

# Co-Variation of Chemical and Mechanical Defenses in Lima Bean (*Phaseolus lunatus* L.)

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**Abstract** Plants usually express multiple chemical and mechanical defenses simultaneously. The interplay of these defenses is still poorly understood, as predictions range from negative associations such as allocation tradeoffs to positive correlations forming synergistic defense syndromes. Surprisingly, little empirical evidence exists on the co-variation of multiple plant defenses. In the present study, we analyzed different genotypes of lima bean (*Phaseolus lunatus* L.) for the expression of two direct chemical defenses [cyanogenic potential (constitutive), polyphenol oxidase activity (inducible)], two indirect chemical defenses [volatiles (VOCs) and extrafloral nectar (EFN; both inducible)] and a constitutive mechanical defense (hook-shaped trichomes). While the occurrence of trichomes was positively correlated with cyanogenesis, these traits showed a tradeoff with polyphenol oxidase activity, release of VOCs, and secretion of EFN. Hook-shaped trichomes were abundantly present in four of 14 genotypes investigated, and were found only in one monophyletic group of an AFLP-based tree, thus indicating a single evolutionary origin within the species. Our findings show that different lima bean genotypes express either one of two defense systems: 1) high constitutive defense via cyanogenesis and trichomes or 2) high inducible defense via VOCs, EFN, and PPO activity.

**Keywords** Tradeoff · Defense syndromes · Trichomes · Indirect defense · Cyanogenesis · Extrafloral nectar

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## Introduction

In accordance with their manifold attackers, plants usually express multiple defenses simultaneously, including chemical and mechanical defenses. While some defenses show tradeoffs due to resource allocation constraints, others are positively correlated forming defense syndromes (Agrawal, 2011). Lima bean (*Phaseolus lunatus*) is a model plant in chemical ecology for which various defenses have been well studied (Kost and Heil, 2006; Ballhorn, 2011). Cyanogenesis, the wound-induced release of hydrogen cyanide (HCN) from cyanogenic glucosides (CNGs) represents a constitutive direct chemical defense against many herbivores. When plant tissue is damaged, preformed vacuolar CNGs react with apoplastic  $\beta$ -glucosidases releasing toxic HCN. Polyphenol oxidases (PPOs) catalyze the oxygen-dependent oxidation of phenols to reactive quinones and represent an inducible direct defense—mostly against pathogens (Ballhorn, 2011). In addition to these direct chemical defenses, indirect defenses of lima bean have been particularly well studied. Herbivore-induced volatile organic compounds (VOCs) serve as an indirect defense against herbivores by chemically signaling to parasitoids; inducible secretion of extrafloral nectar (EFN) is highly efficient in attracting predatory ants (Kost and Heil, 2006). In our previous studies, we showed distinct tradeoffs between cyanogenesis and VOCs as well as cyanogenesis and PPO-activity (Ballhorn et al., 2008; Ballhorn, 2011). The interaction of cyanogenesis and PPO-activity is unique in that there is direct interference of these traits as cyanide released from damaged cells inhibits PPO-activity (Ballhorn et al., 2010).

In contrast to the well characterized chemical defenses of lima bean, their co-variation with mechanical defenses has not been studied. Here, for the first time, we report the occurrence of defense-associated hook-shaped trichomes, analyze the co-

variation of this mechanical defense with chemical defenses, and discuss the expression of these defenses in a phylogenetic context. Our study provides insight into the evolution of different defense syndromes within a single plant species.

## Methods and Materials

Lima bean plants were grown from seeds provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK; Gatersleben, Germany), the Max Planck Institute for Chemical Ecology (Jena, Germany), as well as from seeds collected from natural populations in Colombia and Mexico (see Table 1 for details). Plants were cultivated under greenhouse conditions according to Ballhorn (2011).

