

Effects of domestication and agronomic selection on phytoalexin antifungal defense in *Phaseolus* beans

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Systems of wild and cultivated relatives are good experimental systems to test chemical defense theory because they provide closely related varieties that differ in discrete traits. To determine the relationship between resistance and chemical defense diversity among wild, landrace and cultivar accessions of *Phaseolus vulgaris*, we measured resistance to fungal infection in laboratory and field experiments, quantified phytoalexin diversity, and assessed the effectiveness of phytoalexin mixtures extracted from live tissue. Results show a gradient of resistance to fungal infections between wild, landrace and cultivar varieties. In the laboratory, wild seedlings were more resistant (93% non-infected) than landrace seedlings (80% non-infected) and modern cultivar seedlings (68% non-infected). Under field conditions wild seedlings were more resistant (97% non-infected) than cultivar seedlings (71% non-infected). Wild seedlings presented the highest phytoalexin diversity ($H' = 1.11$), while those of the landrace presented an intermediate level ($H' = 0.97$) and cultivar seedlings presented the lowest diversity ($H' = 0.93$). No differences were found in total concentrations. The *in vitro* inhibitory properties on hyphal growth of the isoflavonoid mixtures produced by individual seedlings showed a similar trend. Our results are consistent with similar gradients in other species of *Phaseolus* beans and resistance to *Colletotrichum sublineolum* in sorghum.

Key words: domestication; isoflavonoids; *Phaseolus vulgaris*; phytoalexins; resistance.

INTRODUCTION

Chemical defense hypotheses in plants assume that defenses are costly because they divert resources from other plant functions, particularly from growth and reproduction (Herms & Mattson 1992). This assumption is supported by strong evidence of inverse relationships between resource allocation to growth or reproduction and defense (Mooney *et al.* 1983; Bazzaz *et al.* 1987), between yield and resistance to consumers (Pimentel 1977; Rosenthal & Dirzo 1997), and trade-offs between primary and secondary metabolism *in vitro* (Collin 1987). Two major kinds of chemical defenses are known to occur in plants: constitutive defenses, which are present continuously in plant tissues,

and induced defenses. Induced defenses are produced as a response to an environmental stimulus such as pathogens or herbivory (Janzen 1979). Plant defense theory predicts higher costs for constitutive defenses than for induced defenses because the former continuously divert resources from primary metabolism to secondary metabolism and the latter only after stimuli (Clark & Harvell 1992; Herms & Mattson 1992). Moreover, the reliance of plants on constitutive or induced defense has been hypothesized to be determined by the probability of attack by consumers: plants with high probability of attack should display constitutive defenses and no induced defenses whereas plants with low probability should display the inverse pattern (Zangerl & Bazzaz 1992).

Domesticated plants have increased yields when compared to their wild relatives (Tivy 1990; Araujo *et al.* 1998), but domesticated plants have lowered levels of chemical defenses Hawkes 1983; Krischik & Denno 1983; Roddick 1993; Sotelo *et al.* 1995). Low levels of defenses in non-harvested

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parts of cultivated plants are attributed to the reallocation of limited resources within the plant to increase yields (Evans 1984; Rosenthal & Dirzo 1997). During most of the history of agriculture, plant domestication has been carried out under local conditions and has coexisted with natural selection pressures from herbivores and pathogens; this process gave rise to landraces. In contrast, selection of modern cultivars for yield occurs under conditions that greatly reduce pressure from herbivores and pathogens, therefore the selection pressure for defenses is considerably lowered. Consequently, a gradient of defense levels can be hypothesized following the negative resource reallocation relationship between chemical defenses and yield. In this scheme, modern cultivars have the lowest defense levels but the highest yield, followed by landraces with higher defenses and intermediate yields and, finally, wild types with the highest levels of defense but the lowest yields (Rosenthal & Dirzo 1997). Nevertheless, this relationship should only occur for costly constitutive defenses and not for induced defenses. Previous experiments with *Phaseolus coccineus* and *Phaseolus lunatus* suggested that such a gradient in induced phytoalexin defenses occurred for these species (Lindig-Cisneros *et al.* 1997), contradicting current chemical defense theory, but it was either confounded by the production of other defenses (cyanogenic glucosides in *P. lunatus*) or the lack of a modern cultivar (*P. coccineus*). As *P. vulgaris* cultivars have been subjected to intense breeding programs we measured resistance to infection under field and laboratory conditions and phytoalexin diversity in a system of wild, landrace and modern cultivar of *P. vulgaris* varieties. These comparisons, which have been suggested as useful but seldom used for induced defense studies (Hammerschmidt 1999), allowed us to test if the weak gradient found for the two other main domesticated species in the genus was stronger in *P. vulgaris*, as suggested by domestication pressure on this species.

