Journal of Ecology

British Ecological Society

Journal of Ecology 2010, **98**, 226–236 doi: 10.1111/j.1365-2745.2009.01591.x

Direct trade-off between cyanogenesis and resistance to a fungal pathogen in lima bean (*Phaseolus lunatus* L.)

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Summary

1. Plants are simultaneously attacked by multiple herbivores and pathogens. While some plant defences act synergistically, others trade-off against each other. Such trade-offs among resistances to herbivores and pathogens are usually explained by the costs of resistance, i.e. resource limitations compromising a plant's overall defence.

2. Here, we demonstrate that trade-offs can also result from direct negative interactions among defensive traits. We studied cyanogenesis (release of HCN) of lima bean (Fabaceae: *Phaseolus lunatus*) and effects of this efficient anti-herbivore defence on resistance to a fungal pathogen (Melanconiaceae: *Colletotrichum gloeosporioides*).

3. Leaf tissue destruction by fungal growth was significantly higher on high cyanogenic (HC) lima bean accessions than on low cyanogenic (LC) plants. The susceptibility of HC accessions to the fungal pathogen was strongly correlated to reduced activity of resistance-associated polyphenol oxidases (PPOs) in leaves of these plants. LC accessions, in contrast, showed high PPO activity, which was correlated with distinct resistance to *C. gloeosporioides*.

4. Experimentally applied, gaseous HCN reduced PPO activity and significantly increased the size of lesions caused by *C. gloeosporioides* in LC leaves.

5. Field observations of a wild lima bean population in Mexico revealed a higher infection rate of HC compared to LC plant individuals. The types of lesions observed on the different cyanogenic plants in nature were similar to those observed on HC and LC plants in the laboratory.

6. *Synthesis.* We suggest that cyanogenesis of lima bean directly trades off with plant defence against fungal pathogens and that the causal mechanism is the inhibition of PPOs by HCN. Our findings provide a functional explanation for the observed phenomenon of the low resistance of HC lima beans in nature.

Key-words: *Colletotrichum gloeosporioides*, direct defence, multiple defence syndrome, plant–pathogen interaction, plant resistance, polyphenol oxidase, PPO, trade-off

Introduction

In natural systems, plants defend themselves against attack from multiple herbivores and pathogens with various resistance strategies. Some defences against herbivores are effective also against pathogens, while others are specific to particular attackers (Mitchell-Olds *et al.* 1995; Juenger & Bergelson 1998; Stout *et al.* 1998; Tiffin & Rausher 1999; Stinchcombe & Rausher 2001). However, in many cases the functional interplay of different defence mechanisms is little understood and experimental approaches that quantitatively analyse interacting plant defences against herbivores and pathogens are rare (Felton & Korth 2000; Paul, Hatcher & Taylor 2000). To fill this gap, we focused on two direct defensive traits of lima bean – cyanogenesis and activity of polyphenol oxidases (PPOs). Polyphenol oxidases are enzymes that are involved in a broad array of defence-associated plant responses including responses to pathogen attack (Campos *et al.* 2004).

Cyanogenesis is the enzymatically accelerated release of toxic hydrogen cyanide (HCN) from pre-formed cyanide-containing precursors in response to cell damage (Møller & Seigler 1999). This trait is widely distributed among higher plants and is a highly efficient plant defence against herbivores (e.g. Bernays *et al.* 1977; Bernays 1991; Ballhorn, Lieberei & Ganzhorn

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2005; Ballhorn, Heil & Lieberei 2006; Ballhorn & Lieberei 2006; Ballhorn *et al*. 2007, 2008a, 2009). In contrast to studies on herbivore–plant interactions, comparably little research has been conducted on the role of cyanogenesis in plant interactions with fungal pathogens (Lieberei *et al*. 1989; Lieberei, Fock & Biehl 1996; Nielsen *et al*. 2005).

Hydrogen cyanide is highly toxic to all eukaryotes due to inhibition of the mitochondrial respiration pathway by blocking the cytochrome a/a3-dependent oxidase (Solomonson 1981). During infection by pathogens, gaseous HCN diffuses from destroyed plant cells through the tissue surrounding the infected area and, thus, both the plant itself and the pathogen are in direct contact with the released HCN (Lieberei, Fock & Biehl 1996). Strikingly, despite the strong and general toxicity of cyanide to eukaryotic cells, no consistent functional association between cyanogenesis and resistance of plants to pathogenic fungi has been found so far (Rissler & Millar 1977; Fry & Myers 1981; Lieberei 1988; Lieberei *et al*. 1989; Nielsen *et al*. 2005). Partly, this is due to the effective HCN detoxification in some pathogenic fungi, e.g. by cyanide hydratases (Osbourn 1996). However, several studies suggest that highly cyanogenic plants are even more susceptible to fungal pathogens than conspecifics with lower cyanogenic properties (Dirzo & Harper 1982; Lieberei *et al*. 1989; Lieberei, Fock & Biehl 1996; Osbourn 1996).

This apparent contradiction can be explained by negative effects of cyanide on plant enzymes involved in anti-pathogen defence (Solomonson 1981; Lieberei 2007). Hydrogen cyanide quantitatively affects many enzymatic activities, most of all those of metal-containing enzymes. In addition to catalases and peroxidases, PPOs represent an important group of plant enzymes involved in plant defences against pathogens and herbivores. Polyphenol oxidases oxidate phenolic compounds to reactive quinones (Felton *et al*. 1989, 1992; Bi *et al*. 1995; Stout, Workman & Duffey 1996; Thaler *et al*. 2001, 2002; Kranthi, Kranthi & Wanjari 2003). These enzymes have been reported to be involved in plant defence against arthropod (e.g. Felton *et al*. 1989, 1992; Stout, Workman & Duffey 1996) and nematode herbivores (e.g. Mayer 2006) as well as to provide resistance to bacterial and fungal pathogens (e.g. Lieberei *et al*. 1989; Nicholson 1992; Campos *et al*. 2004; Thipyapong, Stout & Attajarusit 2007).

In our present study, we used lima bean (Fabaceae: *Phaseolus lunatus* L.) as experimental plant. Lima bean is well-studied concerning inducible indirect defences against herbivores (e.g. Dicke 1999; Dicke & Vet 1999; Arimura, Kost & Boland 2005; Mithöfer, Wanner & Boland 2005; Choh, Kugimiya & Takabayashi 2006a, b; Heil & Silva Bueno 2007; Kost & Heil 2008). However, in addition to indirect defences, some lima bean genotypes are characterized by strong cyanogenesis and therefore are also efficiently directly defended against herbivores (Ballhorn, Lieberei & Ganzhorn 2005; Ballhorn, Heil & Lieberei 2006, Ballhorn & Lieberei 2006; Ballhorn *et al*. 2007, 2008a, 2009). Although lima bean exhibits these two defence mechanisms, we recently demonstrated that cyanogenesis and total release of volatile organic compounds (VOC) in lima bean are negatively correlated to each other, i.e. a quantitative trade-off occurs (Ballhorn *et al*. 2008b, c). Whether trade-offs also exist between cyanogenesis and anti-pathogen defence of lima bean remains elusive so far.