All plant individuals ( $N=10$  per genotype) were analyzed for all traits considered in this study. The cyanogenic potential (HCNp; concentration of cyanogenic precursors) was quantified by using the Spectroquant® cyanide test (Merck) (Ballhorn et al., 2005). Enzymatic activity of PPOs was measured by quantification of the  $O_2$ -consumption during oxidation of polyphenols and their derivatives to quinones (Ballhorn et al., 2010). Extracts were prepared from leaves inoculated with the generalist fungal plant pathogen *Colletotrichum gloeosporioides* by application of spore solutions adjusted to  $10^5$  spores  $ml^{-1}$  according to Ballhorn et al. (2010). Extraction was carried out in 1.5 ml

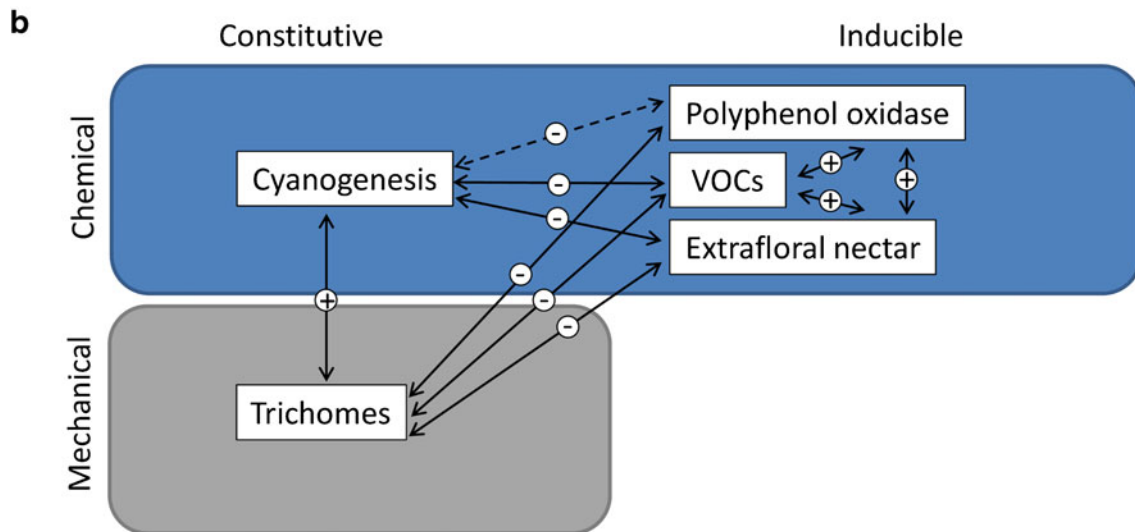
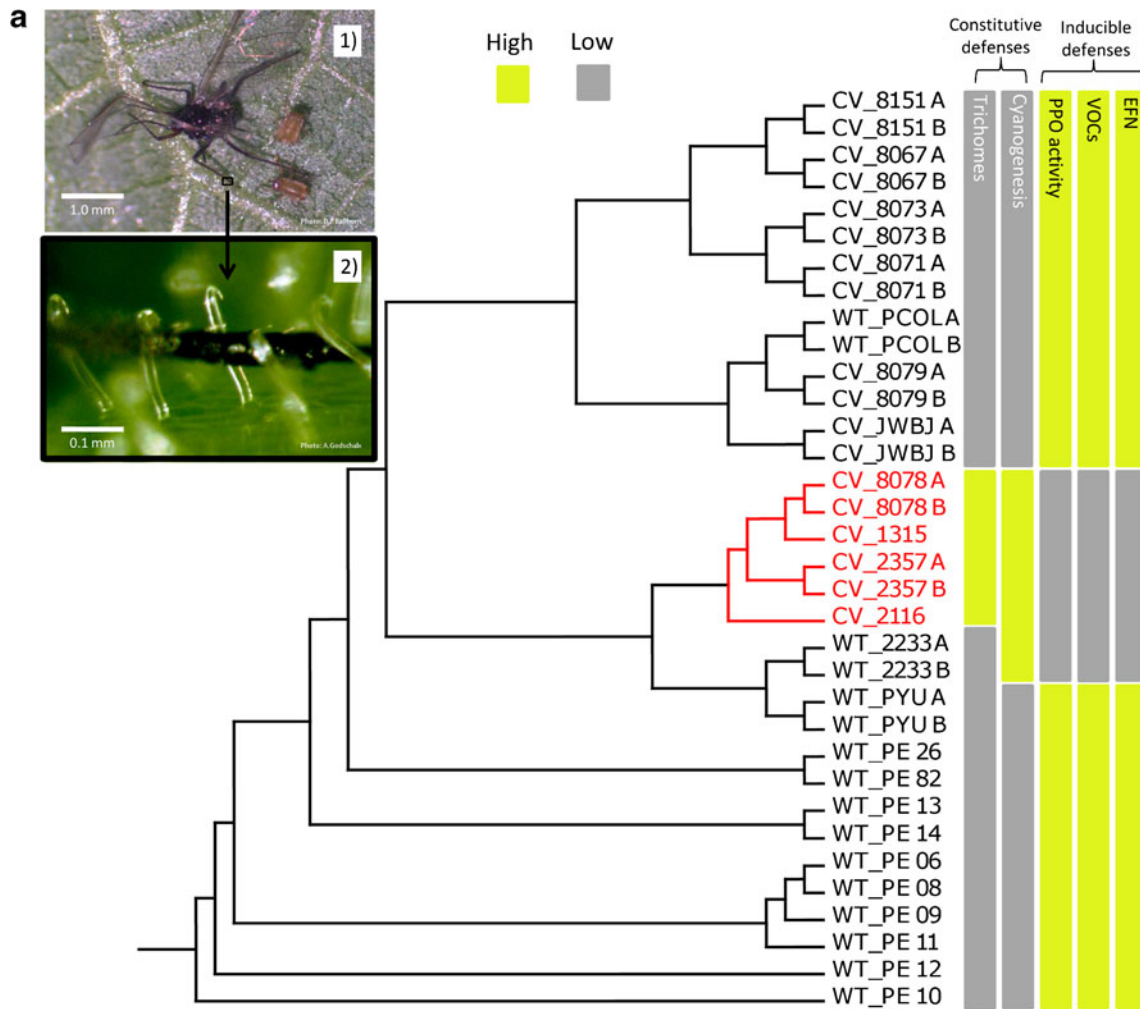
**Fig. 1 a** AFLP-based dendrogram of lima bean genotypes. The tree topology was obtained using PAUP\* (Swofford, 2003) after the Nei–Li model (Nei and Li, 1979) using a subset of samples from Ballhorn et al. (2008). A total of 34 lima bean samples (14 genotypes) with 509 variable bands derived from eight primer combinations were included in the analysis. *Phaseolus microcarpus* Martius was used as outgroup (not shown). The quantitative expression of trichomes, cyanogenesis, polyphenol oxidase activity (PPOs), herbivore-induced volatile organic