua dest. HCl was added for the neutralization of the alkaline sample because pH 7 had to be adjusted to guarantee optimal reaction of Spectroquant® reagents by Merck. The concentration of the chromogenic product was measured spectrophotometrically after 5 min of incubation.

Analysis of Cyanogenic Capacity. We tested the clonal plant material of different accessions for the total quantities of HCN release and the pattern of HCN release, respectively. In these experiments, we also differentiated for the ontogenetic leaf developmental stages "young," "medium," and "mature" of each accession. For this analysis, single trifoliate leaves were treated with 400 μl chloroform. Chloroform disintegrates cell membranes and leads to a breakdown of cellular compartmentation. HCN is released by this chemical tissue rupture from cyanogenic precursors if any active endogenous β-glucosidases with affinity for the cyanogenic precursors are present. Pattern of hydrogen cyanide, which was released to the atmosphere after addition of chloroform, was followed over a time period of 1 hr. The leaf was treated with chloroform a second time later to test for further release of hydrogen cyanide.

HCN Detection System. Both the release of HCN from cyanogenic leaf material as reaction to chloroform treatment and the release from injured leaf tissue in the course of the feeding trials were detected by using the same airflow system (Figure 1). This system was passed by an adjustable airflow provided by a pump with upstream potentiometer for capacity control. The pump was connected with an air humidifier (Figure 1B) consisting of a triple neck flask with a total volume of 2 l filled with 1 l aqua dest. to ensure a sufficient ambient air humidity in the experimental system and to guarantee optimal ambient conditions for locust activity and avoid wilting of the leaf during the bioassays (air humidity was >90%). From the humidifier, air was passed through a 200-ml Erlenmeyer flask (Figure 1C) containing leaf material used for the analysis of the HCNc or for feeding trials with single locust nymphs, respectively. The flask was immersed into a water bath adjusted at 30°C to ensure high activity of the insects in the course of feeding trials, whereas light with an intensity of 200 μmol s^{-1} m^{-2} was provided by a 150-W spot (Philips®). The discharge opening of the flask was connected to a glass capillary inserted into a test tube filled with 0.1 mol/l aqueous NaOH solution (Figure 1D). The capillary released bubbles of a defined volume of 0.83 ml at an immersion depth of 10 cm. The airflow in the equipment was adjusted precisely to 70 bubbles per minute (≈3.5 l/hr). Inflowing air was kept at this relatively low speed because higher speed had a negative impact on locusts. The test tube containing the NaOH solution served as collector of the released HCN and was exchanged at intervals

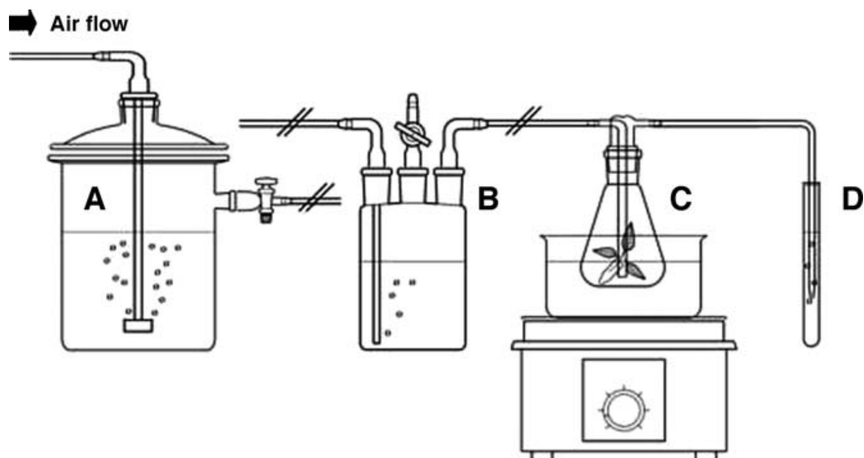

FIG. 1. Equipment for detection of gaseous HCN. The airflow in the vessel system was provided by a diaphragm pump with adjustable capacity. The air passes an air humidifier filled with aqua dest. (**B**) or a HCN generator (**A**) depending on the experiment. The HCN generator was filled with KCN solution (0.025–2.0 mmol/l) to provide a continuous HCN atmosphere within the airflow system for observation of behavior patterns depending on concentration of gaseous hydrogen cyanide. For detection of the cyanogenic capacity (HCNc), single trifoliate leaves were put into an Erlenmeyer flask (**C**) and treated with chloroform. In the course of bioassays, single locust nymphs were put into the flask (**C**) together with a trifoliate leaf. The flask was immersed into a water bath with a temperature of 30°C. Gaseous HCN—provided by the HCN generator, released from leaves as reaction to chemical cell disintegration or released from leaves as a result of tissue damage due to insect feeding—was detected quantitatively at the output of the equipment (**D**).

of 10 min. The pattern of the HCN release from leaf material per unit time was revealed by quantitative measurement of cyanide in the detection solutions by use of the Spectroquant® cyanide test (Merck). A pH of 7 was adjusted by adding 0.1 mol/l HCl to satisfy conditions required for this reagent kit. For analysis of the HCNc by chloroform treatment of particular leaves, the same speed of airflow was chosen, and the air humidifier was kept connected to the vessel system to achieve a similar experimental situation as in the feeding trials.

Bioassays. For no-choice feeding trials, single locust nymphs were placed into the 200-ml Erlenmeyer flask for HCN detection (Figure 1C) containing a trifoliate leaf of a defined accession and developmental stage. *P. lunatus* leaf material was characterized by specific cyanogenic features and was offered to the animals for 1 hr in no-choice feeding trials. In addition to the lima beans, *P. vulgaris* var. Saxa with extremely low cyanogenic concentrations was used

as control. Only locust nymphs of similar size and body weight were chosen for the experiments (mean ± SD, 0.987 ± 0.054 g; $N = 245$). Locust nymphs that molted about 48 hr previously were kept for 24 hr with *ad libitum* access to *Poa annua* leaf material which was part of their normal diet under the conditions mentioned above. They were food deprived for 1 hr prior to the bioassay.

The leaves used in the bioassays were harvested 1 hr after irrigation by the computer-based greenhouse control system. Leaves were put into water-filled Eppendorf® tubes with the petiole immediately after harvest and used in this form in the bioassays to guarantee a constant water supply. The amount of consumed leaf material was determined by weighing the particular leaf before and after the bioassay (MC 1 Analytic AC 210 S, Sartorius®) during constant water supply. This method for estimation of consumed leaf material was favored over leaf area measurements because even slight pressure during scanning in addition to interrupted water supply may lead to premature release of HCN. During the bioassay, release of HCN as a reaction to leaf material consumption by the locusts was consequently detected per unit time, and the duration of feeding on the particular leaf was documented. Detection of released hydrogen cyanide started with the first feeding incidence of the particular locust. Additional binary-choice feeding trials were conducted under the same experimental conditions. In these trials, selected single trifoliate leaves of high and low cyanogenic accessions were presented pairwise to the locusts, and the amount of leaf consumption was determined, respectively.