Thus, the aim of our study was to quantify putative effects of cyanogenesis on anti-pathogen defence. By this approach, we intended to provide new insights into the functional interplay of defences against different attackers. We used six cultivated accessions of lima bean with highly different concentrations of cyanogenic precursors in their leaves and measured their resistance to the fungal pathogen *Colletotrichum gloeosporioides*. To experimentally confirm the observed trade-off between cyanogenesis and anti-pathogen defence, we applied gaseous HCN to leaves inoculated with *C. gloeosporioides*. In the present study, our goal was to demonstrate that quantitative trade-offs between anti-herbivore and anti-pathogen defences have to be considered in functional analyses of plant ecology in networks of interacting species.

Materials and methods

PLANT MATERIAL

For bioassays with pathogens, we used three high cyanogenic (HC) (CV_2357, CV_8078, CV_1315) and three low cyanogenic (LC) (CV_2441, CV_8073, CV_8067) accessions of lima bean (Fabaceae: *Phaseolus lunatus* L.). Selected accessions represented the upper and lower range of cyanide concentration in leaves of wild-type plants measured under natural field conditions (Ballhorn *et al*. 2009). Seeds were provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany.

PATHOGEN MATERIAL

In preliminary experiments, we screened defined strains of the fungal bean pathogen *Colletotrichum lindemuthianum* (Saccardo & Magnus) Briosi & Cavara [strains 12250 and 63144 obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany)] and the closely related generalist plant pathogen *Colletotrichum gloeosporioides* (Penzig) Penzig & Saccardo (strains 62136 and 62146) for their ability to efficiently infect lima bean leaves under laboratory conditions. Inoculation of plants with spore solutions showed that the strain 62146 of *C. gloeosporioides* was most compatible to lima bean genotypes. In addition, this strain was most reliable in producing spores, which was an obligatory precondition for conducting reproducible inoculation experiments. Thus, we selected *C. gloeosporioides* strain 62146 for bioassays.

The cosmopolitan fungus *C. gloeosporioides* is a facultative hemibiotrophic plant pathogen. Infection of plant tissue occurs through wounds or stomata as well as via penetration of intact plant surfaces (Wijesundera, Bailey & Byrde 1984; Bailey *et al*. 1992). At this stage, the fungus lives biotrophically and develops primary infection hyphae (Nicholson 1992; Bergstrom & Nicholson 1999). When the host tissue is destroyed in the course of an infection, the fungus develops secondary necrotrophic hyphae and produces new spores after a few days.

CULTIVATION OF PLANTS AND PATHOGENS

Plants were cultivated under glasshouse conditions (16:8 L:D with a photon flux density of 350–450 μmol m^{-2} s^{-1} at the plant container and 800–950 μmol m^{-2} s^{-1} at the top of the plants, depending on

natural sun light). Additional light was provided by 400 W high-pressure sodium lamps (Son-T Agro 400; Philips®; Hamburg, Germany). To avoid hot spots under the lamps, the position of the plant containers was changed every 3 days. Temperature [25 °C (day): 20 °C (night)] and ambient relative air humidity (60–70%) were controlled by INTEGRO® (Surrey, Canada) software. Plants were fertilized with a nitrogen–phosphate fertilizer (Blaukorn®-Nitrophoska®-Perfekt; Compo GmbH; Münster, Germany) twice a week (3 mg per pot). A 1:1 mixture of standard substrate (TKS®-1-Instant, Floragard®; Oldenburg, Germany) and sand (grain size 0.5–2.0 mm) served for cultivation in plant containers (18 cm in diameter). Twenty-eight plants per accession were grown (full sibs). Plants used for the experiments were 5 weeks old and had developed five to six leaves.

Colletotrichum gloeosporioides was cultivated in Petri dishes (9.5 cm in diameter) on oat medium, pH 5.0 (4% flour, 1.5% agar). Cultures were stored in a climatic chamber at a temperature of 25 °C, a relative air humidity of 85%, and under a light regime of 10 min light (150 μmol m^{-2} s^{-1}) per 12 h to induce development of spores.

LEAF MATERIAL USED FOR BIOCHEMICAL ANALYSES AND BIOASSAYS

Young leaves of a defined developmental stage were used for all biochemical analyses and bioassays to assess effects of cyanogenic features and PPO activity on pathogen growth. From each plant the third trifoliate leaf from the top was selected (Fig. 1). These leaves were characterized by soft leaf tissue, had been unfolded for 5–6 days and did not exceed one-third to one half of the final leaf size. Leaf mass per area (LMA) was considered as an index of sclerophylly to test for similar morphological features of leaves among experimental plants (Table 1).

The aim of this study was to establish a direct quantitative relationship of chemical leaf characteristics and their quantitative effects on pathogen performance. For this purpose, trifoliate leaves were partitioned for the respective analyses (Fig. 1). One leaflet per leaf was analysed for gaseous HCN release after chemical cell disruption (HCNc; cyanogenic capacity). The second leaflet was cut along the midrib. One half was used for analysis of cyanogenic potential (HCNp; concentration of cyanogenic precursors) and for analysis of

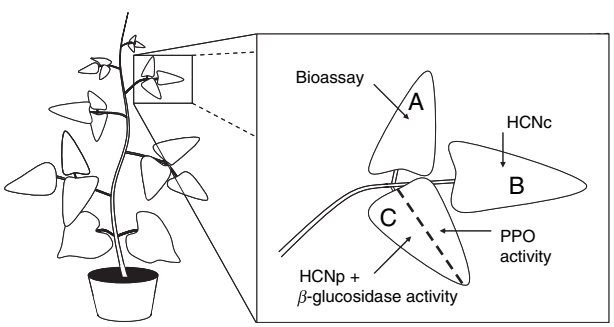

Fig. 1. Sampling of leaf material. Individual trifoliate leaves inserting three positions down the apex were used for quantitative analyses of plant traits and their effects on pathogen performance ($n = 14$ plants per accession with, $n = 1$ leaf per plant). Two intact leaflets per leaf were used for bioassays with *Colletotrichum gloeosporioides* (A) and HCNc analysis (B), respectively. The third leaflet (C) was partitioned and one half was used for analysis of HCNp and β-glucosidase activity (using the same leaf extract), while the other half was used for analysis of polyphenol oxidase (PPO) activity. The selection of leaflets (terminal or lateral) for the respective analysis or experiment was set at random.

