

PHENOTYPIC PLASTICITY OF CYANOGENESIS IN LIMA 2
 BEAN *Phaseolus lunatus*—ACTIVITY AND 3
 ACTIVATION OF β -GLUCOSIDASE 4

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Abstract—Cyanogenesis, the release of toxic HCN from damaged plant 12
 tissues, is generally considered as a constitutive plant defense. We found 13
 phenotypic plasticity of cyanogenesis in young leaves of lima bean *Phaseolus* 14
lunatus based on increased activity of the β -glucosidase in response to 15
 herbivore attack. Two aspects of plant cyanogenesis have to be considered in 16
 ecological analyses: (1) the cyanogenic potential (HCNp), which indicates the 17
 total amount of cyanide-containing compounds present in a given tissue, and 18
 (2) the cyanogenic capacity (HCNc), representing the release of HCN per unit 19
 time. This release is catalyzed by specific β -glucosidases, whose activity is 20
 a crucial parameter determining overall toxicity. Enzymatic activity of 21
 β -glucosidase—and, in consequence, the rate of HCN release—was increased 22
 significantly after 72 hr of incubation with spider mites as compared to 23
 noninfested leaves. Feeding by L1 larvae of Mexican bean beetles also led to 24
 enhanced enzymatic activity, whereas mechanical damage of leaf tissue had 25
 no effect on β -glucosidase activity and the release of HCN. The results place 26
 plant cyanogenesis in the group of induced resistance traits, whose degree of 27
 activity depends on the feeding by a particular herbivore. 28

Key Words—Cyanogenesis, β -glucosidase, chemical defense, constitutive 29
 defense, phenotypic plasticity, *Phaseolus lunatus*, *Tetranychus urticae*, 30
Epilachna varivestis, plant–herbivore interactions. 31

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INTRODUCTION

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Plant cyanogenesis, the release of hydrogen cyanide from cyanogenic precursors as response to cell or tissue disruption, is considered a constitutive plant defense against generalist herbivore attack (Nahrstedt, 1985; Belotti and Riss, 1994; Gleadow and Woodrow, 2002). Most studies focus on the variation of the content of cyanogenic plant compounds; however, cyanogenesis, if defined as the liberation of hydrogen cyanide per unit time, also has a kinetic character. Therefore, three different components have to be quantified to fully describe this phenomenon. These components are (1) the cyanogenic potential (HCNp), which gives the total amount of cyanide-containing compounds present in a given tissue (Lloyd and Gray, 1970), (2) the activity of specific β -glucosidases, and (3) the resulting cyanogenic capacity (HCNc), defined as the release of hydrogen cyanide per unit time (Lieberei, 1988). In contrast to the widely studied HCNp, the capacity of plants or particular plant organs for HCN release following tissue damage is rarely studied, but data available on *Phaseolus lunatus* indicate a substantial role of such kinetics in herbivore deterrence (Ballhorn et al., 2005). In this context, two modes of defense have to be considered. The function of deterrence depends on the plants' ability for fast release of high amounts of HCN during the feeding process of the herbivore. In contrast, extensive consumption of plant material with lower cyanogenic features in the long term leads to an intoxication of the herbivore. This intoxication results from the endogenous release of hydrogen cyanide within the gastrointestinal tract, which appears to be more harmful to the herbivore than the development of gaseous HCN during the feeding process (Ballhorn et al., 2005; Miguel and Alberto, 2005).