Behavior of Insects. A general statement as to whether feeding occurred or not did not seem to be sufficient to characterize the herbivore response. In preliminary studies, behavior of locust was observed by offering leaves of *P. vulgaris* and *Poa annua* as standard fodder. The animals were observed for 1 hr under the experimental setup for HCN detection. The HCNp of *P. vulgaris* var. Saxa was close to zero, and *Poa annua* did not contain any detectable cyanogenic components. In these preliminary experiments, very low and acyanogenic leaf material was used to ensure that insects were not affected by cyanogenic precursors or released HCN. These observed characteristics of behavior were interpreted as "normal." The following feeding trials were carried out under the same experimental conditions, but with leaf material of different cyanogenic accessions of *P. lunatus*. Leaf material of *P. vulgaris* var. Saxa was used as a control. Apart from feeding time, the following behavioral patterns were considered to reflect responses of the locusts to leaf consumption: general agility of the insects including the change of location after feeding, cleaning of the mouthparts, antennae, and legs, presence of flight reaction, and posture of the antennae and of the legs.

Behavioral patterns were defined as follows: 0, "normal" behavior (similar to behavior after consumption of leaf material of *Poa annua* and *P. vulgaris*); 1, no change of location, legs are angled and pressed onto the abdomen; 2, like 1,

but loss of any flight reaction and no cleaning of the mouthparts, antennae, and legs; 3, like 2, but, in addition, the antennae are sloped downwards by 45°.

During and after feeding on leaf material, changes of behavior occurred that were classified as defined above. Normal behavior on leaf material of *P. vulgaris* and *Poa annua* was characterized by high agility of the insects and by complete excitability to visual stimuli or vibrations. The feeding interval of the insects was followed by change of the location on the leaf and cleaning of the antennae, the compound eyes, wings, mouth parts, and legs. After intake of cyanogenic leaf material, the legs were pressed on the abdomen, and the locusts were less agile. This behavior was defined as the first level of negative effects (class 1). At this level, the insect still showed complete excitability with respect to visual stimuli or vibrations. The loss of this ability and the loss of comfort behavior together with the posture described above were interpreted as a more severe level of intoxication (class 2). In some cases, the locusts lowered their antennae downwards by about 45° in addition (class 3). In 1.7% (4 of 230 bioassays), the locust nymph died 1–2 hr after the experiment. These most severe signs of intoxication were included in class 3.

Exogenous Application of HCN. To test if application of exogenous hydrogen cyanide leads to similar effects with regard to locust behavior, we added quantified doses of gaseous HCN into the experimental vessel system by replacing the air humidifier (Figure 1B) by a gas generator (Figure 1A). This generator was made of a glass vessel with a total volume of 5 l that was filled with 3 l HCN-containing buffered solutions (phosphate buffer 6.7 mmol/l, adjusted to pH 5.5) and bubbled by a constant stream of air (≈3.5 l/hr). By use of defined cyanide solutions in the gas generator from 0.025 to 2 mmol/l, defined concentrations of gaseous HCN were constantly applied to *S. gregaria* individuals that had *ad libitum* access to almost noncyanogenic leaves of *P. vulgaris*. Gaseous HCN was added over a time period of 60 min. The speed of the airflow was controlled by detection of bubbles with defined volume that were released into a 0.1 mol/l aqueous NaOH solution by passing a capillary at the output of the equipment. The constancy of the HCN atmosphere within the equipment could be proven by spectrophotometric measurement of the amount of cyanide that was fixed in the NaOH solution per unit time using the Spectroquant® cyanide test (Merck). Experiments were conducted at a room temperature of 25°C, whereas the 200-ml Erlenmeyer flask containing the locust and leaf material was immersed into a water bath with a temperature of 30°C to ensure optimal conditions for locust activity. Light intensity was 200 μmol s^{-1} m^{-2} provided by a 150-W spot (Philips®).

HCN Balance. We calculated a HCN balance to investigate in which way the feeding insect gets in contact with HCN. Contact with released HCN could occur mainly during the feeding process, or large amounts of cyanide in bound form could be ingested and HCN might be released within the gastrointestinal

tract of the locust. Five aspects were considered to evaluate the fate of cyanogenic substances during the experiments: (1) amount of cyanide in bound form present in the particular leaf; (2) amount of cyanide consumed by the insect; (3) released HCN from leaf material during the feeding process of the insect; (4) released HCN from the locust bodies after consumption of leaf material; and (5) amount of cyanide in the feces.

The HCNp of every single leaf used in experiments was estimated. Leaf material that was left behind in the particular bioassay was used for analysis of the HCNp, and from these data, the amount of cyanogenic precursors within the consumed part of the leaf was calculated. The total amount of consumed leaf material was determined by weighing the particular leaf before and after the bioassay during constant water supply (MC 1 Analytic AC 210 S, Sartorius®).

The release of HCN from leaf material during the feeding of *S. gregaria* was measured per unit time with the equipment for HCN detection. For detection of hydrogen cyanide released from locust bodies into the atmosphere after leaf consumption, the insect was taken out of the experimental vessel and put in the center of a Conway's microdiffusional apparatus (semimicro type, Shibata Scientific Technology) with the outer ring filled with 10 ml 0.1 mol/l NaOH. The locust was left in there for 30 min. After that, the cyanide concentration of the surrounding NaOH solution was measured spectrophotometrically. The HCN content of the excrements that were obtained for up to 1 hr after the bioassay was analyzed by the method described for the HCNp determination of leaf material.

Statistics. Statistical analyses were run with Statistica 6.0 (Statistica System Reference, 2001).

RESULTS

Cyanogenic Features of P. lunatus. The clonal plants of all accessions studied were cyanogenic. All plants synthesized and accumulated cyanide-containing compounds in their leaves and were capable of releasing hydrogen cyanide from these compounds as reaction to chemical or mechanical damage.