β-glucosidase activity. For both analyses, the same leaf extract was used. The other half served for quantification of PPO activity. The third leaflet was used for bioassays with *C. gloeosporioides* (Fig. 1). Because the leaflets of the same leaf were used to conduct the biochemical and pathogen growth analyses, it was possible to relate the results on pathogen performance directly to the biochemical characteristics of that same leaf.

QUANTIFICATION OF CYANOGENIC FEATURES

For analysis of cyanogenic potential (HCNp), fresh leaflets were weighed to the nearest 0.001 g and ground with liquid nitrogen and cooled mortar and pestle (4 °C) under addition of the fourfold volume (v fresh weight^{-1}) of ice-cold Na_2HPO_4 solution (67 mmol L^{-1}). Samples were quantitatively analysed for their HCNp by complete enzymatic hydrolysis of cyanogenic precursors with substrate-specific β-glucosidase isolated from rubber tree [Euphorbiaceae: *Hevea brasiliensis* (WILLD. ex JUSS) MUELL. ARG]. We used closed glass vessels (Thunberg vessels) for incubation (20 min at 30 °C in a water bath) of leaf extracts together with enzyme solution adjusted to an activity of 20 nkat. Quantification of released HCN was carried out spectrophotometrically at 585 nm using the Spectroquant® cyanide test (Merck KGaA, Darmstadt, Germany) according to Ballhorn, Lieberei & Ganzhorn (2005).

Activity of β-glucosidase in leaflets was analysed according to Ballhorn, Heil & Lieberei (2006). Leaf extracts were centrifuged for 15 min at 20 000 × *g* and 4 °C (RC5C; Sorvall Instruments, DuPont Co. Bioresearch Systems, Wilmington, DE, USA). The protein-containing supernatant was concentrated by ammonium sulphate fractionation and filtered through membrane caps with a pore size < 10 000 kD (Schleicher & Schuell BioScience GmbH, Dassel, Germany). We used *p*-NP-glucoside (Merck KGaA) as chromogenic artificial substrate. Released *p*-nitrophenol was quantified spectrophotometrically (400 nm, Ultraspec 3000; Amersham Pharmacia Biotech, Freiburg, Germany) after 10 min of incubation at 30 °C and after the reaction was stopped by adding 1 mL ice-cold sodium carbonate solution (1 mol L^{-1}).

Cyanogenic capacity (HCNc; release of gaseous hydrogen cyanide per unit time) was measured using an airflow system according to Ballhorn, Lieberei & Ganzhorn (2005). In this experimental set-up, chloroform (350 μL per leaflet) was pipetted onto intact leaflets and the subsequent release of gaseous HCN from the disintegrated cells was detected quantitatively (Fig. 2). The airflow in the system was adjusted to 7.0 L h^{-1}. In order to test for any remaining non-cleaved cyanogenic precursors in the leaf material after HCNc analysis, we prepared extracts from leaflets, added specific exogenous β-glucosidase and analysed the cyanogenic potential (HCNp) of leaf material in Thunberg vessels as described previously.

QUANTIFICATION OF POLYPHENOLOXIDASE ACTIVITY

We quantified enzymatic activity of PPOs by measuring the O_2 consumption during the oxidation of polyphenols and their derivates to quinones (Richter, Lieberei & von Schwartzenberg 2005). The activity was determined polarographically, using the Clark electrode system (Yellow Springs Instruments, Yellow Springs, OH, USA), by measuring the concomitant oxygen depletion. The electrode was calibrated to 100% O_2 saturation using O_2-saturated Sörensen buffer (phosphate–citrate buffer, pH 5.6). We used 4-methylcathechol (Fluka Chemie GmbH, Deisenhofen, Germany) as standard. The autoxidation of 4-methylcathechol was measured under stirring before each PPO activity analysis. The solution for measuring

Table 1. Cyanogenic and morphological traits of lima bean leaves. Young leaves from different individual plants were screened for their cyanogenic features ($n = 14$ individual plants per accessions). Values shown for leaf characteristics are means ± SD. Significant differences between accessions were calculated by a *post hoc* analysis (Tukey's HSD; $P < 0.05$) after one-way ANOVA and are indicated by different letters in parentheses

	Cyanogenic traits				Morphological traits*	
Accessions	Cyanogenic type† [high (HC) and low (LC)]	Cyanogenic potential HCNp (μmol HCN g^{-1} fresh wt.)	Cyanogenic capacity HCNc (μmol HCN g^{-1} fresh wt. min^{-1})	β-glucosidase activity (μkat g^{-1} dry wt.)	Leaf texture	Leaf mass per area LMA (mg cm^{-2})
CV_2357	HC	67.22 ± 4.62 (a)	0.63 ± 0.07 (a)	1.88 ± 0.23 (ab)	Soft	0.84 ± 0.07 (a)
CV_8078	HC	63.18 ± 5.00 (a)	0.58 ± 0.09 (a)	1.91 ± 0.15 (a)	Soft	0.86 ± 0.13 (a)
CV_1315	HC	58.01 ± 6.41 (ab)	0.52 ± 0.09 (ab)	1.67 ± 0.25 (bc)	Soft	0.85 ± 0.12 (a)
CV_2441	LC	27.78 ± 2.78 (c)	0.12 ± 0.02 (cd)	1.19 ± 0.11 (d)	Soft	0.85 ± 0.11 (a)
CV_8073	LC	22.59 ± 4.38 (cd)	0.20 ± 0.03 (c)	1.48 ± 0.23 (cd)	Soft	0.87 ± 0.10 (a)
CV_8067	LC	19.61 ± 2.51 (d)	0.18 ± 0.04 (c)	1.46 ± 0.16 (cd)	Soft	0.85 ± 0.12 (a)

*We selected lima bean accessions with similar leaf texture from a larger set of experimental plants, since some genotypes are characterized by hard-textured leaves (Ballhorn, Heil & Lieberei 2006).
†Accessions were assigned to different cyanogenic types (HC and LC) according to significant differences in their HCNp and HCNc.

