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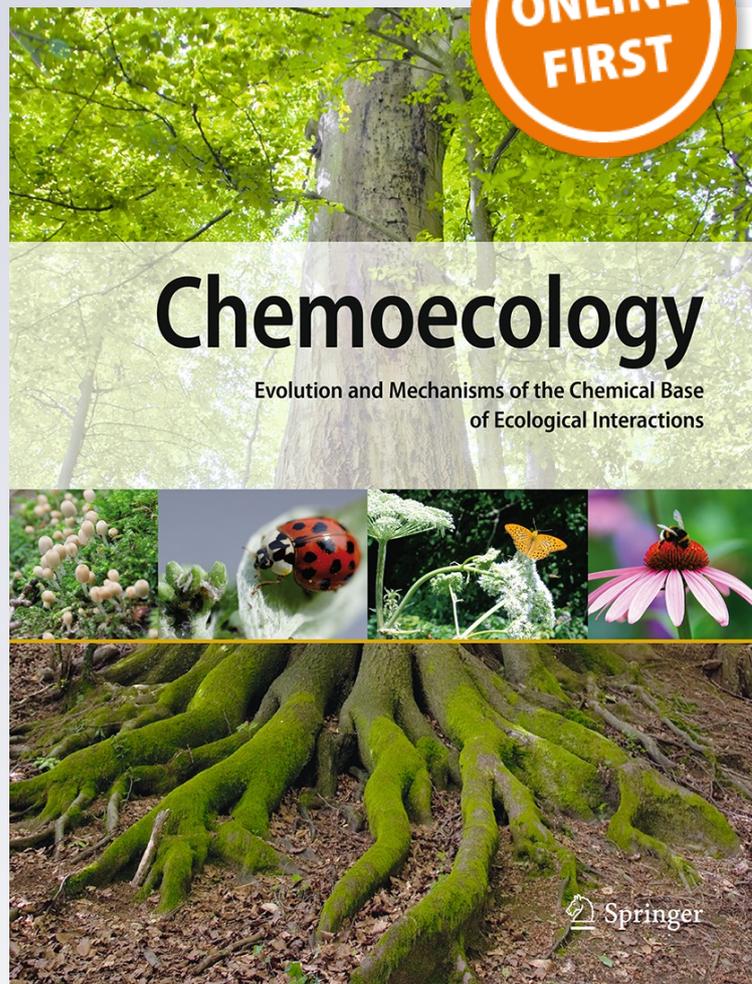
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Herbivore damage induces a transgenerational increase of cyanogenesis in wild lima bean (*Phaseolus lunatus*)

Daniel J. Ballhorn¹ · Stefanie Kautz¹ · Jessie May Laumann¹

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Abstract Plants produce a bewildering diversity of defensive compounds against their herbivore attackers. In addition to permanently expressed (constitutive) chemical defenses, plants generally show an array of inducible defenses which are only formed when the plant is under attack. However, there is increasing evidence that inducible defenses may persist on a long-term time scale, and across generations. Nevertheless, transgenerational studies quantifying multiple plant traits and their impact on plant survival in nature are lacking. In this study, we show for the first time, transgenerationally elevated cyanogenesis (release of hydrogen cyanide from cyanogenic precursors) in wild lima bean plants, *Phaseolus lunatus*, experimentally exposed to damage by natural chrysomelid herbivores, *Gynandrobrotica guerreroensis*. First generation offspring from damaged parents showed higher β -glucosidase activity, released more cyanide and showed significantly lower mortality compared to plants grown from seeds collected from non-manipulated, less damaged parent plants. However, this effect was significant only at the seedling stage of the first offspring generation and within this generation vanished within 4 weeks, indicating a transient nature of transgenerationally enhanced defense. While defense traits remained at elevated levels only for a relatively short period of time, our study shows the importance of a transgenerational inherited defense for seedling survival in nature.