METHODS

A system of wild, landrace and modern cultivar varieties of *P. vulgaris* was assembled. The source of wild seeds was a population located about 3-km southeast from the town of Tepoztlán (State of

Morelos, Mexico) in a heavily disturbed oak forest. The landrace 'Amarillo' was selected together with the cultivar 'Black Valent' (Asgrow Co., Des Moines, IA, USA) because these two varieties are regularly planted in the Tepoztlán area. After collecting or purchasing the seeds, they were stored at 5°C until use.

We tested resistance to infection in an agricultural field. During the first week of April 1996, the site was cleared after a 5-year resting period to avoid the interference of commonly used pesticides and fertilizers. Seeds were surface-sterilized by immersion in a 1% aqueous solution of sodium hypochloride for 5 min. Surface-sterilized wild and cultivar seeds were randomly scattered in two 1.5 m² plots spaced 10 cm from each other. Each seed was covered with an individual plastic mesh to avoid animal predation and allow inspection with minimal disturbance. Plots were covered with a thin layer of soil and watered daily. Eight days after planting, seedlings were inspected and an equal number of wild and cultivar seedlings of similar age were selected to form cohorts. Cohorts were inspected daily after selection until the first true leaves appeared in all non-infected seedlings, as these grew faster than infected ones.

Soil was collected from the wild population site and two agricultural fields, one from the field experiment and one from the Chapingo region of the State of Mexico for a growth chamber experiment to test resistance under different common agricultural soils in the region. Soil samples were sieved through a number 10 mesh, and then homogenized. Homogenized soil (50 g) was placed in polyethylene cups and then filled to approximately one-fifth of the cup capacity. Sterilized distilled water was added to each cup to saturation capacity levels. Seeds of wild, landrace and modern cultivar varieties were superficially sterilized as described. One seed of each variety was placed in each cup under sterile conditions (laminar flow chamber) and covered with a translucent plastic cover, for a total of 50 seeds per variety.

The cups were placed in an environmental chamber in a completely randomized design (Model E15; Conviron, Winnipeg, Manitoba, Canada) with a photoperiod of 8 h (26°C) and a dark period of 16 h (29°C). Inside the plastic cups these settings produced a constant 29°C temperature that favored fungal growth.

Each cup was inspected daily, initially to select the cohorts and later to follow the infection process. Each experiment was considered completed when the first true leaves of the non-infected seedlings appeared.

A binomial response (infected/non-infected) was recorded at the end of the experiments and analyzed as a binomial trial with sample size 1 (Crawley 1993) with GLIM 3.77 (Royal Statistical Society 1985) using a logit link function and a binomial probability distribution. Probabilities were calculated with TAB 1.3 (Ezcurra 1995).

Finally, for all infected seedlings, standard procedures to isolate fungi in malt extract agar media were followed in order to identify the infectious agents (Johnston & Booth 1983).

After surface sterilization with sodium hypochloride (as previously described) 150 seeds of each variety were imbibed in distilled and sterilized water for 8 h and germinated under sterile conditions at 25°C. At 48 h after germination cohorts of 48 seedlings per variety were selected and treated with aqueous 5 mM CuCl₂ for 2 min and incubated for an extra 48 h. The use of CuCl₂ allows standardization and prevents the induction of compounds other than phytoalexins (Sequeira 1983). After incubation, cotyledons were removed from each seedling and the remaining tissue was treated with 20 times the seedling weight of chromatographic grade ethyl acetate. Twenty extracts per variety were analyzed with high-performance liquid chromatography (Waters HPLC system Model 600 MS; Millipore Corp., Milford, MA, USA). Isoflavonoid phytoalexin separation was achieved with a Lichrosphere 10 μ 250 \times 3.2 mm column (Phenomenex, Torrance, CA, USA), using a solvent gradient of ethyl acetate : hexane (85 : 15) to ethyl acetate : hexane : chloroform (85 : 6 : 9) with a flow gradient of 0.7 ml min⁻¹ to 1.2 ml min⁻¹ (curve 6). Chromatograms of each seedling extract of all varieties showed between two and four peaks due to isoflavonoid phytoalexins. Using the peak parameters of the identified isoflavonoids in the chromatograms, chemical diversity for each individual seedling was calculated as Shannon Indices (H'). Statistical analysis was carried out with Kruskal–Wallis ANOVA by ranks (assigned to the individual H' values) due to inequalities of variances.