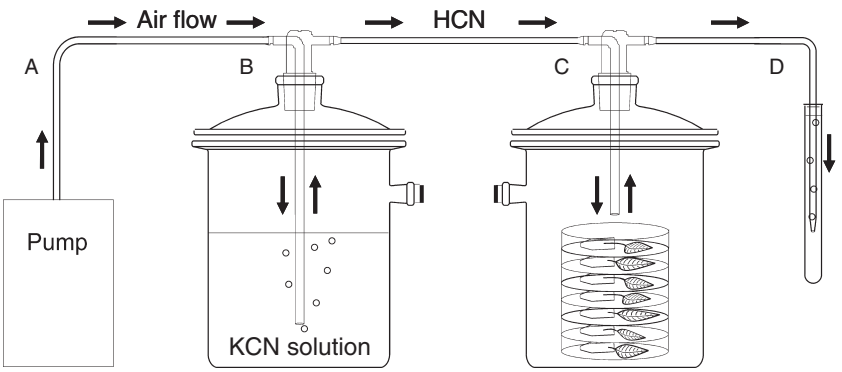

Fig. 2. Application of gaseous HCN to inoculated leaflets. The airflow in the vessel system was provided by a diaphragm pump with adjustable capacity (A). The air passes the HCN generator (B) containing 5 L of an unbuffered, slightly alkaline KCN solution (0.10, 0.50 and 1.00 mmol KCN L^{-1}, respectively), which was adjusted to pH 7.0 with an adequate amount of phosphoric acid. Produced gaseous HCN (0.34, 1.80, and 3.61 μmol HCN L^{-1} air) was transported into the incubation chamber (C) containing inoculated leaflets of lima bean. At the outlet of the system gaseous HCN was detected quantitatively (D) to control for constant ambient cyanide concentration in the incubation chamber.

autoxidation consisted of 2.9 mL Sörensen buffer and 100 μL 4-methylcatechol (225 mmol L^{-1}). For extraction of PPOs, leaflets were homogenized in the threefold volume (v fresh $weight^{-1}$) Sörensen buffer. Extraction was carried out in Eppendorf® tubes (1.5 mL) at 25 °C to allow enzymatically accelerated decomposition of cyanogenic precursors in the plant extract. The concentration of free HCN in the sample was analysed by use of the Spectroquant® cyanide test (Merck). Thus, quantitative effects of released HCN on PPO activity could be evaluated. Subsequently, leaf extracts were centrifuged (8000 × *g*, 10 min, 4 °C) and the supernatant was used for analysis of PPO activity. The solution in the O_2 electrode consisted of 100 μL supernatant and 2.8 mL O_2-saturated buffer. After 1 min of stirring, 100 μL of 4-methylcatechol (225 mmol L^{-1}) was added and O_2 depletion was determined polarographically over a time period of 6 min. This procedure gives relative values of O_2 depletion including enzymatic O_2 depletion in the course of substrate oxidation, autoxidation of the substrate, and other O_2 consuming processes in the leaf extracts. Thus, we subtracted both values of autoxidation of the substrate and O_2 consumption of the samples (measured in the O_2 electrode without sample or substrate solution, respectively) from total O_2 depletion. As prerequisite for a specific PPO assay, we checked whether the system could be inhibited by cyanide. In control experiments, potassium cyanide (KCN) was added to leaf extracts prepared from LC plants (showing high natural PPO activity) in a concentration of 15, 30 and 60 μmol cyanide g^{-1} fresh wt. (60 μmol cyanide g^{-1} fresh wt. corresponds to cyanide concentrations naturally occurring in HC plants).

INOCULATION OF LEAFLETS WITH *COLLETOTRICHUM GLOEOSPORIOIDES*

Leaflets were inoculated with a spore suspension adjusted to a concentration of 10^5 spores mL^{-1}. Spore samples were taken from 4-day-old fungal cultures, diluted with 5 mL aqua dest. and mixed three times for 1 min (Whirl Mix; Eppendorf AG, Hamburg, Germany). Microscopic determination of spore concentration was carried out by use of an Improved-Double-Neubauer counting chamber. Seven samples of each suspension were counted under the microscope (diagonal squares were counted in a row). Leaflets were injured on the lower surface (one injury per leaflet) using a 0.9-mm glass pin,

and 5 μL of spore suspension were pipetted onto the wound. Sterile water instead of spore suspension served as a control. Leaflets were kept at room temperature until the spore suspension had evaporated and/or was absorbed by the leaf tissue.

EVALUATION OF FUNGAL PERFORMANCE

Each inoculated leaflet (n = 14 leaflets per accession and per treatment) was placed in a Petri dish (9.5 cm in diameter) lined with moist filter paper to ensure water supply. Seven Petri dishes were placed in the incubation chamber of the airflow system for 48 h simultaneously (Fig. 2). We used four airflow systems in parallel, which were placed in the climatic chamber under ambient conditions as described above. Positions of the Petri dishes were changed twice a day during exchange of the KCN solution in the HCN generator. After the incubation period, the diameter of lesions (here defined as leaf tissue destroyed by fungal growth) was evaluated. For this purpose, leaves were digitally photographed on a scaled light box (Fleischhacker GmbH & Co. KG, Schwerte, Germany) and lesion size was quantified using the analySIS software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

APPLICATION OF HCN TO INOCULATED LEAVES

We carried out four experimental series using different HCN regimes (i.e. treatments with different concentrations of gaseous HCN) with all six accessions of lima bean. Application of gaseous HCN to inoculated leaves was conducted by use of an airflow system as described in Ballhorn, Lieberei & Ganzhorn (2005) with some modifications. Here, we used two 10-L glass vessels, one as a HCN generator, the other as an incubation chamber for inoculated leaves (Fig. 2). The airflow was adjusted to 3.6 L h^{-1}. The HCN generator contained 5 L of a slightly alkaline non-buffered KCN solution of different concentrations (0.1, 0.5 and 1.0 mmol cyanide L^{-1}) depending on the experimental set-up. The release of gaseous HCN from these KCN solutions resulted in HCN atmospheres in the airflow system of 0.34, 1.80 and 3.61 μmol HCN L^{-1}, respectively. These concentrations covered the range of HCN that naturally can be released from lima bean leaves (Ballhorn, Lieberei & Ganzhorn 2005). For the controls, the HCN generator was filled with 5 L aqua dest. instead of KCN solution. For production of gaseous HCN, KCN solutions were adjusted to pH 7.0 by adding phosphoric acid in equimolar amounts to accelerate release of gaseous HCN. The KCN solution was exchanged twice a day to guarantee a constant concentration of atmospheric HCN. In the airflow system, gaseous HCN released from KCN solution was discharged into the incubation vessel and trapped at the outlet of the airflow system in a test tube containing 10 mL of NaOH solution (0.1 mol L^{-1}). The concentration of HCN in the atmosphere was checked for constancy five times a day by quantifying the HCN trapped per time period (10 min). Quantification was carried out using the Spectroquant® cyanide test (Merck) as described above.

FIELD OBSERVATIONS

In a natural population of wild-type lima beans near Puerto Escondido (15°53.432′ N, 97°07.110′ W, elevation 12 m a.s.l., Oaxaca, southern Mexico) in October 2007, we screened defined leaf developmental stages (fully unfolded leaves located three insertion positions down the apex; n = 21 plants) for the concentration of cyanide-containing precursors. Occurrence and type of lesions (small and dark brown vs. large and light brown, see Fig. S1 in Supporting Information) on individual plants was noted.