Keywords Epigenetics · Plant defense · Legumes · Herbivory · β -Glucosidase

Introduction

Plants are able to produce manifold direct and indirect defenses against their multiple attackers, many of which are inducible and are only expressed in response to herbivore or pathogen attack. Interestingly, some induced defenses have been shown to be expressed at higher levels in offspring from damaged compared to undamaged parent plants, thus indicating a transgenerational transmission of the induced phenotype. Because the maternal environment in which seeds develop might predict the future conditions the progeny will face, it seems intuitive that parents further enhance their net fitness by providing their offspring with phenotypes better adapted to potential future stresses, such as herbivory. In fact, potentially adaptive transgenerational maternal effects have been reported in various organisms (Roach and Wulff 1987; Tollrian 1995; Mousseau and Fox 1998; Thiede 1998). For example, *Nicotiana tabacum* inoculated with tobacco mosaic virus showed elevated systemic acquired resistance (SAR) in the selfed progeny of infected plants, compared with the offspring of virus-free plants (Roberts 1983). Further experiments showed that non-lethal exposure of *Raphanus raphanistrum* plants to herbivores not only induced defenses, but also resulted in the attacked plant producing measurably better defended offspring than progeny from parents not damaged by herbivores (Agrawal et al. 1999). Also in *Arabidopsis*, transgenerational persistence of pathogen-, herbivore- and chemical-induced resistance has been observed (Luna et al. 2012; Rasmann et al. 2012; Slaughter et al. 2012). Strikingly, so

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✉ Daniel J. Ballhorn
ballhorn@pdx.edu

¹ Department of Biology, Portland State University, 1719 SW 10th Ave, Portland, OR 97201, USA

far all experimental studies examining transgenerational effects of herbivore-, pathogen-, and chemically induced resistance have used plants under controlled conditions. The existence and the actual effects of such mechanisms in the interaction with herbivores in nature remain elusive.

In our study, using cyanogenic wild lima bean (*Phaseolus lunatus* L.), we test the novel hypothesis that herbivory can enhance plant defense across generations through elevated cyanogenesis, and that these effects enhance offspring survival in nature. Cyanogenesis is historically considered a constitutive defense (Gleadow and Møller 2014). However, recent data show that lima bean plants express increased cyanogenic capacity (HCNc; release of gaseous cyanide per time) in response to herbivory and jasmonic acid treatment (Ballhorn et al. 2014), due to enhanced activity of specific β -glucosidases. In the present field study, we test the ability of cyanogenesis to be induced and transgenerationally expressed at elevated levels in two subsequent generations, following exposure to herbivore damage in the parental generation.

Methods

Plants

This study was conducted in a large wild lima bean population (Fabaceae: *Phaseolus lunatus* L.) in southern Mexico (Oaxaca). Twenty randomly selected plants were exposed to experimentally enhanced herbivory by covering plants with anti-aphid nets and introducing 10 specialist herbivorous beetles (Chrysomelidae: *Gynandrobrotica guerreroensis*) into the enclosures. In nature, *G. guerreroensis* is feeding on a small range of fabaceous host plants but shows distinct preference for cyanogenic lima bean. Despite its preference for a cyanogenic food plant, given the choice to select, these beetles prefer low over high cyanogenic lima bean individuals and there is no evidence for sequestration of host plant-derived defense chemicals (Ballhorn et al. 2009a, b). In our experiment, dead or disappearing beetles were replaced over a period of 9 weeks until plants formed flowers and fruit. Based on estimation of missing leaf area, the resulting herbivore damage on the experimental plants was higher (20–25 %) than on non-manipulated plants (< 10 %) (data not shown), but did not exceed levels of natural beetle herbivory observed in years with high beetle abundance. When ripe, seeds were collected from experimental plants as well as from twenty randomly selected untreated plants, growing in the same wild lima bean population, at least 15 m away from experimental plants.

Transgenerational experiments

Seeds from both the experimental group of plants and the untreated group were germinated on paper towels and, when a healthy root began to develop, were transplanted to a field (previously used for corn cultivation) adjacent to the lima bean population [$N = 300$ seedlings per group; 150 seedlings were randomly assigned to be used for chemical analyses, 150 seedlings served to quantify survival rates over time (6 weeks)]. Position of seedlings (from induced and untreated parents as well as for the different experiments) was randomized and each seedling was labeled and provided a bamboo stick (2.20 m) to climb on. Distance of seedlings from each other was 1.5 m. Plants were cultivated until development of new seeds (approx. 15 weeks), and the experiment was repeated with the subsequent (Gen-2) and the following generation (Gen-3). In both transgenerational experiments (Gen-2 and Gen-3), seedlings were collected randomly every week, 16 from each group (treated and untreated parents or grandparents). Seedlings were destructively analyzed for HCNp, β -glucosidase activity, and HCNc. Over the course of the experiments (Gen-2 and Gen-3), survival of seedlings in the field was recorded in weekly intervals starting 2 weeks after planting.