Cyanogenic Potential. The variation of the HCNp was high and ranged from a HCNp of 0.7 μmol HCN/g leaf fw in mature leaves of accession 8071 to 82.0 μmol HCN/g leaf fw in young leaves of accession 2233. In general, the HCNp of young leaves was higher than in leaves of the intermediate or mature developmental stage. This ontogenetic pattern of variation was apparent in all clonal plants of all accessions studied (Table 1; Figure 2). Leaves of the cultivar "Saxa" (*P. vulgaris*) that served as control were characterized by very low concentrations of HCNp. This genotype was almost noncyanogenic.

TABLE 1. CALCULATED BALANCE OF CYANIDE INGESTED BY NYMPHS OF *S. gregaria*

						Percentage of detected HCN (%)			
Accession/ leaf development	Cyanogenic status, H = high, L = low	*N*	HCNp of leaf material (μmol HCN/g leaf fw)	Consumed leaf material (mg leaf fw)	Calculated amount of HCN in the consumed leaf material (nmol HCN)	HCN released to the atmosphere during the feeding process	HCN released from the locust bodies	HCN in the feces	Total loss of HCN
2357, young	H	11	60.5 ± 7.0	10.6 ± 7.5	646.8 ± 478.1	4.4 ± 3.7	0.4 ± 0.5	0.0 ± 0.0	95.1 ± 4.2
2357, medium	H	11	58.4 ± 7.7	15.6 ± 7.5	918.2 ± 522.7	2.1 ± 1.3	0.2 ± 0.1	0.1 ± 0.1	97.6 ± 1.4
2357, mature	H	11	14.9 ± 3.2	53.4 ± 18.3	792.1 ± 313.7	1.4 ± 0.7	0.3 ± 0.1	0.2 ± 0.1	98.2 ± 0.8
2233, young	H	4	60.0 ± 3.1	0.5 ± 0.1	27.0 ± 7.2	6.5 ± 4.4	1.9 ± 2.4	0.0 ± 0.0	91.6 ± 2.4
2233, medium	H	3	24.6 ± 2.7	1.2 ± 1.1	31.6 ± 31.9	15.9 ± 7.1	5.0 ± 7.6	0.0 ± 0.0	79.2 ± 8.4
2233, mature	H	3	6.8 ± 2.1	0.6 ± 0.4	4.3 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0
1315, young	H	11	51.7 ± 3.3	10.0 ± 5.3	514.5 ± 263.3	5.1 ± 2.8	0.3 ± 0.2	0.0 ± 0.1	94.5 ± 2.9
1315, medium	H	11	33.5 ± 4.4	18.3 ± 11.2	593.0 ± 321.0	3.1 ± 1.5	0.3 ± 0.1	0.0 ± 0.0	96.5 ± 1.5
1315, mature	H	10	18.1 ± 6.6	42.6 ± 21.1	677.1 ± 266.7	1.8 ± 0.9	0.4 ± 0.2	0.1 ± 0.1	97.7 ± 1.0

1259, young	H	11	30.5 ± 7.0	18.6 ± 8.5	544.5 ± 213.2	2.7 ± 1.3	0.2 ± 0.1	0.3 ± 0.2	96.9 ± 1.4
1259, medium	H	11	27.9 ± 5.1	23.1 ± 14.2	634.5 ± 401.8	3.6 ± 3.6	0.4 ± 0.4	0.4 ± 0.5	95.8 ± 4.1
1259, mature	H	11	18.0 ± 5.9	32.1 ± 17.5	588.2 ± 330.6	2.1 ± 1.5	0.3 ± 0.2	0.0 ± 0.0	97.1 ± 1.9
2441, young	L	11	18.8 ± 3.3	36.5 ± 25.0	635.6 ± 402.2	3.1 ± 3.5	0.3 ± 0.4	2.1 ± 1.4	94.5 ± 4.0
2441, medium	L	11	13.1 ± 3.9	52.3 ± 35.4	658.3 ± 434.5	2.6 ± 2.7	0.7 ± 1.2	14.3 ± 34.4	82.4 ± 38.1
2441, mature	L	11	6.2 ± 2.1	73.5 ± 26.1	444.1 ± 230.4	1.3 ± 0.7	0.6 ± 0.3	5.5 ± 2.1	92.8 ± 2.4
8079, young	L	8	9.4 ± 1.0	90.4 ± 22.6	849.3 ± 237.1	1.3 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	98.6 ± 0.3
8079, medium	L	8	8.6 ± 0.7	96.4 ± 20.0	835.8 ± 214.7	1.3 ± 0.4	0.1 ± 0.0	0.0 ± 0.0	98.6 ± 0.4
8079, mature	L	7	8.1 ± 1.4	90.7 ± 22.1	726.3 ± 194.5	0.9 ± 0.5	0.2 ± 0.1	0.0 ± 0.0	98.9 ± 0.5
8071, young	L	11	6.5 ± 0.9	78.5 ± 18.3	505.4 ± 127.6	1.1 ± 0.9	0.1 ± 0.1	0.0 ± 0.0	98.8 ± 0.7
8071, medium	L	11	5.1 ± 0.6	52.8 ± 36.0	276.1 ± 200.2	1.0 ± 0.9	0.2 ± 0.3	0.0 ± 0.0	98.7 ± 1.2
8071, mature	L	11	1.2 ± 0.4	64.4 ± 25.1	77.2 ± 37.8	2.4 ± 1.9	0.5 ± 0.6	0.0 ± 0.0	97.1 ± 2.3
Saxa, young	Control	11	0.8 ± 0.2	84.9 ± 30.1	68.6 ± 27.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Saxa, medium	Control	11	0.9 ± 0.1	79.7 ± 26.0	71.5 ± 27.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Saxa, mature	Control	11	0.7 ± 0.1	87.2 ± 22.5	63.7 ± 20.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0

Notes: Cyanide ingestion by locust is calculated from the weight of consumed fresh leaf material and the cyanogenic potential (HCNp) of the particular leaf. The HCNp of every trifoliate leaf that was used in the bioassays was measured ($N = 230$). Data in percentage are related to the calculated amount of ingested cyanide that was considered as 100%.