STATISTICAL ANALYSES

We applied a nested ANOVA design using 'Cyanogenic Type' (fixed factor) and 'Accession' (random factor) to analyse the data set for differences in constitutive PPO activity, induced PPO activity and pathogen damage between HC and LC lima bean accessions. 'Accession' was nested within 'Cyanogenic Type' (i.e. HC or LC), since we used six lima bean accessions of which three were HC and three were LC accessions. The effect of 'Cyanogenic Type' was tested against the effect of 'Accession'. To test for correlations between cyanogenic features and constitutive PPO activity (i.e. to search for genetic correlations), we applied Pearson's correlations. To analyse effects of exogenous cyanide application on PPO activity and size of developing lesions on lima bean leaves, we applied a nested generalized linear model (GLM) with 'Cyanogenic Type', 'Accession' (nested within 'Cyanogenic Type') and 'Treatment' as factors. The effect of 'Cyanogenic Type' and its interaction with 'Treatment' was tested against 'Accession' and its interaction, respectively. All statistical analyses were carried out using SPSS 17 (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Results

CYANOGENIC FEATURES OF EXPERIMENTAL PLANTS

Cyanogenic features of lima bean leaves [cyanogenic potential (HCNp) and cyanogenic capacity (HCNc)] were significantly lower for LC than for HC accessions. Within groups of HC accessions, HCNp showed no significant differences, while in the group of LC plants the least cyanogenic accession CV_8067 exhibited a significantly lower HCNp than the two other LC accessions [according to *post hoc* analysis (Tukey's HSD, $P < 0.05$) after ANOVA; n = 14 plants per accession; Table 1]. The activity of β-glucosidases, which crucially determines the kinetics of hydrogen cyanide release from the cyanogenic precursors in case of cell damage, was higher for HC accessions as compared to LC accessions. However, while the differences were significant for HC accessions CV_2357 and CV_8078, β-glucosidase activity of the HC accession CV_1315 was not significantly different from the LC accessions CV_8073 and CV_8067 (Table 1). Concerning HCNc, no significant differences were observed within the groups of HC and LC plants, respectively. In contrast to strong quantitative differences of cyanogenic features between HC and LC plants, leaves showed distinct homogeneity concerning their LMA. No significant differences were found for this trait among accessions (Table 1).

CONSTITUTIVE POLYPHENOL OXIDASE ACTIVITY

Polyphenol oxidase activity in untreated leaves of HC accessions was lower as compared to LC genotypes (Fig. 3). 'Cyanogenic Type' (HC or LC) and 'Accession(Cyanogenic Type)' had a significant effect on constitutive PPO activity (according to nested ANOVA design; Table 2). Among the three HC accessions, differences of constitutive PPO activity were not significant [according *post hoc* analysis (Tukey's HSD; $P < 0.05$) after one-way ANOVA, Table S1], whereas

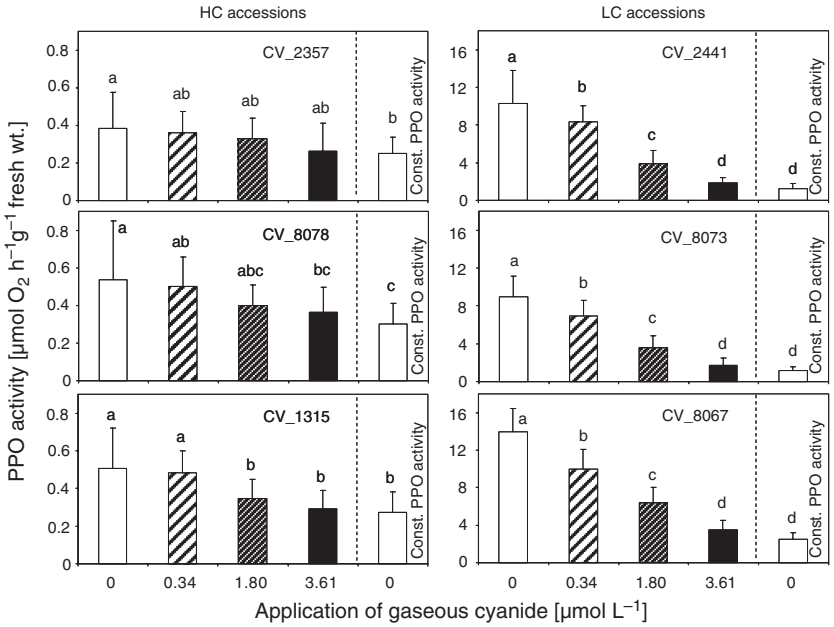

Fig. 3. Polyphenol oxidase activity in leaflets under different HCN-atmospheres. Activity of polyphenole oxidases (PPOs) in leaflets of different low cyanogenic (LC) and high cyanogenic (HC) lima bean accessions was measured under different experimental conditions. PPO activity was measured in inoculated leaflets incubated under different ambient cyanide concentrations (□ 0, ▨ 0.34, ▨ 1.80 and ■ 3.61 µmol HCN L^{-1} air). Constitutive PPO activity represents enzymatic activity in non-inoculated leaves incubated under HCN-free conditions in the airflow system (last column). Values shown for different treatments represent means + SD (n = 14 leaves per treatment and accession; each one leaf was derived from one individual plant). Different letters indicate significant differences among treatments [according to *post hoc* analysis (Tukey's HSD, $P < 0.05$) after one-way ANOVA].

Table 2. Effect of cyanogenic type and accession of plants on polyphenol oxidase activity and lesion size. We measured constitutive polyphenol oxidase (PPO) activity and PPO activity after inoculation with *Colletotrichum gloeosporioides* in leaflets of different cyanogenic lima bean accessions ('Cyanogenic Type' = HC or LC accessions). In addition, we measured the size of lesions occurring on leaves. Effects of 'Cyanogenic Type' and 'Accession(Cyanogenic Type)' were calculated using a nested ANOVA design. The term 'Accession(Cyanogenic Type)' is nested because each measure comes from only one combination of the 'Accession' and 'Cyanogenic Type'. The effect of 'Cyanogenic Type' was tested against the mean squares of 'Accession(Cyanogenic Type)'

Source	Dependent variable	*SS*	d.f.	*F*	*P*-value
Cyanogenic Type	Constitutive PPO activity	38.051	1	9.376	< 0.037
Accession(Cyanogenic Type)		16.232	4	24.086	< 0.001
Error		13.142	78		
Cyanogenic Type	Pathogen-induced PPO activity	2342.148	1	49.956	< 0.01
Accession(Cyanogenic Type)		187.538	4	11.674	< 0.001
Error		313.249	78		
Cyanogenic Type	Lesion size	380.759	1	572.120	< 0.001
Accession(Cyanogenic Type)		7.237	4	4.918	< 0.01
Error		28.692	78		

among LC accessions, CV_8067 (the least cyanogenic accessions among LC plants) showed significantly higher PPO activity than the other two LC accessions (Table S1). Among all six accessions, constitutive activity of PPOs was negatively correlated to plant cyanogenic features [Pearson's correlation: HCNp: $r = -0.659$, $P < 0.001$; β-glucosidase activity: $r = -0.490$, $P < 0.001$; HCNc: $r = -0.691$, $P < 0.001$]. Within each accession, we found no significant correlations between constitutive PPO activity and cyanogenic traits (data not shown). This finding is due to the high quantitative consistency of both constitutive PPO activity and cyanogenic traits among plants of a given accession.