Chemical analyses

We conducted chemical analysis of leaves derived from both wild plants with experimental and natural herbivore damage. In addition, we measured leaf traits of second and third generation offspring from both treatment groups. In both Gen-2 and Gen-3 plants, traits were measured 2, 3, 4, 5 and 6 weeks after germination. Individual leaves of a defined developmental stage (young but fully unfolded) were used for chemical analyses to reduce variability of traits due to differences in leaf age (Ballhorn et al. 2008). The three leaflets of the trifoliate leaves were portioned randomly for analyses of cyanogenic potential (HCNp; amount of cyanogenic precursors), β -glucosidase activity, cyanogenic capacity (HCNc; release of gaseous cyanide per time), and soluble protein. HCNp was quantified spectrophotometrically using the Spectroquant[®] cyanide test (Merck) according to Ballhorn et al. (2013). The quantification of β -glucosidase activity in the extracts was conducted according to Ballhorn et al. (2006). The assay is based on the detection of *p*-nitrophenol released by hydrolysis of the chromogenic artificial substrate *p*-NP-glucoside by β -glucosidases. HCNc was measured with airflow equipment for the detection of gaseous HCN (Ballhorn and Lieberei 2006; Ballhorn et al. 2010a) and soluble protein was determined according to Ballhorn et al. (2009a). Leaf chemical characteristics were assumed to be similar among all three leaflets (Ballhorn et al. 2010b).

Results

Experimentally enhanced herbivore damage of wild lima bean plants growing in nature resulted in significantly increased β -glucosidase activity, HCNC and foliar soluble protein concentration (Fig. 1a–c), compared to non-

manipulated plants exposed to natural herbivory. In the following generation (Gen-2), offspring from these heavily damaged experimental plants showed significantly elevated β -glucosidase, HCNC and leaf protein compared to offspring from non-manipulated plants, which did not show any significant variation (Fig. 1e–g). In fact, in