The activity of specific β -glucosidases represents the most important parameter determining the kinetics of hydrogen cyanide release (Swain et al., 1992). However, studies on possible changes of specific β -glucosidase activity and resulting changes of the cyanogenic capacity in response to herbivore or fungal attack are lacking. Only a study on the cyanogenic rubber tree (*Hevea brasiliensis*) reported a distinct increase of β -glucosidase activity in response to local, mechanical damage of leaf tissue (Voß, 2001). For many cyanogenic plants such as rubber tree, cassava (*Manihot esculenta* Crantz), sorghum (*Sorghum bicolor* (L.) Moench), clover (*Trifolium repens* L.) and *Lotus corniculatus* L.), bracken fern (*Pteridium aquilinum* (L.) Kuhn), and lima bean (*P. lunatus* L.) there is evidence for the protective effect of plant cyanogenesis (Jones, 1962; Cooper-Driver and Swain, 1976; Hruska, 1988; Bernays, 1991). Furthermore, experiments with genetically modified plants have underlined the effectiveness of cyanogenesis as an antiherbivore defense system. Transferring the complete pathway for synthesis of cyanogenic glycosides and a corre-

sponding, specific β -glucosidase into *Arabidopsis thaliana* provided resistance against *Phyllotreta nemorum*, a chrysomelid beetle that is specialized on cruciferous plants (Tattersal et al., 2001).

However, a broad range of factors on both sides of the plant–herbivore interaction affects the effectiveness of cyanogenesis as an herbivore deterrent. Focusing on the herbivores, the mode of feeding, the availability of alternative foods, as well as mechanisms of adaptation may affect the defensive potential (Walling, 2000; reviewed by Gleadow and Woodrow, 2002).

In the last decades, cyanogenesis has received considerable interest in analyses of ecological costs of plant defense (Kakes, 1989; Blaise et al., 1991; Lieberei et al., 1996; Hayden and Parker, 2002). A number of authors have described genetically based variation in cyanogenesis among populations (Lieberei, 1988; Shore and Obrist, 1992; Caradus and Forde, 1996). In addition to genotypic variability, the state of cyanogenesis may be correlated with the ontogenetic stage of the particular plant or specific plant organs (Lloyd and Gray, 1960; Till, 1987; Coley, 1980, 1988; Thayer and Conn, 1981; Schappert and Shore, 2000).

There seem to be some counteractive forces selecting against cyanogenesis because populations of some potentially cyanogenic plants are partly or even almost entirely composed of acyanogenic genotypes (Hughes, 1991; Schappert and Shore, 1995, 1999). Distribution of cyanogenesis within and among populations may depend on the presence or absence of herbivores, which lowers or increases the net fitness effects of this constitutive plant defense trait, respectively (Abbott, 1977; Ellis et al., 1977; Bokanga et al., 1994; Gleadow and Woodrow, 2000a,b). In addition, abiotic conditions, such as temperature and humidity, and their seasonal variation, as well as nutrient availability may have an impact directly on the status of cyanogenesis within a given plant and, therefore, can have the potential to affect the distribution of plant cyanogenesis at the level of populations (Jones, 1962, 1966, 1972; Cooper-Driver et al., 1977; Kakes, 1989; Calatayud et al., 1994; Calatayud and Le Rü, 1996). Thus, general problems of estimating the relevance of cyanogenesis in an ecological context arise from the highly dynamic nature of this trait that also comprises the putatively different responses of its separate parameters, i.e., HCNp, β -glucosidase activity, and HCNc.

In the present study, we use lima bean (*P. lunatus*) as a presumably obligate cyanogenic plant with high intraspecific and ontogenetic variability in its cyanogenic features (Baudoin et al., 1991; Debouck, 1991; Ballhorn et al., 2005). Spider mites (a generalist cell-content feeder) and larvae of the Mexican bean beetle (a specialist herbivore with chewing mouthparts) were used for analysis of phenotypic plasticity of β -glucosidase activity and the corresponding HCNc.

METHODS AND MATERIALS

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Plants. The experiments were carried out with 12 accessions of *P. lunatus* including 11 domesticated lines and a wild type. For further characterization of the accessions see (Table 1). Trifoliolate leaves of these accessions were characterized by different concentrations of cyanide-containing compounds (Ballhorn et al., 2005). The cyanogenic potential (HCNp) of young leaves ranged from 7.4 ± 2.1 (accession 8071) to 65.3 ± 13.4 (accession 2357) $\mu\text{mol HCN per gram leaf fresh weight (fw; mean} \pm \text{SD; } N = 11 \text{ clonal plants per accession)}$. Seed material was provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany.