In control experiments conducted to assess the effect of cyanide on PPO activity in leaf extracts during preparation, adding KCN at a concentration of 60 µmol cyanide g^{-1} fresh wt. (corresponding to the mean HCNp of HC plants) to aliquots of extracts prepared from LC leaves (n = 42 extracts, i.e. n = 14 extracts per accession) reduced constitutive PPO activity by 90.87 ± 2.39% (mean ± SD; Table S2). Adding half the concentration (30 µmol cyanide g^{-1} fresh wt.) reduced PPO activity in leaf extracts by 52.35 ± 3.52% (n = 42), whereas

cyanide added at a concentration of 15 μmol g^{-1} fresh wt. resulted in an inhibition by 27.85 ± 5.82% (n = 42) of the constitutive PPO activity in LC leaf extracts (Table S2).

POLYPHENOL OXIDASE ACTIVITY IN INOCULATED LEAFLETS

Inoculation with *C. gloeosporioides* resulted in a significant increase of PPO activity in both, HC and LC leaves (according to Wilcoxon Signed Ranks test for two dependent variables; HC leaves: $P = 0.02$, $Z = -5.189$; LC leaves: $P < 0.001$, $Z = -5.645$; n = 14 replications per treatment and accession; Fig. 3). However, the increase of PPO activity in response to pathogen inoculation was lower for HC than for LC plants. Differences in induced PPO activity depending on 'Cyanogenic Type' and 'Accession(Cyanogenic Type)' were significant according to nested ANOVA design (Table 2). Within the group of HC plants, we found no significant differences in enzymatic activity [according to *post hoc* analysis (Tukey's HSD, $P < 0.05$) after one-way ANOVA; Table S1]. Among LC plants, induced PPO activity was significantly higher for the least cyanogenic accession CV_8067 as compared to the other (higher cyanogenic) LC accessions CV_8073 and CV_2441 (Table S1).

PATHOGEN DAMAGE OF HC AND LC LEAFLETS

After experimental inoculation, mean size of lesions caused by *C. gloeosporioides* under control, i.e. HCN-free conditions, was 6.6 times higher for HC than for LC accessions. Differences in mean lesion size depending on 'Cyanogenic Type' and 'Accession(Cyanogenic Type)' were significant (according to nested ANOVA design; Table 2). Among HC accessions (CV_2357, CV_8078 and CV_1315), destruction of leaflet tissue by the fungus was significantly lower for accession CV_1315 (the least cyanogenic accessions among HC plants) compared to CV_2357 (the highest cyanogenic accession) while mean lesion size on accession CV_8078 took an intermediate position and was not significantly different to both other HC accessions [according to *post hoc* analysis (Tukey's HSD, $P < 0.05$) after one-way ANOVA; Table S3]. Among LC plants we found no significant differences in lesion size (Table S3).

EFFECT OF HCN TREATMENT ON POLYPHENOL OXIDASE ACTIVITY

According to GLM, HCN treatment of inoculated leaves was a significant source of variance for PPO activity (Table 3). In addition, the factor 'Treatment × Cyanogenic Type' had a significant effect on PPO activity, indicating quantitatively different responses in HC and LC accessions to HCN treatment. Compared to HC accessions, reduction of PPO activity in LC accessions in response to HCN treatments was stronger.

Among HC accessions, enzymatic activity of PPOs in inoculated leaves treated with intermediate and high concentrations of external HCN was slightly reduced by the factors 0.98 ± 0.15 (1.80 μmol HCN L^{-1}) and 0.75 ± 0.10 (3.61 μmol HCN L^{-1}; mean ± SD, n = 14 replications per treatment and accession), whereas PPO activity in inoculated leaves of HC accessions under the lowest HCN regime (0.34 μmol HCN L^{-1}) was similar to PPO activity measured under control conditions, i.e. inoculated leaves under cyanide-free atmosphere (factor: 1.02 ± 0.19; n = 14 replications per treatment and accession).

Table 3. Treatment effects on polyphenol oxidase activity. Results obtained using the GLM analysis of variance after a univariate design using polyphenol oxidase activity as variable. The term 'Accession(Cyanogenic Type)' is nested because each measure comes from only one combination of the 'Accession' and 'Cyanogenic Type'. The effect of 'Cyanogenic Type' and its interaction with 'Treatment' was tested against the mean squares of 'Accession(Cyanogenic Type)' and its interaction with 'Treatment', respectively

Source	*SS*	d.f.	*F*	*P*-value
Cyanogenic Type	1283.927	1	33.113	< 0.01
Treatment	613.098	3	136.358	< 0.001
Cyanogenic Type × Treatment	558.919	3	124.309	< 0.001
Accession(Cyanogenic Type)	155.095	4	25.871	< 0.001
Accession(Cyanogenic Type × Treatment)	17.985	12	1.876	< 0.05
Error	249.256	312		

For all LC accessions, we found distinct quantitative effects of gaseous HCN application on pathogen-induced PPO activity under all HCN regimes (Fig. 3). At the lowest ambient HCN concentration of 0.34 μmol HCN L^{-1}, PPO activity was reduced by factor 0.85 ± 0.11 as compared to controls. Higher concentrations led to substantial reduction of enzymatic activity by the factors 0.46 ± 0.06 (1.80 μmol HCN L^{-1}) and 0.23 ± 0.04 (3.61 μmol HCN L^{-1}).