compounds (VOCs), and extrafloral nectar (EFN) is indicated with bars. Genotypes that possess hooked-shaped trichomes are highlighted in red and are restricted to one monophyletic group. The dashed line between ‘Cyanogenesis’ and ‘PPO-activity’ in B indicates that these traits interact on a physiological level as PPO-activity is inhibited by cyanide. Photo insert 1 shows a female aphid trapped by trichomes on the lower surface of a lima bean leaf (genotype CV\_8078). Before its death, the aphid gave birth to two offspring. The upper juvenile is trapped as well. Photo insert 2 shows close-up of an aphid leg trapped by hook-shaped trichomes on the leaf vein. **b** Co-variation and trade-offs of various plant defensive traits in lima bean. Cyanogenesis and trichomes are positively correlated, while these two defenses are negatively correlated with the inducible defenses PPOs, VOCs and EFN

Eppendorf® tubes at 25 °C using Sörensen buffer allowing for enzymatic decomposition of cyanogenic precursors in the plant extracts. For further details, see Ballhorn et al. (2010). Trichomes were quantified microscopically (Leica MZ16) by counting their number along a 0.5 cm segment of a first-order leaf vein of mature leaves. One segment per leaf was evaluated ( $N=10$  leaves per genotype, each derived from a different plant individual).