Growing and Treatment of Plants. Single plants of the different accessions were vegetatively propagated (28 plants per accession) to reduce genetic variability of the experimental plants. These plants were used for the experiments when they were 8 wk old and had developed two fully expanded leaves. Clonal plant material was obtained by preparing one-node cuttings from the mother plant. Cuttings were rooted in water supplemented with Rhizopon AA 1%[®] (Rhizopon bv, Hazerwoude, Holland). The mother plants obtained from seeds as well as the cuttings were cultivated under green house conditions at a light regime of 16:8 hr light–dark period by a photon flux density of $400 \mu\text{mol sec}^{-1} \text{ m}^{-2}$ at the plant container and $900 \mu\text{mol sec}^{-1} \text{ m}^{-2}$ on the top of the plants. Supplementary 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, and the ambient relative air humidity ranged between 60 and 70%. Plants were fertilized with a nitrogen–phosphate fertilizer (Blaukorn[®]–Nitrophoska[®]–Perfekt, Compo GmbH & Co. KG, Münster, Germany) twice a week and rooted in standard substrate (TKS[®]–1-Instant, Floragard[®], Oldenburg, Germany) that was mixed with two thirds of washed sand with different grain size (0.3–0.7 and 1–2 mm). Plant containers with a diam of 18 cm were used for cultivation.

Selection of Leaf Material. Young leaves were selected for the experiments. Leaves of this developmental stage revealed the highest homogeneity of structural parameters among the accessions and the highest constancy of physiological traits (HCNp and general β -glucosidase activity) within the lines. This leaf developmental stage was classified by the position of leaf insertion at the stem as well as by leaf morphological parameters. By definition, “young leaves” are inserted near the top of the stem or a side stem. They were fully unfolded for at least 4 d, but no longer than 6 d, and the midrib of the central leaflet of these trifoliolate leaves was about 4 cm long. Leaves of this ontogenetic stage were characterized by a bright green color and a soft texture.

Arthropods. Two-spotted spider mites (Acari: Tetranychidae: *Tetranychus urticae* Koch) and Mexican bean beetles (Coleoptera: Coccinellidae: *Epilachna*

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varivestis Mulsant) were used as activating agents. Both the maintenance culture of the spider mites as well as of the Mexican bean beetles were kept on *Phaseolus vulgaris* cv. "Saxa" under identical ambient conditions as experimental plants. The progenitors of the Mexican bean beetle culture were provided by C.P.W. Zebitz, Department of Applied Entomology, University of Hohenheim, Otto-Sander Str. 5, Germany.

Infestation of Leaves with Spider Mites. Fourteen clonal plants were used per accession. The terminal leaflet of one trifoliate leaf per plant was infested with 10 adult females that had been kept on *P. vulgaris* before. Experiments were carried out in the green house under identical ambient conditions as adjusted for plant cultivation. For analysis of plant responses to spider mite attack, the infested leaflets of selected trifoliate leaves were bagged with perforated, transparent polyethylene foil (approx. 9 holes/cm², each with a diam of 0.5 mm), whereas the other two leaflets of the leaf were bagged but remained uninfested. In addition, leaves of control plants were bagged in the same way. These plants were cultivated in a separate chamber under identical ambient conditions. In parallel experimental settings, mites were left on the leaflets for 24, 48, and 72 hr, respectively. The β -glucosidase activity of the infested leaflets was quantitatively compared to the two untreated leaflets of the same leaf, which were pooled for analysis, as well as to leaves of the control plants characterized by the same ontogenetic stage. Leaflets of these control leaves were pooled for analysis of the β -glucosidase activity.