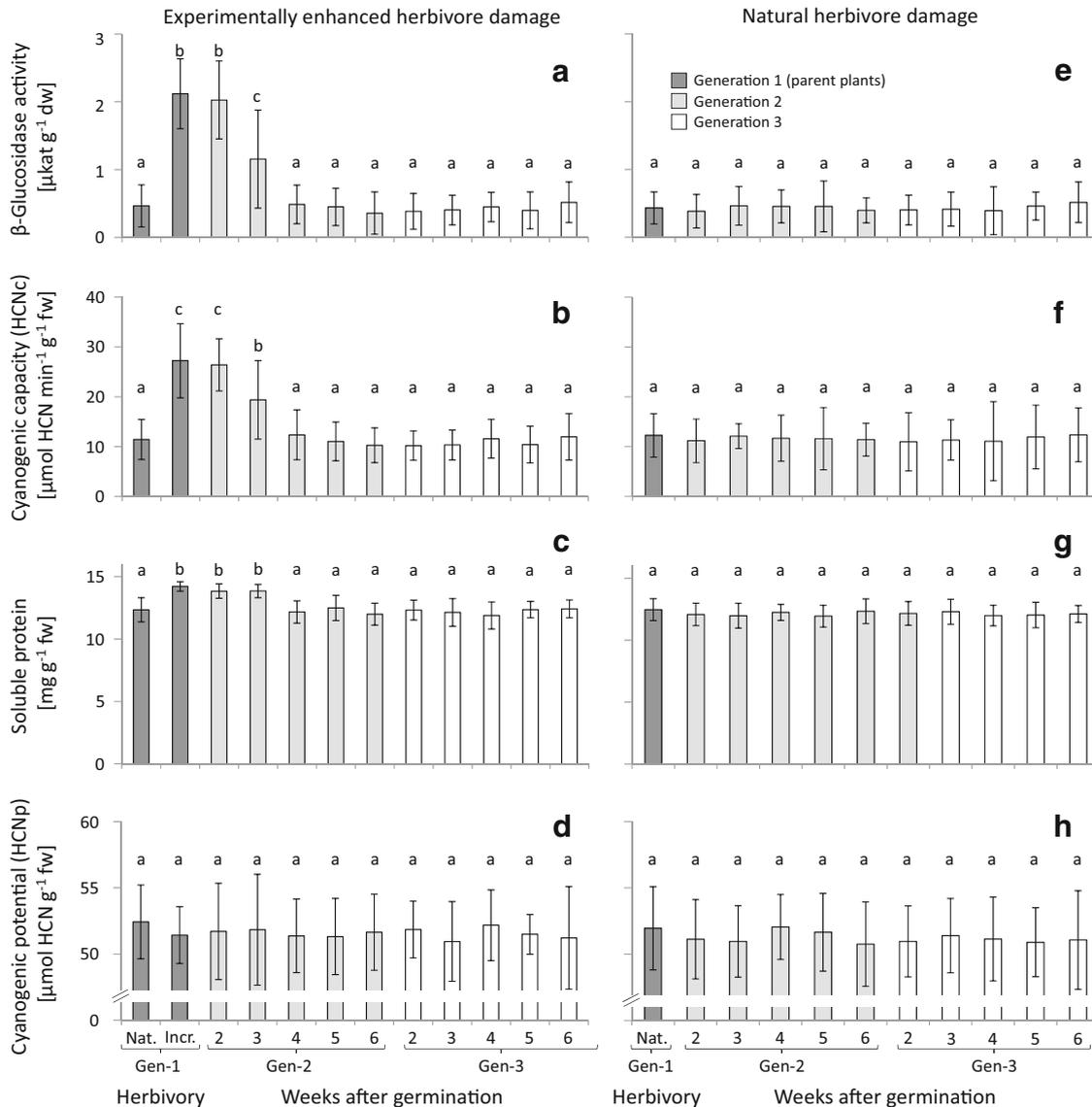


Fig. 1 Transgenerational expression of defense traits in lima bean plants. The *left panel (a–d)* shows defense-associated leaf traits in wild lima bean plants exposed to natural (Nat.) and experimentally increased herbivore damage (Incr.) and their offspring covering two consecutive offspring generations (generation 2 and 3). Chemical leaf traits in offspring plants of both generations were measured in weekly intervals over 6 weeks starting at 2 weeks after germination. The *right panel (e–h)* shows leaf traits of plants and two offspring generations that were exposed to only natural leaf damage. Please note that groups “Nat.” in panel 1 and 2 represent the same “treatment” (=no treatment) but different plant individuals. In the *left panel*, transgenerational effects of herbivory on leaf traits, that is significant variation across generations, were observed for β -

glucosidase activity, **a** $F_{11,180} = 44.493$; $P < 0.001$; HCNC, **b** $F_{11,180} = 27.158$; $P < 0.001$ and soluble protein concentration, **c** $F_{11,180} = 15.144$; $P < 0.001$ but not for HCNp, **d** $F_{11,180} = 0.311$; $P = 0.983$. Transgenerational effects were observed in Gen-2 but not Gen-3. In the *right panel*, no significant transgenerational variation was observed for either of the traits tested (β -glucosidase activity, **e** $F_{10,165} = 0.434$; $P = 0.928$; HCNC, **f** $F_{10,165} = 0.162$; $P = 0.998$; soluble protein concentration, **g** $F_{10,165} = 0.482$; $P = 0.900$, and HCNp, **h** $F_{10,165} = 0.400$; $P = 0.945$; Columns are means \pm SD. *Small-typed letters on top of the columns* represent significant differences according to post hoc analyses (Tukey’s HSD; $P < 0.05$) after one-way ANOVA