**Table 1** Quantitative variability of defensive traits in lima bean (*Phaseolus lunatus*)

| Genotype             | Cyanotype (HC or LC) | HCNp ( $\mu\text{mol HCN g}^{-1}$ fw) | PPO-activity ( $\mu\text{mol O}_2 \text{ h}^{-1}\text{g}^{-1}$ fw) | VOCs ( $\text{ng g}^{-1}$ fw) | EFN ( $\text{mg g}^{-1}$ dw) | Trichomes (# per 5 mm leaf vein) |
|----------------------|----------------------|---------------------------------------|--|-------------------------------|------------------------------|----------------------------------|
| CV_2116 <sup>1</sup> | HC                   | 79.9±3.4 <sup>a</sup>                 | 0.3±0.1 <sup>a</sup>   | 1.4±0.1 <sup>a</sup>          | 0.7±0.2 <sup>a</sup>         | 111.6±7.1 <sup>a</sup>           |
| CV_2357 <sup>1</sup> | HC                   | 73.3±3.5 <sup>ab</sup>                | 0.4±0.1 <sup>a</sup>   | 1.6±0.1 <sup>a</sup>          | 0.9±0.2 <sup>a</sup>         | 112.0±8.7 <sup>a</sup>           |
| WT_2233 <sup>1</sup> | HC                   | 70.5±3.8 <sup>ab</sup>                | 0.6±0.1 <sup>a</sup>   | 1.7±0.1 <sup>a</sup>          | 0.9±0.2 <sup>a</sup>         | 7.7±1.0 <sup>b</sup>             |
| CV_8078 <sup>1</sup> | HC                   | 69.4±3.2 <sup>ab</sup>                | 0.6±0.1 <sup>a</sup>   | 1.9±0.1 <sup>a</sup>          | 0.7±0.2 <sup>a</sup>         | 104.8±7.9 <sup>a</sup>           |
| CV_1315 <sup>1</sup> | HC                   | 63.2±3.1 <sup>b</sup>                 | 0.5±0.1 <sup>a</sup>   | 1.9±0.1 <sup>a</sup>          | 1.0±0.1 <sup>a</sup>         | 92.2±8.2 <sup>a</sup>            |
| CV_8151 <sup>1</sup> | LC                   | 17.1±1.3 <sup>c</sup>                 | 8.9±0.7 <sup>b</sup>   | 9.0±1.4 <sup>b</sup>          | 3.2±0.2 <sup>b</sup>         | 6.2±1.1 <sup>b</sup>             |
| CV_8073 <sup>1</sup> | LC                   | 16.8±1.8 <sup>c</sup>                 | 10.0±0.4 <sup>bc</sup>   | 6.7±0.9 <sup>b</sup>          | 3.7±0.3 <sup>b</sup>         | 6.7±0.9 <sup>b</sup>             |
| WT_PE <sup>2</sup>   | LC                   | 12.9±3.0 <sup>c</sup>                 | 9.7±1.5 <sup>bc</sup>  | 8.6±1.4 <sup>b</sup>          | 3.4±0.2 <sup>b</sup>         | 6.7±1.5 <sup>b</sup>             |
| WT_PCOL <sup>3</sup> | LC                   | 12.2±2.3 <sup>c</sup>                 | 9.1±1.1 <sup>b</sup>   | 8.5±1.1 <sup>b</sup>          | 3.4±0.3 <sup>b</sup>         | 4.2±1.3 <sup>b</sup>             |
| CV_8067 <sup>1</sup> | LC                   | 11.6±1.1 <sup>c</sup>                 | 12.1±0.4 <sup>bc</sup>   | 10.5±1.1 <sup>b</sup>         | 3.5±0.4 <sup>b</sup>         | 8.0±1.2 <sup>b</sup>             |
| WT_PYU <sup>4</sup>  | LC                   | 11.4±2.6 <sup>c</sup>                 | 8.7±1.5 <sup>b</sup>   | 7.1±1.2 <sup>b</sup>          | 3.1±0.2 <sup>b</sup>         | 7.5±1.1 <sup>b</sup>             |
| CV_8071 <sup>1</sup> | LC                   | 9.2±1.2 <sup>c</sup>                  | 12.8±0.6 <sup>c</sup>  | 9.5±1.0 <sup>b</sup>          | 3.8±0.2 <sup>b</sup>         | 7.3±1.2 <sup>b</sup>             |
| CV_JWBJ <sup>5</sup> | LC                   | 8.2±0.9 <sup>c</sup>                  | 11.1±0.4 <sup>bc</sup>   | 9.4±1.0 <sup>b</sup>          | 3.3±0.3 <sup>b</sup>         | 6.6±0.9 <sup>b</sup>             |
| CV_8079 <sup>1</sup> | LC                   | 8.1±1.1 <sup>c</sup>                  | 12.0±0.4 <sup>bc</sup>   | 10.2±1.0 <sup>b</sup>         | 3.9±0.3 <sup>b</sup>         | 4.7±1.1 <sup>b</sup>             |