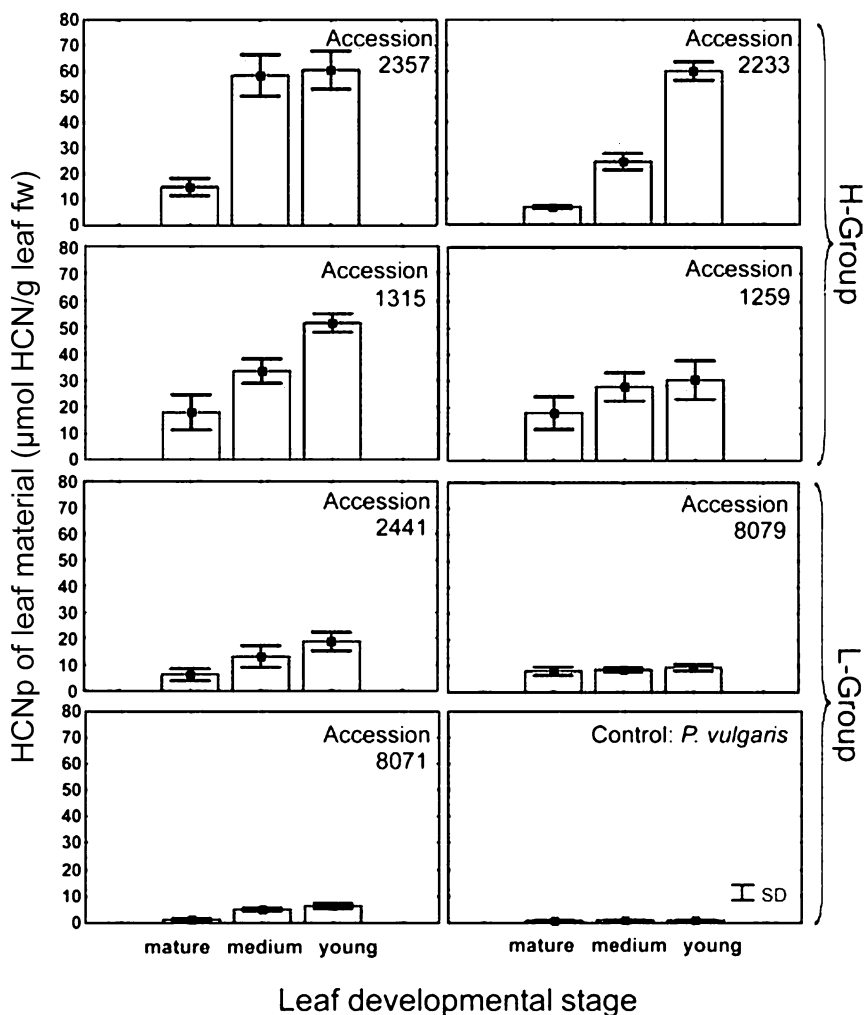

FIG. 2. Cyanogenic potential (HCNp) of *P. lunatus* accessions. Leaves of defined ontogenetic developmental stages of clonal plant material were analyzed. The clearly varying HCNp of the clonal lines allowed to distinguish between a high (H-group) and a low cyanogenic group (L-group) of plants. The control plants (*P. vulgaris* var. Saxa) revealed no substantial HCNp. Values are means ± SD. For number of observations, see Table 1.

Cyanogenic Capacity. The HCNc was estimated by measuring the kinetics of HCN release from leaves as reaction to chemically induced tissue disintegration. The pattern and the total amount of HCN released varied considerably between the accessions and the leaf developmental stages used for analysis (Figure 3).

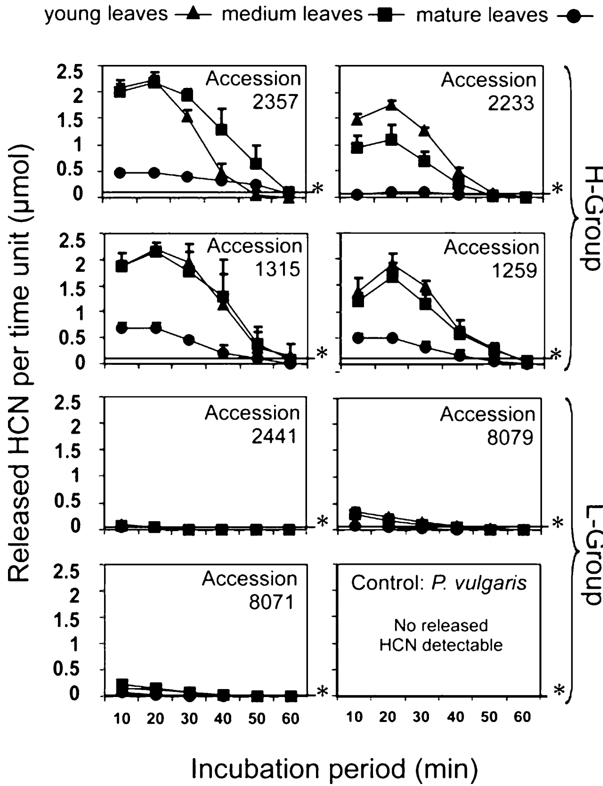

FIG. 3. Cyanogenic capacity (HCNc) of *P. lunatus* accessions. Leaves of defined developmental stages were analyzed for their capacity to release HCN from endogenous cyanide-containing precursors as reaction to chemical tissue disintegration due to chloroform application ($N = 5$ per accession and leaf developmental stage). Values given in the figure are means + SD. The release of HCN was quantitatively estimated per time periods (10 min) between the measurements. The experiments were carried out using the airflow system for detection of gaseous HCN and span a total time period of 1 hr. Application of chloroform to the same leaf a second time later in the experiment revealed no substantial release of hydrogen cyanide. Mean values of released HCN as reaction to this further treatment were below the level marked with an asterisk. The almost noncyanogenic *P. vulgaris* var. Saxa served as control.

Young and intermediate leaf developmental stages of the high cyanogenic accessions 2357, 2233, 1315, and 1259 revealed a rapid release of HCN and also a fast decrease of measurable hydrogen cyanide within 50 min when treated with chloroform. Mature leaves of these accessions were characterized by a lower and more continuous release of HCN that lasted as long as 60 min. No distinct maximum of hydrogen cyanide release was observed for these leaves. HCN was also released from leaves of all developmental stages of the low cyanogenic accessions of *P. lunatus* (2441, 8079, and 8071), but the release of HCN especially from mature leaves was close to zero. Control plants (*P. vulgaris*) had no capacity to release HCN from leaves. Thus, the pattern of HCN release from leaf material of *P. lunatus* depends on the cyanogenic status of the accessions as well as on the developmental stages of the leaves. No additional HCN loss was observed when chloroform was added to the leaves a second time later in the experiments. In addition, grinding the remaining leaf tissue under application of exogenous β-glucosidase isolated from *H. brasiliensis* revealed no substantial release of HCN (Figure 3).