Leaf Damage by Mexican Bean Beetles. In experiments using the Mexican bean beetle as attacking herbivore, three freshly hatched L1 larvae were placed on the terminal leaflet of a trifoliate leaf. Running time of the feeding trials and bagging of leaflets were consistent with the experiments using spider mites as herbivores. Noninfested control plants were used in the same way as mentioned above.

Effect of Artificial Damage. Artificial damage was applied to the leaflets using a plastic (PE) pin with a diam of 1.5 mm. Initially, 15 injuries were set per leaflet. In parallel experimental settings, additional 15 injuries were set every 24 hr to achieve an increasing damage of leaf tissue visually similar to the leaf area damaged by the Mexican bean beetles. The damaged leaflets were bagged in the same way, and untreated but bagged control plants were used as described for the plants wounded by herbivores.

Harvest of Leaves for Analysis. Leaves were cut off with a razor blade after the respective period of incubation. The three leaflets per leaf were separated by the criteria "treated" and "untreated" and were immediately prepared for analysis of β -glucosidase activity. Leaf material of control plants was prepared in the same way.

Extraction and Purification of β -Glucosidase. Whole leaflets were weighed and homogenized in a fourfold volume of 67 mmol l⁻¹ phosphate buffer adjusted

to pH 6.4. The extract was filtered through cotton fabric and centrifuged at 196
20,000 $\times g$ and 4°C (RC5C, Sorvall). The protein-containing supernatant was 197
concentrated by ammonium sulfate fractionation and filtered through membrane 198
caps with a pore size <10,000 kD (Schleicher & Schuell BioScience GmbH, 199
Dassel, Germany). 200

Constitutive β -Glucosidase Activity. The quantification of β -glucosidase 201
activity in the extracts was based on the detection of *p*-nitrophenol released by 202
hydrolysis of the chromogenic artificial substrate *p*-NP-glucoside (Merck; Hösel 203
and Nahrstedt, 1975; Selmar, 1981, 1986). The standard incubation mixture for 204
analysis of the enzymatic activity contained 1-ml substrate solution (2 mmol) in 205
citrate buffer adjusted to pH 5.6. The incubation mixture was made up by citrate 206
buffer (pH 5.6) to a volume of 4.9 ml, and finally 0.1 ml extract was added. 207
After 10 min of incubation at 30°C, the reaction was stopped by adding 1 ml 208
ice-cold sodium carbonate solution (1 mol/l), and the released *p*-nitrophenol 209
was quantified spectrophotometrically at 400 nm (Pharmacia Biotech, Ultraspec 210
3000). The β -glucosidase activity was calculated per gram leaf dry weight as 211
katal (kat). An enzyme activity of 1 kat is defined as a substrate conversion rate 212
of 1 mol substrate per second under standard temperature and pressure. The 213
calculation of the enzyme activity was carried out by use of a coefficient of 214
extinction for *p*-nitrophenol ($\epsilon_{400 \text{ nm}} = 16,159 \text{ l/mol cm}$; Voß, 2001). 215

HCN Detection System. The kinetic analysis of HCN release from 216
experimentally treated lima bean leaves was carried out using an airflow system 217
(Ballhorn et al., 2005). This vessel system was passed by a constant airflow 218
adjusted at 7.0 l/hr. Infested and noninfested leaflets of the experimental plants 219
as well as terminal leaflets of the control plants were placed in the equipment, 220
respectively. The leaflets were treated with chloroform (250 μl /leaflet) to 221
achieve complete tissue disintegration and, in consequence, the release of gaseous 222
hydrogen cyanide from the accumulated cyanogenic precursors. At the discharge 223
opening of the equipment, the air together with the transported HCN was led into 224
a test tube containing 0.1 mol/l NaOH solution. Thus, cyanide was fixed as NaCN 225
and then was spectrophotometrically quantified at 585 nm as a polymethine dye 226
that was formed by use of Spectroquant[®] cyanide test (Merck). We used a 227
coefficient of extinction of ($\epsilon_{585 \text{ nm}} = 131,600 \text{ l/mol cm}$) for calculation of the 228
cyanide concentration in the samples following the product description. 229