Values shown for plant traits are means ( $\pm$  SD;  $N=10$  individual plants per genotype). Significant differences between genotypes were calculated by a *post hoc* test (Tukey’s HSD;  $P<0.001$ ) after one-way ANOVA [cyanogenic potential (HCNp) ( $F_{13,126}=139.01$ ;  $P<0.001$ ), polyphenol oxidase (PPO) activity ( $F_{13,126}=50.98$ ;  $P<0.001$ ), volatile organic compounds (VOCs) ( $F_{13,126}=15.83$ ;  $P<0.001$ ), extrafloral nectar (EFN) ( $F_{13,126}=34.56$ ;  $P<0.001$ ), and hook-shaped trichomes ( $F_{13,126}=111.50$ ;  $P<0.001$ )] and are indicated by different superscript letters. Source of seeds for plant cultivation is indicated by superscript numbers in parentheses: <sup>(1)</sup> Leibniz Institute of Plant Genetics and Crop Plant Research (IPK; Gatersleben, Germany), <sup>(2)</sup> Oaxaca, Mexico (15.925532,-97.151151); <sup>(3)</sup> Colombia (Pacific coast, exact location unknown), <sup>(4)</sup> Yucatan, Mexico (19.832924,-87.466335), <sup>(5)</sup> Max Planck Institute for Chemical Ecology (Jena, Germany)



VOCs released from plants induced by spraying jasmonic acid (JA; 1 mmolL<sup>-1</sup> aqueous solution) were collected over 24 h on charcoal filters in a closed loop stripping set-up (circular airflow in a closed headspace) and were

qualitatively and quantitatively analyzed on a GC-Trace mass spectrometer (Trace GC Ultra DSQ; Thermo Electron, Austin, TX, USA). For detailed information on the program used for separation and for information on

compound identification and quantification, see Ballhorn et al. (2008). EFN production was quantified according to Kost and Heil (2006). After spraying plants (24 h) with JA and subsequent mechanical damage of leaves (15 holes per cm<sup>2</sup> leaf blade, diameter of individual holes 0.2 mm), EFN production was quantified as amounts of soluble solids by using microcapillaries (PCR Micropipets 1–10 µl; Drummond) for determination of volume and a brix refractometer for determination of sugar concentration.

We generated a dendrogram of all 14 lima bean genotypes by using AFLPs, and we mapped the level of each defense on the tree. The dataset was based on a subset of samples that had previously been published, and methods are described in detail in Ballhorn et al. (2008).