Grouping of Plants for Analysis. Considering the results for the HCNp (Figure 2) and the corresponding HCNc (Figure 3), the accessions were assigned into a high (H-group) and a low cyanogenic category (L-group). Plants of the H-group were characterized by a HCNp of 34.4 ± 18.4 μmol HCN/g leaf fw (mean ± SD; $N = 108$). These plants (accessions 2357, 2233, 1315, and 1259) release more than 1 μmol HCN per trifoliate leaf within the first 10 min after treatment with chloroform. The plants of the L-group (accessions 2441, 8079, and 8071) showed a HCNp of 8.5 ± 5.7 μmol hydrogen cyanide/g leaf fw ($N = 89$). The H- and L-groups differed significantly (ANOVA, $F = 154.97$, $df = 1$, $P < 0.001$). The control *P. vulgaris* var. Saxa was almost noncyanogenic.

No-Choice Bioassays. Different effects of cyanogenesis on the susceptibility of leaf material to *S. gregaria* were observed in the feeding trials in relation to differences of HCNp and HCNc between the accessions of *P. lunatus* and their leaf developmental stages. Below a HCNp of 15 μmol HCN/g leaf fw that corresponds to a release of about 5 nmol HCN/10 min from the beginning of feeding under experimental conditions, there was no deterrent effect of cyanogenic leaf material observed (Figure 4). When feeding on plants of the H-group, locusts consumed less leaf material than when feeding on the L-group ($F = 197.02$, $df = 1$, $P < 0.001$). The intake of plants assigned to the H-group was 22.3 ± 19.6 mg fresh leaf material ($N = 108$). Within the H-group, substantial leaf consumption occurs exclusively on mature leaves with an HCNp near the threshold range of 15 μmol HCN/g leaf fw and a low capacity for HCN release. Leaves of accession 2233 were barely consumed despite the wide range of HCNp from 5.9 μmol HCN/g leaf fw in mature leaves up to more than 81 μmol HCN/g leaf fw in leaves of the young developmental stage. It is assumed that feeding deterrence of these plants is partly because of factors not

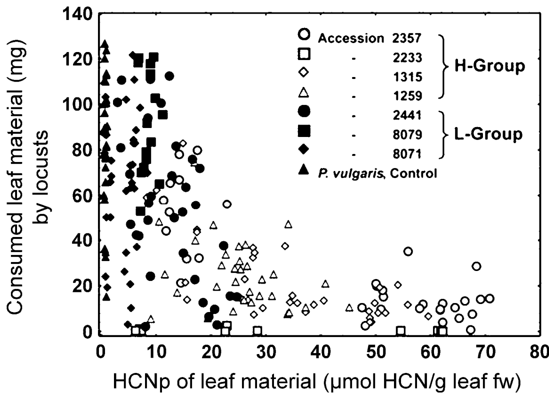

FIG. 4. Cyanogenic potential (HCNp) vs. leaf consumption. Leaves of high (H-group) and low (L-group) cyanogenic accessions of *P. lunatus* were presented to locusts over a time period of 1 hr within the airflow equipment for detection of gaseous HCN. Leaf consumption decreased with increasing HCNp of the presented leaf material. In this figure, there is no discrimination among ontogenetic leaf developmental stages. Results of statistical analyses are given in the text. For number of observations, see Table 1. The almost noncyanogenic *P. vulgaris* var. Saxa served as control.

considered in the present study, but may be a consequence of their outstanding high capacity for HCN release as reaction to feeding (Table 1). The intake of L-group plants of *P. lunatus* was 65.7 ± 32.9 mg fresh leaf material ($N = 89$). A tendency of reduced feeding was observed on the L-group accession 2441 where young leaves revealed the highest HCNp within this group and reached the threshold level of 15 μmol HCN/g leaf fw (Table 1). There was no measurable effect of the HCNp observed within the L-group of *P. lunatus* accessions with regard to leaf consumption. The leaf consumption by locusts on the *P. vulgaris* control plants (83.9 ± 26.6 mg fresh leaf material; $N = 33$) was significantly higher than the leaf intake on plants of the L-group of *P. lunatus* ($F = 7.99$, $df = 1$, $P = 0.006$). The HCNp and the corresponding HCNc affected the feeding behavior of locusts depending on threshold levels.

Binary-Choice Bioassays. Young leaves of the highest and the lowest cyanogenic accession (2357 vs. 8071) and leaves of the second highest and second lowest cyanogenic accession (1315 vs. 8079) were presented pairwise to the locusts (Figure 5). The choice tests between these leaves yielded a significant preference for the lower cyanogenic leaf material ($F = 88.62$, $df = 1$, $P < 0.001$).

Release of HCN as Reaction to Insect Feeding. Similar to the chemically induced release of HCN, the pattern of hydrogen cyanide released from leaf

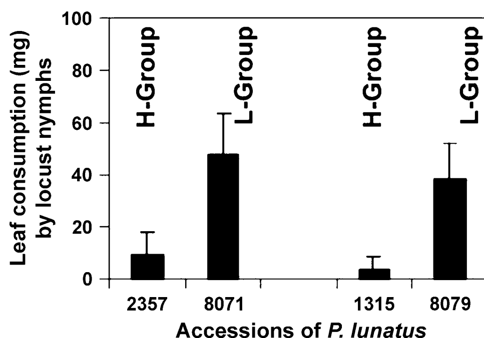

FIG. 5. Binary-choice bioassay. Leaves of H- and L-group plants were offered pairwise to single locust nymphs. Values are means and standard deviations ($N = 9$). Results of statistical analyses are given in the text.

material as reaction to insect feeding revealed differences depending on the general status of the HCNp, the accession, and the ontogenetic leaf developmental stage (Figure 6). Furthermore, the liberation pattern and amount of released HCN depended on the duration of feeding on selected leaf material. The time of feeding on H-group leaf material was 5 ± 4 min ($N = 108$) over the total observed period of 1 hr. On leaves of L-group plants, the locusts fed 18 ± 9 min ($N = 89$). The duration of feeding on H- and L-group plant materials was significantly different (ANOVA, $F = 164.87$, $df = 1$, $P < 0.001$). On high cyanogenic leaf material, further feeding incidences of a few locusts were observed over the time period of 1 hr. These further feeding incidences started 30–60 min after the beginning of the experiment; they were very short, and were not considered as time of feeding, but resulted in visible additional peaks of HCN release (Figure 6).

An interesting fact is that young and intermediate leaves of the L-group accession 2441 showed a substantial capacity for release of HCN as a reaction to feeding (Figure 6). This finding is in contrast to the very low HCNc detected as reaction to chemical tissue disintegration by chloroform treatment (Figure 3) and may indicate the existence of endogenous β-glucosidases in the locusts' saliva.