Statistics. Statistical analyses were carried out with Statistica 6.0 (Statistica 230
System Reference, 2001). 231

RESULTS

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Variability of β -Glucosidase Activity. Substantial interaccession variability 234
of β -glucosidase activity in young leaves was found among the 12 accessions of 235

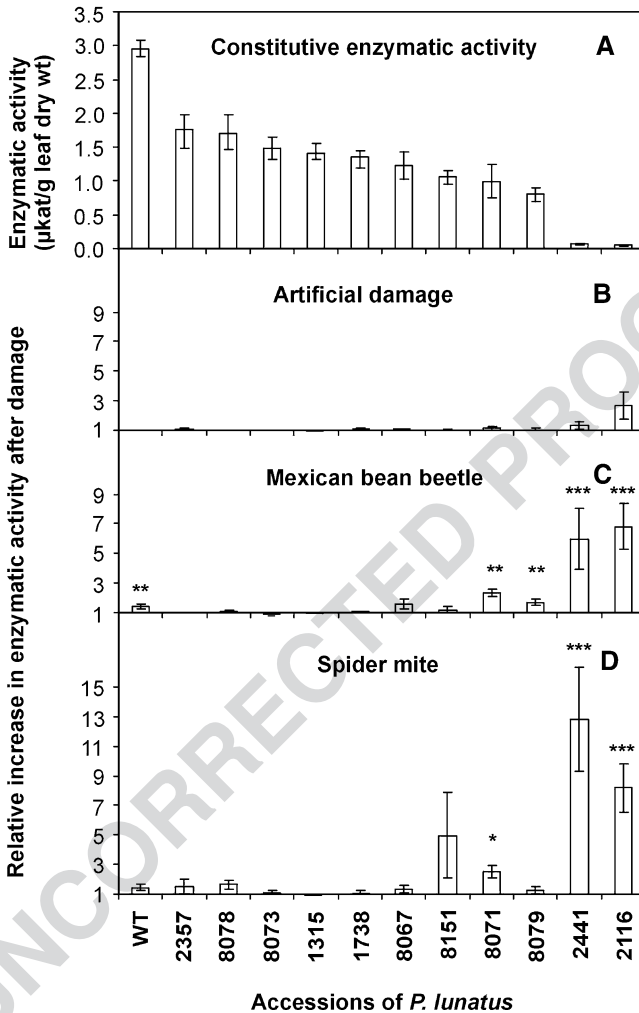


FIG. 1. Increase in β -glucosidase activity following leaf damage. Presented is the constitutive β -glucosidase activity in young *Phaseolus lunatus* leaves (A) and relative increases in enzyme activity in response to attack from artificial damage (B), larvae of Mexican bean beetles (C), and spider mites (D). Values are means \pm SE [($N = 15$ leaves per accession (A) and $N = 7$ leaflets of different plant individuals per accession (B–D)]. Asterisks represent significant increase in enzymatic activity ($***P < 0.001$; $**P < 0.01$; $*P < 0.05$).

P. lunatus (Figure 1A). Among these lines, the enzymatic activity ranged from 0.016 to 3.765 μ kat per gram leaf dry weight ($N=9$ per accession) and varied significantly depending on the genotype (ANOVA: $F=21.861$, $df=11$, $P<0.001$).

Attacking Period. In experimental settings that used spider mites as well as Mexican bean beetles as damaging agents, substantial increase of β -glucosidase activity was found after 72 hr of incubation, whereas no distinct increase of the enzymatic activity was observed after an incubation period of 24 and 48 hr. Thus, results presented hereafter refer to an incubation period of 72 hr (Figure 1B–D).