## Results and Discussion

Lima bean genotypes included here showed different but distinct combinations of defensive traits. Five genotypes were high cyanogenic (HC) (HCNp between 49 and 101 µmol HCN g<sup>-1</sup> fw), whereas nine showed significantly lower HCNp (Table 1). HCNp of these low-cyanogenic (LC) plants was between 3 and 31 µmol HCN g<sup>-1</sup> fw. In line with our previous studies, HC-plants released quantitatively less volatiles upon JA-induction (Table 1). These two defenses were significantly negatively correlated (*Pearson's correlation*:  $r=-0.787$ ,  $P<0.001$ ,  $N=40$ ). Furthermore, HCNp and EFN ( $r=-0.843$ ,  $P<0.001$ ) as well as HCNp and activity of PPOs ( $r=-0.909$ ,  $P<0.001$ ) were negatively correlated. While cyanogenesis showed a tradeoff with all other chemical defenses considered, this trait was significantly positively correlated to density of defense-associated trichomes ( $r=0.796$ ,  $P<0.001$ ). Four out of five HC-genotypes showed significantly higher numbers of trichomes than LC-genotypes (Table 1). Only WT\_2233 had significantly lower numbers of trichomes than other HC-genotypes. However, regarding all other chemical traits analyzed (PPO-activity, EFN, and VOCs), this genotype showed the typical defense system of HC-plants (Fig. 1). The combination of high chemical defense through cyanogenesis and mechanical defense by trichomes was found exclusively in one monophyletic group of an AFLP-based tree indicating a single evolutionary origin of trichomes within the species (Fig. 1). Thus, despite variation in the combination of individual traits, lima bean expresses either one of two defense systems: 1) High levels of cyanogenesis, a direct chemical defense against herbivores, usually coupled with mechanical anti-herbivore defense via trichomes; or 2) high levels of VOCs and EFN, as indirect defenses against herbivores, coupled with high levels of PPOs, enzymes mainly involved in resistance to pathogens (Fig. 1). Defense system 1) is constitutive, whereas defense system 2) is composed of inducible traits.

One genotype (WT\_2233) was high cyanogenic and was nested within a low cyanogenic clade and had no trichomes. Otherwise, high cyanogenic plants with trichomes occurred only in an exclusive clade; genetic conservation of this trait combination seems likely. However, the basis of the observed tradeoffs and positive correlations that we report here remains unknown. Constraints in resource allocation or evolutionary adaptations to suites of attackers dominating under distinct environmental conditions are potentially underlying causes of such contrasting defense systems. In the case of HCNp and PPO-activity, negative associations between plant defenses on the physiological level have been demonstrated (Lieberei et al., 1989; Ballhorn et al., 2010; Ballhorn, 2011). Free cyanide released upon cell damage quantitatively inhibits PPOs but also many other metal-containing enzymes involved in plant defense against herbivores and pathogens such as catalases and peroxidases (Kranthi et al., 2003). Thus, in the light of multiple inhibiting effects of cyanogenesis on other plant defenses, the combination of this trait with trichomes, which do not interfere with chemical defenses or synthesis pathways, represents a possibility for HC-lima beans to rely on an efficient second type of defense besides cyanogenesis. In particular, this combination of high HCNp and trichomes may provide simultaneous resistance to chewing herbivores, which cause the release of significant amounts of cyanide, and sucking herbivores, which cause minimal tissue damage and thus limited release of HCN, but are susceptible to trapping by hook-shaped trichomes (Fig 1). In support of this hypothesis, these trichomes are found mostly along the leaf veins on the lower surface of leaves, representing the favored feeding location for many sucking insects.

Future studies are needed to understand how these different defense systems function in nature when plants are under simultaneous attack by multiple pathogens and insect herbivores. Furthermore, analyzing variation of defense systems on spatial scales and identifying correlations to specific habitat characteristics in combination with transplanting experiments would yield a deeper understanding of the function of these defense systems. Nevertheless, despite further questions, our study shows for the first time, even at the intraspecific level, complex and fundamentally different defense systems occur.

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