Effects of Leaf Development. Young leaves of the high cyanogenic accessions that were characterized by the highest HCNp and HCNc were consumed in small amounts. To clarify whether these cyanogenic features were acting as the effective components of plant defense, or if any other chemical or physical factors could be correlated with leaf maturation and might have influenced the feeding behavior of locusts quantitatively, we applied a post hoc test after ANOVA to the data set (Tukey's HSD for unequal N, $P < 0.05$;

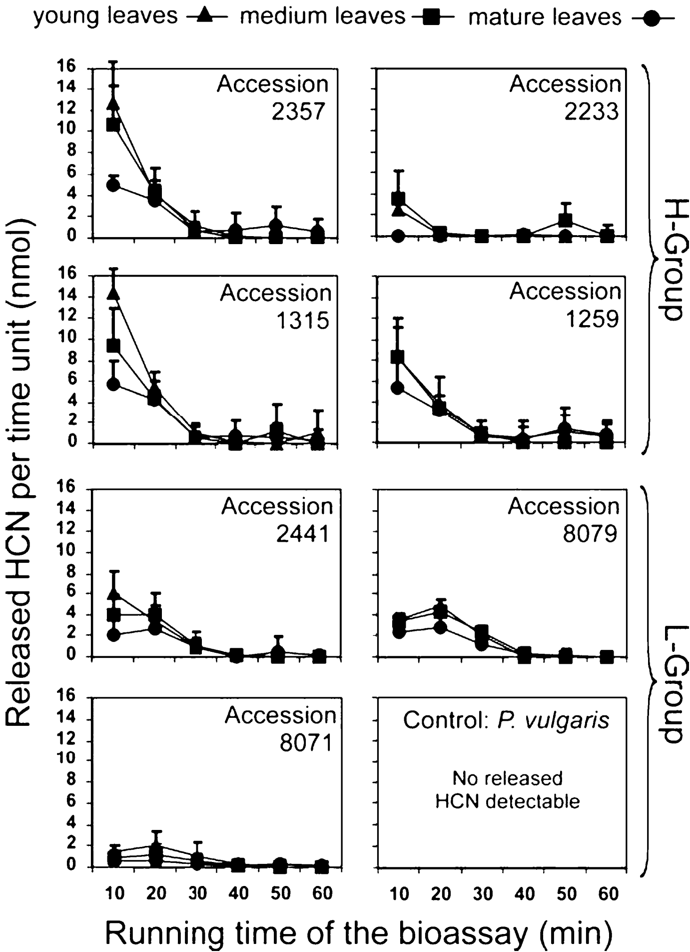

FIG. 6. Release of HCN from leaves as reaction to herbivory. Illustrated is the quantitative release of HCN as reaction to leaf consumption. This experiment was run with the airflow system for detection of gaseous HCN. The total period of detection of HCN release was 1 hr. The experiments started with the first feeding incidence of the locusts where duration is significantly different between the H- and the L-groups of plants. Peaks of HCN release, 30–60 min after start of the feeding trial, were results of further feeding incidents of single locusts. Values given in the figure are means + SD. The duration of the first feeding incidence and results of statistical analyses are given in the text; for number of observations, see Table 1. The almost noncyanogenic *P. vulgaris* var. Saxa served as control.

H-group, N = 108; L-group, N = 89). Within the H-group, no significant differences in consumption of young and intermediate leaves were found, but there was a significant difference between these leaves and the mature developmental stage characterized by lower HCNp and HCNc. Within the L-group, there was no significant difference in leaf consumption between the developmental stages. Thus, among the H-group plants, leaf consumption decreased with the increase of the cyanogenic features in younger leaves. Young and intermediate leaves of the high cyanogenic plants were not consumed less than mature leaves just because of the leaf developmental stage itself. Deterrent effects on locusts appeared to depend on the high HCNp and HCNc that occurred at this ontogenetic leaf developmental stages.

Effects of Cyanogenesis on Locust Behavior. The state of cyanogenic features affected locust behavior and led to different symptoms of intoxication. The reactions of the locusts feeding on *P. vulgaris* var. Saxa were defined as normal (behavioral pattern 0). This behavior contrasted with reactions of the insects after feeding on cyanogenic plant material of *P. lunatus*. These deviating patterns were interpreted as symptoms of intoxication (behavioral patterns 1–3). Strong symptoms of intoxication occurred after extensive consumption of leaf material of accessions 2441, 8071, and 8079 with a low HCNp and a corresponding low HCNc, whereas feeding on high cyanogenic leaf material (accessions 2357, 2233, 1315, and 1259) resulted in fewer or no symptoms of intoxication (Figure 7). The severity of intoxication strongly interacted with the

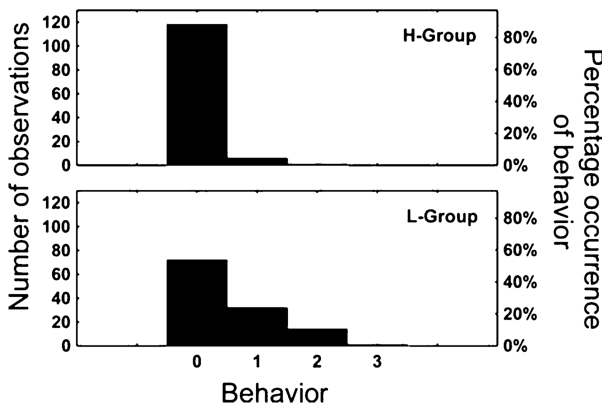

FIG. 7. Behavior of locusts. The behavioral patterns as reaction to consumption of high (H-group) and low (L-group) cyanogenic leaf material were documented and assigned to defined categories. These categories represent different levels of intoxication that range from normal behavior (0) to massive impairment of the herbivore (3). Presented are the total number of observations and the percentage occurrence of defined behavioral patterns among the H-group and L-group of plants.

amount of consumed leaf material ($F = 27.96$, $df = 3$, $P < 0.001$). Furthermore, the release of HCN from leaf material as a reaction to locust feeding was significantly higher for bioassays in which low symptoms of intoxication occurred ($F = 62.94$, $df = 3$, $P < 0.001$). Thus, the results indicate detrimental effects because of extensive ingestion of cyanogenic leaf material and a strong repellent action of high HCNc.