EFFECTS OF HCN APPLICATION ON LESION SIZE

Experimental application of gaseous HCN to inoculated leaflets of HC and LC accessions increased fungal growth, i.e. lesion size. Effects of HCN treatment on lesion size were significant according to GLM (Table 4). Corresponding to

Table 4. Treatment effects on lesion size. Results obtained using the GLM analysis of variance after a univariate design using lesion size as variable. The term 'Accession(Cyanogenic Type)' is nested because each measure comes from only one combination of the 'Accession' and 'Cyanogenic Type'. The effect of 'Cyanogenic Type' and its interaction with 'Treatment' was tested against the mean squares of 'Accession(Cyanogenic Type)' and its interaction with 'Treatment', respectively

Source	*SS*	d.f.	*F*	*P*-value
Cyanogenic Type	297.661	1	141.056	< 0.001
Treatment	820.380	3	141.338	< 0.001
Cyanogenic Type × Treatment	191.116	3	32.926	< 0.001
Accession(Cyanogenic Type)	8.441	4	1.091	0.404
Accession(Cyanogenic Type × Treatment)	23.217	12	2.220	< 0.05
Error	249.256	312		

PPO activity, the factor 'Treatment × Cyanogenic Type' had a significant effect on lesion size, indicating quantitatively different responses of the fungal pathogen depending on cyanogenic type (HC or LC) of host plants. Application of gaseous cyanide in a concentration of 0.34 μmol HCN L^{-1} increased lesion size on HC leaves by a factor of 1.16 ± 0.08, whereas lesion size of LC plants was increased by a factor of 5.97 ± 1.00. Higher HCN atmospheres (1.80 and 3.61 μmol HCN L^{-1}) resulted in substantially increased lesion sizes by factors 1.28 ± 0.09 and 1.50 ± 0.08 for HC plants and factors 8.56 ± 1.26 and 11.39 ± 1.59 for LC plants (Fig. 4).

FIELD OBSERVATIONS

Defined leaf developmental stages of wild-type lima bean plants in nature showed distinct variability of cyanogenic features. Cyanogenic potential (HCNp) of young leaves ranged from 11.52 to 70.06 μmol cyanide g^{-1} leaf fresh wt. and thus covered the range of HCNp expressed by lima bean genotypes selected for laboratory experiments. High cyanogenic plants at the natural site were more commonly colonized by pathogenic fungi than lower cyanogenic wild-type plants. Cyanogenic potential and occurrence of lesions on leaves were highly significantly correlated (according to Pearson's correlation: $r = 0.718$, $P < 0.001$). Lesions observed on HC plants were larger and less brown than the sporadically occurring lesions observed on the lower cyanogenic individuals (Figs 5 and S1).

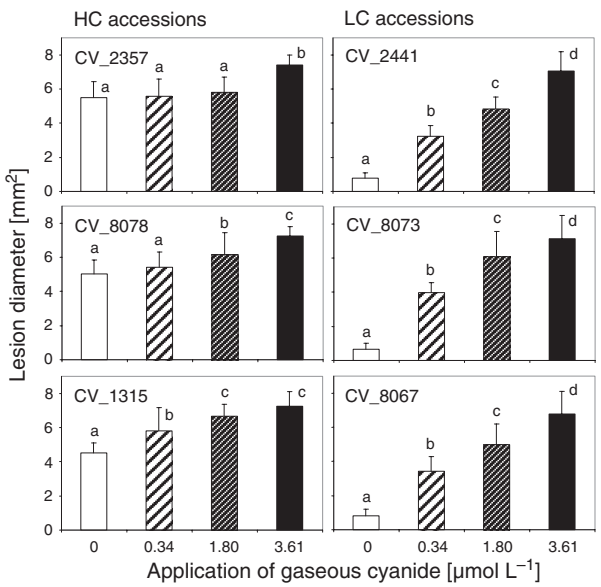

Fig. 4. Lesion size on leaflets under different HCN atmospheres. Leaf damage of low cyanogenic (LC) and high cyanogenic (HC) accessions of lima bean by *Colletotrichum gloeosporioides* was measured as size of lesions after 48 h of incubation under control (□ HCN-free) and different ambient cyanide concentrations (▨ 0.34, ▨ 1.80 and ■ 3.61 μmol HCN L^{-1} air). Values represent means + SD ($n = 14$ leaves per treatment and accession; each one leaf was derived from one individual plant). Different letters indicate significant differences among treatments [according to *post hoc* analysis (Tukey's HSD, $P < 0.05$) after one-way ANOVA].

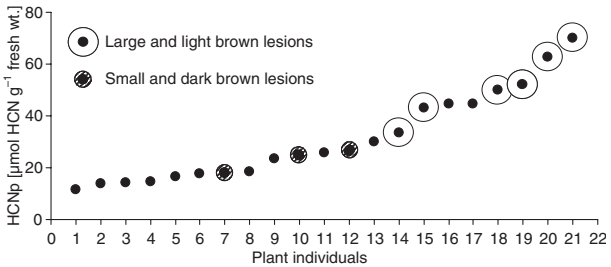

Fig. 5. Lesion type vs. cyanogenic potential of lima bean plants in nature. In a natural population of wild-type lima bean plants we analysed defined leaf stages of individual plants ($n = 21$ plants) for cyanogenic potential (HCNp). Leaves were screened for occurrence of lesions caused by fungal pathogens and type of lesions (small and dark brown/large and pale to light brown) was recorded.

Discussion

Cyanogenesis is an efficient defence against insect herbivores, which represent a major group of plant antagonists (e.g. Bernays *et al.* 1977; Ballhorn, Lieberei & Ganzhorn 2005; Agrawal 2006; Ballhorn *et al.* 2009). However, in addition to herbivores, plants have to face almost ubiquitously occurring pathogens (Osbourn 1996). Thus, the general question arises whether defences against herbivores are also effective against pathogens or vice versa, or if trade-offs among such defences occur (Thaler *et al.* 1999; Thaler, Owen & Higgins 2004).

We used a set of lima bean plants with defined cyanogenic features (Table 1), which allowed utilizing the natural variability of cyanogenesis for functional analyses in plant–pathogen interactions. Cyanogenic potential of accessions corresponded to the HCNp of wild-type lima bean plants in nature (Fig. 5). In previous studies, the HC lima bean accessions used here (CV_2357, CV_8078 and CV_1315) were demonstrated to be efficiently defended against herbivores, whereas LC accessions (CV_2441, CV_8073 and CV_8067) were only weakly defended (Ballhorn, Lieberei & Ganzhorn 2005; Ballhorn & Lieberei 2006; Ballhorn *et al.* 2007).

Here, as an additional component of lima beans' defence syndrome, we considered polyphenol oxidase (PPO) activity. We found low constitutive PPO activity in leaves of HC accessions, whereas LC plants showed substantial constitutive PPO activity (Fig. 3) suggesting a genetic trade-off or constraint for these two defence pathways. Furthermore, HC accessions showed only modest increase in PPO activity following infection, whereas the naturally high PPO activity in LC accessions was strongly enhanced in response to infection with *Colletotrichum gloeosporioides* (Fig. 3). Although reactive quinones provided by PPO activity can generally affect both herbivores and pathogens (Krischik, Goth & Barbosa 1991; Constabel 1999; Biere, Marak & van Damme 2004), in previous comparative studies on the same LC and HC lima bean accessions used here, we demonstrated that efficiency

of extensive PPO activity in LC plants as an anti-herbivore defence must be low (Ballhorn *et al*. 2007). This is in accordance to other recent studies, in which limited effects of PPOs on herbivores have been reported (e.g. Barbehenn *et al*. 2007).