Estimation of total isoflavonoid concentration of 16 seedling extracts per variety was as follows. After inducing phytoalexin production by the seedlings and removing the cotyledons, the remaining tissue was extracted without maceration (as described for the HPLC analyses) to allow for a minimum of substances other than the phytoalexins to be diluted. Extracts were lyophilized and the remaining solid weighed. Total concentration estimates are grams of isoflavonoid phytoalexins per gram of fresh weight of seedling tissue. Kruskal–Wallis ANOVA by ranks was used for data analysis.

With 12 extracts per variety, an *in vitro* assay was performed for assessing the inhibitory properties of phytoalexin mixtures produced by each seedling. For this assay we used an isolate of *Aspergillus* sp. previously obtained from an infected seedling. Three cultures were prepared by placing a 5-mm plug of inoculum at the center of each Petri dish containing malt extract agar media. Phytoalexin extracts of individual seedlings were lyophilized and the remaining solids were dissolved in distilled sterilized water with the equivalent of two times the fresh weight of the extracted seedling. An equal quantity of malt extract agar was later added. Controls were prepared in the same way but without phytoalexins. Sterilized capillary tubes (0.4 \times 100 mm) were filled with 0.040 ml of each of the phytoalexin agar dissolution. The open end of each tube was sealed with paraffin wax. To prepare the assay, Petri dish covers were concentrically perforated (16 holes per cover at 1.0 cm from the border). The capillary tubes were randomly placed to the cover through the perforations allowing the media in the tubes to be in contact with the growing hyphae in the cultures. The spaces between the edge of the perforations and the side of the capillary tubes were sealed with epoxy resin to avoid contamination and loose tubes. The experiments were incubated at 25°C for 6 days. Each *Aspergillus* culture was a complete experiment with four replicates per treatment (wild, landrace, cultivar and control). Growth of the hyphae inside the capillary tubes was measured as the linear distance (in mm) between the surface of the agar in the Petri dish and the growing end of the mycelia inside the capillary tube. ANOVA (GLM procedure, SAS Institute 1988) analysis was applied to the data. Multiple comparisons were

Table 1 Number of healthy and infected seedlings of wild and cultivated varieties of *Phaseolus vulgaris* under experimental field conditions

	Wild seedlings			Cultivar seedlings		
	Healthy	Infected	Total	Healthy	Infected	Total
Block A	52	1	53	40	14	54
Block B	46	2	48	37	17	54

Table 2 Deviance comparisons between blocks from the same variety

	Deviance (d.f. = 1)	<i>P</i>
Wild seedlings	0.5	0.47
Cultivar seedlings	0.41	0.52

made with the Sidak *T*-test (GLM procedure, SAS Institute 1988).

RESULTS

In all field experiments, the cultivar seedlings were more susceptible to fungal infection (Table 1). Wild seedlings performed better in all the experiments, showing lower infection percentages than the landrace or the cultivar varieties. No plot effects were detected in the field experiment (Table 2). Significant differences in infection rates were detected between wild (13%) and cultivar seedlings (29%) (Table 3). Results of the environmental chamber experiment were similar. Infection rates of wild seedlings were the lowest in two of the three soils (Table 4) and infection rates were highest in the cultivar in all soils. Infection rates of the landrace seedlings were intermediate between those of the wild and cultivar varieties. Interestingly, the infection rates of the landrace were, in two of the experiments, closer to those of the cultivar seedlings (no statistically significant differences (Table 5), but in the experiment with the soil with less organic matter (Chapingo), the infection rate of the landrace was closer to that of the wild variety.

From the 94 infected seedlings from all experiments, seven genera of fungi were identified. Two genera with many pathogenic species were isolated (*Pythium*, *Fusarium*), three genera with pathogenic

Table 3 Deviance analysis for the complete dataset

	Non-infected seedlings	Deviance (d.f. = 1)	<i>P</i>
Wild seedlings	0.97	29.1	<0.00001
Cultivar seedlings	0.71		

Table 4 Proportions of non-infected seedlings of *Phaseolus vulgaris* in environmental chamber experiments carried out in soils of different origin and use

Soil type	Non-infected seedling		
	Wild (%)	Landrace (%)	Cultivar (%)
Tepoztlán (wild population)	94.3	74.3	62.8
Chapingo	91.4	91.4	68.6
Xochimilco	93.7	75.0	71.9

and non-pathogenic species (*Aspergillus*, *Sporotrichum*, *Cladosporium*) and two genera with opportunistic species (*Geotricum* and *Sepedonium*).