Exogenous Application of HCN. Locusts kept under a HCN atmosphere of 0.260–0.876 μmol/l revealed normal behavior (0). Agility, feeding behavior, and flight reactions were unaffected throughout the 1 hr of incubation under these conditions (Table 2). At a HCN concentration of 1.805 μmol/l in the gas phase, which corresponds to a cyanide concentration in the liquid phase of the generator of 0.5 mmol/l, the locusts showed reduced movements and angled legs that were pressed on the abdomen, whereas other aspects of behavior remained unchanged. This behavior corresponded to pattern 1 that was observed in the bioassays with cyanogenic *P. lunatus* leaf material. At the level of 3.612 μmol/l, the locusts showed reduced movements and, in addition, less feeding on the leaf material and loss of flight reaction. Therefore, this behavior was similar to pattern 2. At the highest HCN concentration of 7.518 μmol/l, the insects stopped feeding on leaf material and the antennae were sloped downwards as was characteristic for behavioral pattern 3 in the feeding experiments. The symptoms of intoxication caused by application of exogenous HCN at higher levels were the same as observed during or after feeding on cyanogenic leaf material. However, an additional characteristic of behavior was the frequent cleaning of the antennae at HCN concentrations above 3.612 μmol/l. All symptoms were completely reversible and disappeared about half an hour after taking the locusts out of the equipment and putting them on fresh leaf material.

In this experiment, we chose relatively low concentrations of cyanide compared to the conditions in cyanogenic leaf material. Even the highest concentrations of hydrogen cyanide in the HCN generator were in the lowest

TABLE 2. LOCUST BEHAVIOR UNDER EXPERIMENTAL HCN APPLICATION

KCN solution (mmol/l)	HCN atmosphere (μmol/l)	Locust Behavior	*N*
0.025	0.260	0	5
0.100	0.312	0	5
0.250	0.876	0	5
0.500	1.805	1	5
1.000	3.612	2	5
2.000	7.518	3	5

Notes: The HCN generator of the airflow system (A, Figure 1) was filled with adjusted KCN solutions to provide defined concentrations of atmospheric HCN. Behavioral patterns of locust on leaf material of control plants (*P. vulgaris* var. Saxa) were documented under these experimental conditions. The meaning of the codes for assignment of locust behavior is given in the text.

range of cyanogenic precursor variation present in *P. lunatus* leaf material. However, the concentration of gaseous HCN in the atmosphere at which first intoxication effects could be observed was 60 times higher than the highest concentrations of released HCN that was measured in the course of the feeding trials as reaction to leaf injury by the locusts.

HCN Balance. The amount of cyanide-containing compounds that were consumed together with the leaf tissue was calculated for every bioassay (N = 230). In addition, the amount of cyanide in the feces of the particular locust and the HCN released to the atmosphere from the locusts' bodies after the bioassay was included into the HCN balance (Table 1). The total calculated intake of cyanide-containing compounds present in the consumed parts of the leaves was not significantly different among all *P. lunatus* accessions ($F = 2.44$, $df = 1$, $P = 0.120$). But, when feeding on leaves on the mature developmental stage of the L-group accession 8071, the intake of hydrogen cyanide was low because of the extreme low HCNp of these leaves. HCN intake was even lower when locusts were feeding on the H-group accession 2233 with a high HCNp because of the small amounts of leaf material that were consumed from this accession. Within the H-group of plants, the intake of leaf material and, therefore, the intake of HCN tended to be lower by locusts feeding on young leaves with high cyanogenic features than by feeding on intermediate and mature leaves with lower cyanogenic properties. Considering the L-group of plants, in contrast, the intake of HCN by feeding on young leaves tended to be higher than when feeding on intermediate and mature leaf developmental stages because of the similar intake of leaf material but the higher HCNp of the young leaves compared with intermediate and mature leaves. The percentage release of HCN to the atmosphere during the feeding process was higher in the H-group than in the L-group of plants ($F = 475.16$, $df = 1$, $P < 0.001$). The young and medium leaf developmental stages especially revealed a high percentage of HCN release compared to the intake of hydrogen cyanide. A special situation was observed in bioassays with the H-group accession 2233. Here, the percentage of HCN released was 6.5 and 15.9% for the young and medium leaf developmental stages, respectively, higher than for any other accession.

Within the L-group, only young and intermediate leaves of accession 2441 and the mature leaves of accession 8071 revealed a considerable percentage release of HCN as reaction to feeding by the locusts. The relatively high percentage release of the latter was because of the fact that the intake of cyanogenic precursors by locust feeding on these leaves was extremely low corresponding to the very low HCNp that was not much higher than in the case of the control plants (*P. vulgaris*). Therefore, even low amounts of released HCN represent a relatively high percentage release. The release of HCN to the atmosphere from the locusts bodies for a time period of 1 hr after the bioassay was low for all accessions with the exception of locust that fed on young and

intermediate leaves of the H-group accession 2233. Here, the mean release of HCN was clearly higher than for the other bioassays.

Considerable amounts of hydrogen cyanide in locust excrement was detected after feeding on leaves of accession 2441 (Table 1). This might depend on the obviously low activity of the endogenous β-glucosidase that resulted in extraordinary low release of HCN as reaction to chemical tissue disintegration (Figure 3).

Among all bioassays, the total loss of HCN in this balance was high. Only for young and intermediate leaves of the H-group accession 2233 and the L-group accession 2441 was the loss lower. In bioassays using this leaf material, a high percentage release of HCN to the atmosphere occurred as a reaction to insect feeding, and high contents of HCN were found in the excrement, respectively.

DISCUSSION

There is substantial evidence that plant cyanogenesis acts as an effective herbivore deterrent against generalist herbivores (Jones, 1962; Hughes, 1991; Schappert and Shore, 1999c, 2000). The potential of cyanogenic glycosides in plants to act as a chemical defense was recently demonstrated by transferring the entire pathway for the synthesis of the aromatic tyrosine-derived cyanogenic glycoside dhurrin from *Sorghum bicolor* to *Arabidopsis thaliana*. The accumulation of dhurrin resulted in resistance of the transformed host plant to the flea beetle *Phyllotreta nemorum*, a Chrysomelid beetle, which is a natural pest on members of the cruciferous plants (Tattersal et al., 2001).

Furthermore, cyanogenesis seems to be beneficial for plants under specific environmental conditions (Kakes, 1989). The distribution of cyanogenesis in populations of *Lotus corniculatus* L. and *Trifolium repens* L. depends on abiotic factors that are also determining the occurrence and activity of snails, the important herbivores of clover (Jones, 1966, 1972, 1988). Hayden and Parker (2002) regarded this plasticity in *T. repens* as an important mechanism to reduce fitness-related defense costs.

According to Bernays et al. (1977), leaves of the cyanogenic tuber crop Cassava (*Manihot esculenta* Crantz) are consumed more frequently by nymphs of *Zonocerus variegatus* (Acrididae) in a senescent or wilted state than young and fresh leaf tissue. These wilted leaves showed a reduced release of HCN from their leaf tissues, but it should be kept in mind that it is not known to what extent other factors also change in a wilted leaf of *M. esculenta*.