In contrast to the limited efficiency of PPO activity as an anti-herbivore defence of lima bean, PPO activity has been described as a trait crucially affecting the resistance of beans to the fungal pathogen *Colletotrichum lindemuthianum* (Campos *et al*. 2004). In our present study, the growth of the closely related generalist plant pathogenic fungus *C. gloeosporioides* in leaf tissue was significantly correlated to both PPO activity (negative correlation) and concentration of cyanide in leaf tissue (positive correlation). Thus, in contrast to potentially limited efficiency of PPO activity in anti-herbivore defence, our bioassays with *C. gloeosporioides* revealed high efficiency of PPOs in anti-pathogen defence, confirming the results of Campos *et al*. (2004). However, since correlations between PPO activity, cyanogenesis and susceptibility to pathogens do not necessarily reflect functional coherences, we experimentally applied HCN at different regimes to leaves inoculated with *C. gloeosporioides* to support our findings. Gaseous external HCN quantitatively inhibited PPO activity (Fig. 3) – and resulted in significantly increased lesion sizes (Fig. 4). In addition, high PPO activity in extracts of LC leaves could be inhibited by adding cyanide (KCN) to leaf extracts (Table S2) in concentrations resembling HCNp of HC leaves. It currently remains elusive whether other enzymes in addition to PPOs (e.g. catalases and peroxidases) involved in plant resistance to pathogens are also inhibited by extensive cyanogenesis (Solomonson 1981). However, inhibition of other enzymes, in addition to PPOs, would increase the impact of defensive trade-offs on plant fitness.

In addition to direct inhibitory effects of cyanide on PPO activity, limiting effects of cyanide on plant resistance to pathogens might result (indirectly) from inhibiting ATP synthesis (Solomonson 1981). Induced or active resistance to pathogens is generally an energy-requiring process, which thus can be constrained by interrupted production of energy equivalents. In addition, the activation of alternative cyanide-insensitive oxidases (that do not contribute to efficient respiratory ATP production) may entail metabolic costs that limit resources for efficient resistance to pathogens (Vanlerberghe & McIntosh 1997). However, since cyanide experimentally added to leaf extracts (Table S2) immediately inhibited PPO activity quantitatively, our results strongly suggest that PPO activity is directly involved in limiting pathogen resistance of lima bean (Fig. 4). In addition, direct positive effects of cyanide on fungal growth, as observed for *Microcyclus ulei* (ascomycetes) (Lieberei *et al*. 1983), a pathogen of the cyanogenic rubber tree (Euphorbiaceae: *Hevea brasiliensis*), could be also excluded here, since we found no effects of cyanide application on the development of *C. gloeosporioides* cultures on pure media (D.J.B., pers. obs.).

Our results indicate substantial quantitative trade-offs between anti-herbivore and anti-pathogen defence inferred by the functional interplay of PPO activity and plant cyanogenesis. Under evolutionary aspects, such trade-offs between plants resistances to multiple enemies might be of high importance. For some plant species genetically based qualitative variation of cyanogenic features has been described (Jones 1962, 1966) and populations of some cyanogenic species may partly or almost entirely be composed of acyanogenic genotypes (Daday 1954a, b; Schappert & Shore 1995; Jones 1998). Thus, some counteractive forces can select against cyanogenesis. The increased susceptibility to fungal pathogens of HC individuals in a population may be one of the crucial pressures selecting against cyanogenesis under natural environmental conditions specifically favouring pathogen development (Dirzo & Harper 1982).

So far the question remains unanswered whether the trade-off we observed here in the laboratory holds true under natural field conditions. However, it seems very likely that the results of our study can be transferred to plant–pathogen interactions in natural systems since damage by (unknown) pathogens on wild-type lima bean plants in a natural population in South Mexico were observed mainly on HC plant individuals, whereas LC genotypes – although damaged strongly by herbivores (Ballhorn *et al*. 2009) – showed significantly less and smaller lesions (Fig. 5). In addition, the lesions observed on differently cyanogenic plants in nature were highly similar in colour and shape to the type of lesions (small and dark brown on LC plant vs. large and light brown to colourless on HC plants; Fig. S1) developing on lima bean leaves in response to inoculation with *C. gloeosporioides* under laboratory conditions (D.J.B., pers. obs.). Furthermore, in preliminary experiments conducted to assess suitability of different pathogens as experimental organisms for this study, we found that lesions on HC and LC plants caused by *C. lindemuthianum* (a commonly occurring pathogen on lima bean; Campos *et al*. 2004) showed the same characteristics as lesions resulting from inoculation with *C. gloeosporioides* (D.J.B., pers. obs.). Last but not least, the concentrations of cyanogenic precursors in the HC and LC cultivars we had selected for our study covered the minimum and maximum range of HCNp of wild-type lima bean plants in nature (Table 1; Fig. 5) and the concentrations of gaseous HCN used for experimental application to inoculated leaves corresponded to concentrations of HCN that naturally can be released by lima bean leaves in response to damage (Ballhorn, Lieberei & Ganzhorn 2005).

Since we carefully selected experimental organisms and adjusted experimental conditions to reflect those in nature, and since our results are supported by field observations, the findings of our study are most likely transferable to natural systems.

However, in future studies, a knock-out plant lacking PPO activity would be useful in establishing the final proof of a causal link between PPO activity and resistance to fungal pathogens under laboratory conditions. In parallel field studies at natural sites, the ecological relevance of lima beans' intraspecific variability of investment in either cyanogenesis or PPO activity should be analysed considering multi-species networks.

Acknowledgements

The authors are thankful to the IPK for providing seed material for experimental plants and to the Universities of Hamburg and Duisburg-Essen for financial support of this research project. Todd Widhelm (Chicago) and Dr. Martin Schädler (Marburg) are gratefully acknowledged for comments on earlier versions of this manuscript. We further would like to thank Martin Heil (Irapuato) for financial support in Mexico (grant He 3196/4-2).

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Received 12 May 2009; accepted 11 September 2009
Handling Editor: Fergus Massey

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Lesions on lima bean leaves.

Table S1. Constitutive and pathogen-induced polyphenol oxidase activity of different lima bean accessions.

Table S2. Quantitative effect of cyanide on polyphenol oxidase activity in extracts prepared from low cyanogenic (LC) lima bean leaves.

Table S3. Lesion sizes on different lima bean accessions.

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