Effect of Spider Mite Infestation on β -Glucosidase Activity. A factor of β -glucosidase activity increase was calculated by dividing the enzyme activity (μ kat) of the infested leaflets by the values of activity of the noninfested leaflets belonging to the same leaves. Enzyme activity in untreated leaflets of treated leaves was not significantly different from activity in completely untreated leaves of control plants (ANOVA: $F=0.012$, $df=1$, $P=0.917$). Relative increases in β -glucosidase activity are therefore expressed only as a factor of β -glucosidase activity increase comparing the damaged and undamaged leaflets of single leaves. These factors generally gave values of 1 or more and indicated an increase of β -glucosidase activity following spider mite attack. Only the accession 1315 showed a slight decrease of enzymatic activity after infestation with spider mites (Figure 1B). The accessions 2441 and 2116 showed a substantial increase of enzymatic activity by the factor 12.8 ± 3.6 and 8.2 ± 1.6 (mean \pm SE), respectively. For these accessions, as well as for the accession 8071, the enzymatic activity in infested leaflets increased significantly in response to spider mite attack (ANOVA; 2441: $F=130.614$, $df=1$, $P<0.001$; 2116: $F=317,091$, $df=1$, $P<0.001$; and 8071: $F=5477$, $df=1$, $P<0.05$). However, none of the other accessions showed significantly enhanced enzymatic activity following spider mite attack.

Effect of Damage by Mexican Bean Beetle Larvae on β -Glucosidase Activity. The β -glucosidase activity of selected *P. lunatus* accessions was increased by feeding of Mexican bean beetle larvae (Figure 1B). Exceptions are only the accessions 2357, 1315, and 8073, which showed a slight decrease of the enzymatic activity. Leaflets of the accessions 2116, 2441, 8071, and 8079 exhibited significantly increased β -glucosidase activity in response to leaf damage by these insects as compared to the nondamaged leaflets of the same leaf (2116: $F=31.131$, $df=1$, $P<0.001$; 2441: $F=23.968$, $df=1$, $P<0.001$; 8071: $F=13.320$, $df=1$, $P<0.01$; 8079: $F=14.052$, $df=1$, $P<0.01$; WT: $F=10.397$, $df=1$, $P<0.01$). These nondamaged leaflets, in turn, never showed significant differences of β -glucosidase activity when compared to leaves of the control plants ($F=0.006$, $df=11$, $P=0.994$).

Artificial Damage of Leaves. Among all accessions, injuries of leaflets set with a plastic pin had no effect on β -glucosidase activity in these leaflets as compared to the remaining leaflets ($F=0.055$, $df=1$, $P=0.815$). However,

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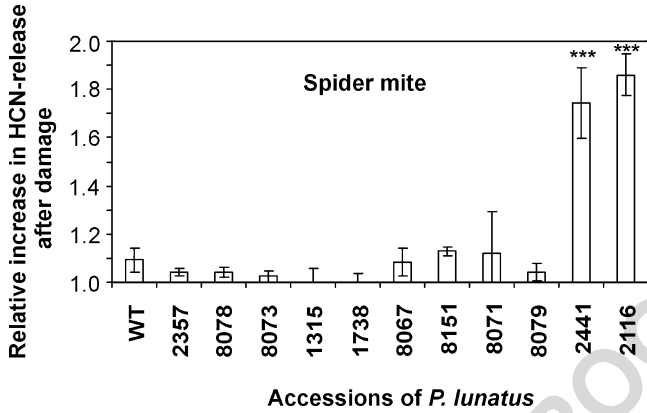


FIG. 2. Increase in HCN liberation by enhanced β -glucosidase activity. The relative increase of gaseous hydrogen cyanide release per unit time was accelerated in response to increased β -glucosidase activity provoked by spider mite attack. Values are means \pm SE ($N = 7$ leaflets of different plant individuals per accession). Asterisks represent significant increases in HCN liberation (** $P < 0.001$).