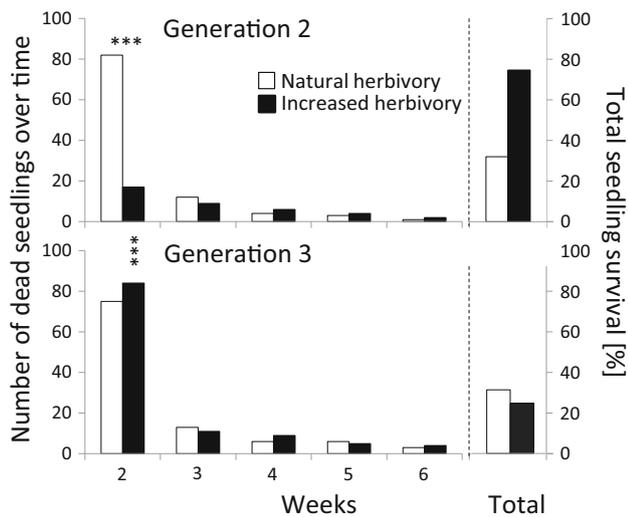


Fig. 2 Seedling survival. Survival of seedlings derived from naturally damaged parent plants (white bars) and parent plants exposed to experimentally increased herbivory (black bars) was measured for two subsequent generations over a period of 6 weeks. Bars right of the dashed lined represent total seedling survival in the different groups. Horizontally arranged asterisks indicate a significant difference in survival between seedlings from untreated versus treated parents, whereas vertically arranged asterisks show a significant difference between seedlings from damaged ancestors between generation 2 and 3 (t test; $P < 0.001$)

young Gen-2 seedlings (from experimentally damaged parent plants), none of these traits showed significantly lower expression than in the herbivore-damaged parents (Fig. 1a–c), indicating Gen-2 plants inherited the fully induced defense of their parents. However, after 4 weeks, Gen-2 plants showed baseline levels of defense similar to non-manipulated parent plants and their offspring (Fig. 1a–c, e–g). Thus, the phenotypically detectable elevated inherited defense was limited to the seedling and young plant stage. Plants of the following generation (Gen-3), originating from extensively herbivore-damaged grandparents, did not show significant enhanced defenses at all. In contrast to β -glucosidase activity, HCNC and soluble protein concentration, the cyanogenic potential (HCNp) (Fig. 1d, h) was not affected by insect damage in parent plants and showed no significant variation among Gen-1 to Gen-3 plants.

In Gen-2 plants, offspring from extensively damaged parent plants showed significantly higher survival during early seedling stages (week 1–3 after germination) compared to offspring from naturally damaged plants, while we did not observe differences in survival at later developmental stages (Fig. 2). In the Gen-3 plants, survival of seedlings from damaged versus untreated grandparents showed no significant difference at any time point.

Discussion

Our findings indicate that the defense-enhancing effects of herbivory on the transgenerational expression of β -glucosidase activity, HCNC and soluble leaf protein (potentially defense-associated enzymes) in wild lima bean are present only in direct offspring of heavily damaged plants (Gen-2), and only for a limited time before these traits return to baseline levels. Increased defense trait expression was reflected by enhanced Gen-2 seedling survival during early growth stages.

From an ecological perspective, this time wise limited expression of elevated defense in offspring of heavily attacked parents makes intuitive sense. While there is a chance that herbivore pressure persists beyond one generation and thus would select for plants with an already enhanced defense, a long-term elevated defense might be costly under low herbivore conditions (Agrawal 2001). Annual or seasonal variation in herbivore densities is common in most ecosystems, making an inherited continuous—and potentially multi-generational—high-level defense a risky investment. A further aspect supporting an only short-lived transgenerational defense expression is the distinct ability of lima bean plants to instantly upregulate various inducible defenses, ranging from VOCs, over extra-floral nectar and polyphenol oxidase activity, to β -glucosidase activity and HCNC (Ballhorn et al. 2007, 2013, 2014). Thus, there seems to be no strong benefit for transgenerationally enhanced defenses. At the seedling stage, however, high levels of preformed defense seem particularly important as herbivory at this stage is likely fatal (Thiede 1998). This is supported by our data on enhanced survival in Gen-2 seedlings (Fig. 2).

The mechanism behind our observation remains to be discovered, but epigenetic processes, which include inherited DNA methylation and histone modifications (Chinnusamy and Zhu 2009), are a likely mechanism for retaining stress memory in subsequent generations (Hamilton and Baulcombe 1999; Matzke et al. 2001; Lee et al. 2004; Chitwood and Timmermans 2010). Epigenetic enhancement of plant defenses is considered a potential way to sustainably produce crop plant seeds that develop into resistant plants (Worrall et al. 2011; Molinier et al. 2006). However, our study using a wild plant species in nature suggests that only certain life stages benefit from transgenerationally enhanced defense. The effects of such early defense barriers on long-term plant performance and fitness remain to be tested.

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