Wild seedlings presented the highest chemical diversity, followed by the landrace and cultivar seedlings (Fig. 1). The results of the Kruskal–Wallis ANOVA by ranks show significant differences among treatments ($H_{(2,n=60)} = 6.424$, $P = 0.0403$). There were no significant differences in total phytoalexin concentrations (Fig. 2; $H_{(2,n=48)} = 2.16$, $P = 0.3396$).

Growth of the hyphae inside the capillary tubes was clearly different between treatments (Fig. 3). There was no significant effect from either replicate or block, but a highly significant effect of variety (Table 6).

When comparing the resistance and phytoalexin diversity in *P. vulgaris* with those previously

Table 5 Deviance (*P*) for among-varieties multiple comparisons in the three soils

Soil type	Wild vs Landrace	Wild vs Cultivar	Landrace vs Cultivar
Tepoztlán (d.f. = 68)	2.1371 (0.036)	2.852 (0.006)	1.025 (0.310)
Chapingo (d.f. = 68)	0 (1)	2.225 (0.027)	2.225 (0.027)
Xochimilco (d.f. = 62)	2.152 (0.035)	2.152 (0.035)	0 (1)

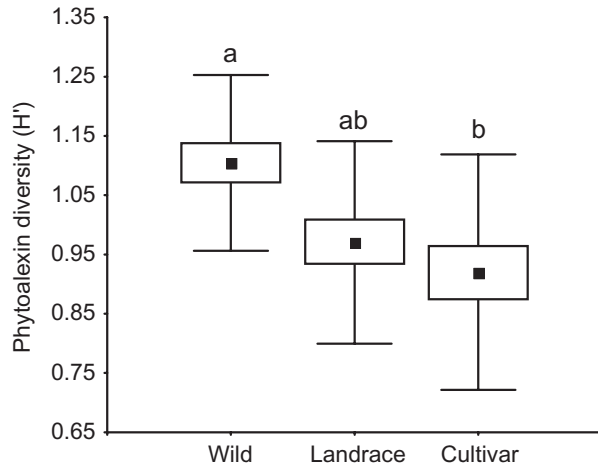


Fig. 1. Mean (\pm SE [box], SD [bar]) chemical diversities of wild, landrace and cultivar seedlings of *Phaseolus vulgaris* after treatment with CuCl_2 . Means were calculated as the Shannon index for each individual seedling. The same letters indicate no difference among treatments (Mann–Whitney *U*-test; $P < 0.05$).

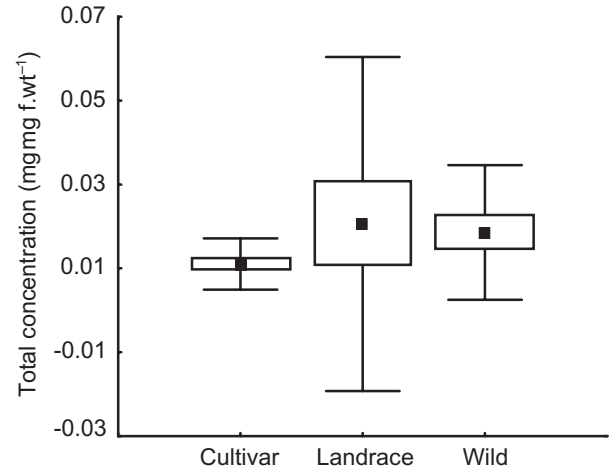


Fig. 2. Mean (\pm SE [box], SD [bar]) total concentration estimates expressed as grams of solids extracted per gram of fresh seedling tissue of the three varieties studied. No differences between treatments were detected. (Kruskal–Wallis ANOVA by ranks ($H_{(2,n=48)} = 2.16$, $P = 0.3396$).

obtained for *P. coccineus* and *P. lunatus* (Lindig-Cisneros *et al.* 1997) a clear pattern of resistance versus phytoalexin diversity that follows domestication status can be appreciated (Fig. 4). Cultivars of all species are in the left lower corner of the graph (low phytoalexin diversity and low resistance). Landraces are in the central to lower left areas of the graph and wild varieties are mostly in the right upper area (high phytoalexin diversity and high resistance).