8 among 12 accessions of *P. lunatus* showed factors of β -glucosidase activity increase higher than 1. 278 279

Effect of Increased β -Glucosidase Activity on HCNc. The increase in β -glucosidase activity in spider-mite-infested leaflets compared to untreated leaflets of the same leaves was correlated with increased HCN release from these leaflets as reaction to breakdown of cell integrity (Figure 2). Similar to the relative increase of enzymatic activity, we calculated a factor of relative increase of HCN release per unit time by dividing the amount of released HCN of the infested leaflets by the values of HCN release of noninfested leaflets belonging to the same leaves. The cyanogenic capacity of spider-mite-infested leaflets of the accessions 2116 and 2441 was accelerated compared to noninfested leaflets of these *P. lunatus* lines (2116: $F = 41.850$, $df = 1$, $P < 0.001$ and 2441: $F = 45.984$, $df = 1$, $P < 0.001$). The other accessions showed no increase of HCN release in response to spider mite attack. However, the increase in HCN release of the accession 2357 was close to statistical significance ($F = 4.52$, $df = 1$, $P = 0.051$). 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296

DISCUSSION

Plant cyanogenesis represents a complex trait in herbivore defense because of high variability of the cyanogenic system. Besides the heterogeneous distribution of cyanogenesis among or within plant populations, the cyanogenic 297 298 299

status of an individual plant is not static (Till, 1987). Therefore, analyses testing only for the presence or absence of cyanogenic glycosides and the general ability for HCN release do not address the variability of this constitutive herbivore defense.

In this study, enzymatic activity of β -glucosidase, a crucial factor limiting the rate of HCN release, increased in response to herbivore attack. Cyanogenesis thus comprises an inducible component, a finding placing it in the group of induced plant responses to herbivores. We found substantial interaccession variability because significant increases in β -glucosidase activity were observed only in some of the accessions tested. Furthermore, the responses of these accessions depended on the type of herbivores causing the damage. Spider mite attack generally led to a higher increase in β -glucosidase activity than leaf damage caused by larvae of the Mexican bean beetle (Figure 1C and D). This result most likely is a consequence of the different modes of feeding of these herbivores. Spider mites are cell-content feeders and damage single cells or limited areas of epidermal and mesophyll cells per feeding incident (Raven, 1983), whereas Mexican bean beetles remove larger areas. Furthermore, elicitors may be present in the herbivores' saliva, which can have varying effects on resistance-related plant enzymes such as pectinases, cellulases, amylases, proteases, lipases, alkaline and acidic phosphatases, and peroxidases (Miles, 1999). Support for this interpretation comes from the observation that pure mechanical damage of similar leaf areas as damaged by the herbivores did not significantly induce β -glucosidase activity.

To test whether increased β -glucosidase activity, in fact, leads to increased HCN release, we used spider mite-infested leaves. The highly significant increases in β -glucosidase activity of the accessions 2116 and 2441 indeed were correlated with significant increases in HCN release (Figure 2). This finding underlined the impact of β -glucosidase activity on kinetic patterns of cyanogenesis. However, the increase in HCN release was quantitatively lower than the increase in β -glucosidase activity. Accessions 2116 and 2441 showed increases in β -glucosidase activity by factors greater than 8 and 12 (Figure 1D), whereas the magnitude in the relative increases in HCN release was less than twofold (Figure 2). This result can be explained when we assume that increased β -glucosidase activity occurred only in limited areas directly adjacent to the cells affected by spider mite feeding. In experiments with rubber tree, areas of enzymatic activation following leaf damage appeared to be local (Voß, 2001). Thus, increased enzymatic activity and enhanced HCN liberation because of herbivory might not affect the whole leaf. Another explanation for the non-proportional increase of β -glucosidase activity and HCN release could be the activity of hydroxynitrile lyases, which are catalyzing the dissociation of hydroxynitriles as the second step of cyanogenesis and, therefore, have the potential to limit the release of HCN (Selmar et al., 1989).