In obligate cyanogenic plants such as *M. esculenta* and the lima bean *P. lunatus*, the precursors and the cleaving enzymes are present in the plant without any induction by abiotic stress or attack by pathogens and herbivores.

However, for these obligate cyanogenic plants, the release of HCN during the feeding process of a herbivore has never been quantified. There are several studies which analyze the amount of cyanide-liberating compounds in plants, its spatial distribution, and its dependence on environmental conditions (Jones, 1966; Cooper-Driver and Swain, 1976; Abbott, 1977; Cooper-Driver et al., 1977; Ellis et al., 1977a; Till, 1987; Caradus et al., 1990; Blaise et al., 1991; Hughes, 1991; Shore and Obrist, 1992; Calatayud et al., 1994; Caradus and Forde, 1996; Schappert and Shore, 1999a; Gleadow and Woodrow, 2000a). However, the central aspect of cyanogenesis, the capacity to release hydrogen cyanide from these endogenous compounds to the atmosphere, was just roughly classified by "yes" or "no" or semiquantitative estimation by Feigl–Anger test paper methods (Hayden and Parker, 2002). In addition, the additional measurement of β-glucosidase activity together with analysis of the content of cyanogenic precursors in plant material is not a conclusive measure for cyanogenesis occurring under natural conditions because there may be variation in tissue distribution of both factors (Gleadow and Woodrow, 2000b). Apart from the general ability for release of hydrogen cyanide and its total amount, the kinetics of HCN release may be an important aspect of plant cyanogenesis as protection against herbivores. It has to be assumed that the velocity of HCN release will affect the rate of herbivore deterrence. The fast movement of gaseous HCN in leaf tissue has been pointed out by Lieberei et al. (1996).

P. lunatus is an obligate cyanogenic species with quantitative variation of its cyanogenic properties. The status of HCNp and HCNc of leaves varied considerably depending on genotype, physiological state, and ontogenetic leaf development. Quantitative variation was evident in leaves of defined developmental stages of clonal plant material under controlled greenhouse conditions. Young leaves are generally characterized by a higher HCNp and HCNc than leaves of intermediate and mature developmental stages. Young tissues and organs of reproduction are mostly, but not in general, characterized by high contents of cyanogenic precursors. It may be assumed that these tissues need special protection because of their essential functions (Poulton, 1983, 1988; Gleadow and Woodrow, 2002). Alternatively, there might be investment decisions based on the expected life span of leaves (e.g., Coley, 1980, 1988; Coley et al., 1985). An attractive hypothesis would be that these cyanogenic substances are first used as feeding deterrents by young leaves before nitrogen is being metabolized (Selmar, 1986). Previous studies on the effects of cyanogenic glycosides on *Schistocerca americana* (grasshopper, Neoptera) and *Hypera brunneipennis* (alfalfa weevil, Coleoptera) revealed that these compounds deterred insects from feeding, but were not detrimental if ingested. Grasshoppers and weevils seemed to be more sensitive in detecting cyanogenic glycosides within food plants (Bernays, 1991).

The present study indicates a more complex action of cyanide-containing compounds and plant cyanogenesis. We were able to analyze the effect of

cyanogenesis concerning the herbivore–plant interaction between nymphs of the generalist locust *S. gregaria* and the cyanogenic plant *P. lunatus*. This was accomplished by the precise measurement of the HCNp of the particular leaf presented to the locusts and by quantitative detection of the HCN released during the feeding process of the insects. However, in consideration of these data and by additional observation of the locusts behavior during and after leaf consumption, the results suggest that plant cyanogenesis in this experimental system is involved in at least two levels of plant defense. On the one hand, high HCNp and HCNc result in a clear repellent effect. On the other hand, consumption of leaves with low HCNp and HCNc leads to severe intoxication. So, this complex action is based both on the presence of cyanide-containing compounds and the kinetics of their bioavailability.

The identification of a certain threshold level at which the HCNp and the HCNc become effective is of special interest. Certain thresholds of cyanogenic precursors and released HCN must be reached to achieve repellent action against *S. gregaria*. Severe intoxication of locusts occurred after extensive consumption of low cyanogenic leaf material. Obviously, the insects did not react to the small amounts of HCN released during the feeding process, and, thus, the total intake of cyanogenic leaf material was above a critical threshold for intoxication. It cannot be excluded that factors other than the HCN releasing compounds may be toxic to the locusts and may have caused the detrimental effects. But, the time sequence and symptoms of intoxication were the same regardless of whether we applied exogenous HCN to locusts feeding on almost noncyanogenic leaf material or whether they were feeding on the cyanogenic varieties. During feeding on high cyanogenic leaf material—especially on young leaves—considerably more HCN was released per unit time than during feeding on low cyanogenic leaves. This physiological feature of immediate HCN release in high amounts obviously represents a potent repellent effect. Thus, high and low cyanogenic leaves revealed different modes of action on the herbivore. Calculation of a HCN balance based on the consumed cyanogenic leaf material revealed that the amount of cyanide in the excrements and the amount of HCN released from the locust bodies after feeding were always low. Accordingly, the total loss of hydrogen cyanide within this balance was always high, indicating a metabolization of cyanide by the locust or an interaction of this highly reactive substance with the insect tissues. The HCN balance prepared from feeding trials with leaves of the H-group accession 2233 (wild type) indicated the impact of the HCNc as a feature with high repellent potential. Concerning this accession, bioassays with leaves of all developmental stages revealed extraordinary high percentage rates of HCN release as reaction to locust feeding compared to the other high and low cyanogenic accessions. However, leaves of all developmental stages of this accession were consumed in very low amounts independent of the widely varying HCNp.

This study demonstrates for the first time a quantified protective effect of the HCNp and of the corresponding HCNc against herbivorous insect attack depending on threshold levels. The different modes of action of high and low cyanogenic accessions of *P. lunatus* deserve further discussion, and experiments with respect to the biological function and with special regard to the ecological costs of these systems. *P. lunatus* is an agricultural crop of increasing importance as source for human food and green fodder for livestock (Debouck, 1991). It is important to evaluate possible agroecological advantages or disadvantages of cyanogenesis. HCN release is not only acting on plant–herbivore interaction, but it is also influencing plant–microbe balances (Lieberei et al., 1983) and may also give an allelochemical input to plant–soil relations. The insights gained from this experimental system could easily be transferred to other cyanogenic plants of global economic importance such as cassava (*M. esculenta* Crantz).

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