A further point to be regarded in this context is the multifunctional character of β -glucosidases as pointed out by Selmar et al. (1987), particularly the putative involvement of β -glucosidases in mechanisms belonging to systemic acquired resistance against pathogens (SAR). SAR represents a systemic and multigenic plant response to a local infection that leads to a broad-spectrum resistance to subsequent infections (Lamb and Dixon, 1997). SAR is primarily induced by pathogens, but tissue damage by piercing and sucking insects and mites induces similar responses (Russo et al., 1997; Bostock, 1999; Walling, 2000; Grinberg et al., 2005). Two important aspects of SAR are the accumulation of phytoalexins and enhanced local lignification of cell walls (Hammerschmidt, 1999a,b; Bruxelles and Roberts, 2001), and β -glucosidases are involved in both processes. Garcia et al. (1995) reported an essential function of β -glucosidases for the release of the phytoalexin scopoletin from its glycoside scopolin in leaves of the cyanogenic rubber tree (*H. brasiliensis*). The role of β -glucosidases for lignin biosynthesis in the course of defense reactions of *Triticum aestivum* against the fungal pathogen *Puccinia graminis* was demonstrated by Kogel et al. (1991). The induction of β -glucosidase reported is likely to have functions beyond the increase in HCN release.

Taken together, the results of our study underline the plastic character of plant cyanogenesis caused by phenotypic plasticity of β -glucosidase activity. This enzyme is an essential agent for the kinetics of HCN release, but further functions of the β -glucosidase present in *P. lunatus* cannot be excluded. Nevertheless, inducibility of β -glucosidase activity further adds to the ecological complexity in the interaction of cyanogenic plants with their biotic environment.

Mechanisms involved in the activation of specific β -glucosidases remain elusive. Voß (2001) reported immediate activation of specific β -glucosidase of the cyanogenic rubber tree (*H. brasiliensis*) as response to mechanical injury of leaves. This activation takes place within seconds. Hence, mechanisms of gene activation and mRNA synthesis could be excluded for this HCN-releasing plant, and posttranslational processes affecting the β -glucosidase activity have to be assumed. In *P. lunatus*, a significant increase of the enzymatic activity was measured at the earliest after 72 hr of incubation. This time lag of activation may be a result of mRNA synthesis in the course of gene activation.

The plastic character of enzymatic activity in *P. lunatus* (and therefore the plasticity of cyanogenesis) appeared to be local because feeding of spider mites and L1 larvae of the Mexican bean beetle on one single leaflet had no inductive effect on β -glucosidase activity in the other two leaflets of the same leaf. This was verified by comparing β -glucosidase activity of these untreated leaflets with leaves of entirely untreated clonal control plants, which were not significantly different. Thus, the increase in β -glucosidase activity of leaflets damaged by herbivores was only detected in the damaged leaflets. The lima bean represents

an experimental plant that is well established for analyses of plant responses to herbivore damage. The focus of research lies on the mechanisms of VOC emission and the processes induced on the molecular level (Arimura et al., 2000) and, more recently, on the activation of extrafloral nectar in the field of indirect defenses (Heil, 2004). Cyanogenesis of the lima bean as a direct defense with high intraspecific and ontogenetic variability, as well as a certain state of phenotypic plasticity, represents an aspect that has to be considered in ecological analysis and evaluation of ecological costs of plant defense in this model system. Plant cyanogenesis of *P. lunatus* appears not to be a constitutive defense in the classical sense.

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- Q1. Table 1 was cited, but not provided. Please check.
- Q2. Please provide publisher's location for the Refs. Bokanga et al. 1994, Selmar 1981, 1986, and Voß 2001.
- Q3. Please provide volume no. for the reference Loyd and Gray 1970.

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