DISCUSSION

The results show the expected gradient in defense levels (wild < landrace < cultivar) as phytoalexin diversity that corresponds to a gradient in resistance both under field and laboratory conditions. Lack of differences among total isoflavonoid concentrations suggests that this trait is not a deter-

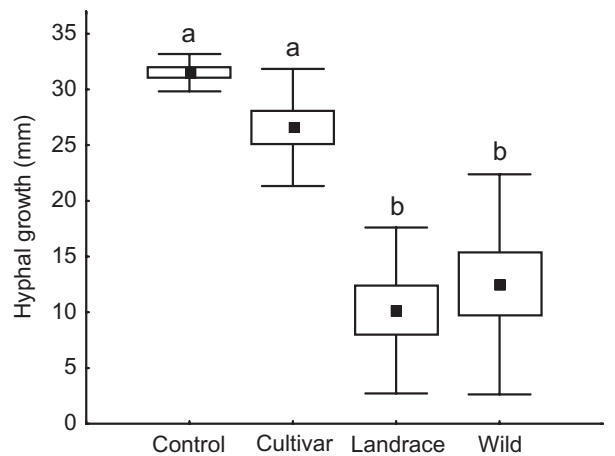
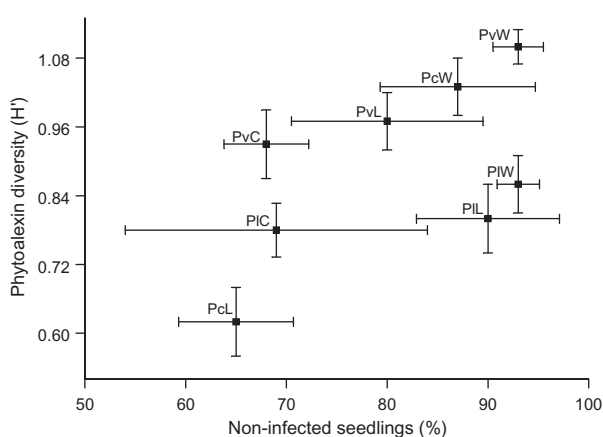


Fig. 3. Hyphal growth (\pm SE [box], SD [bar]) as an indication of the inhibitory effects of the isoflavonoid mixtures produced by the three varieties studied. *Aspergillus* sp. mycelia growth inside the capillary tubes was inhibited in all seedling treatments. The same letters indicate no difference among treatments (Sidak test).

Table 6 Analysis of variance of hyphal growth from *Aspergillus* sp. exposed *in vitro* to wild, landrace and cultivar seedling phytoalexin extracts (see Fig. 3)

Source	d.f.	Type III SS	MS	F	Pr > F
Treatment	3	3940.729	1313.576	27.35	0.0001
Block	2	26.375	13.187	0.27	0.761
Replicate	3	116.895	38.965	0.81	0.495

**Fig. 4.** Phytoalexin chemical diversity and resistance under all experimental conditions (means and SE) in *Phaseolus vulgaris* (PvW = wild, PvL = landrace, PvC = cultivar), *Phaseolus coccineus* (PcW = wild, PcL = landrace) and *Phaseolus lunatus* (PIW = wild, PIL = landrace, PIC = cultivar).

minant of the resistance capability, as is diversity. Wild seedlings presented the highest isoflavonoid phytoalexin diversity ($H' = 1.11$), whereas those of the landrace presented an intermediate level ($H' = 0.97$) and cultivar seedlings presented the lowest diversity ($H' = 0.93$).

Results from the *in vitro* assay show the importance of phytoalexin diversity in the resistance to infection in beans. Because the same compounds are synthesized in plant tissues in response to a wide range of pathogens, the induced response appears to be non-specific (Adesanya *et al.* 1985); thus, biosynthesis of a set of phytoalexins can be beneficial to the plant because of the increased probability of the pathogen being confronted by at least one toxic compound. It is also possible that diversity itself endows plants with greater resistance capabilities due to synergistic effects of the phytoalexins. In addition, variation in metabolites and/or their relative representation may render the plant less susceptible to its consumers (Dolinger

et al. 1973). Such variation is more likely to be displayed with a diverse profile of defensive secondary compounds.

Lo *et al.* (1999) tested 12 wild and 18 cultivated varieties of *Phaseolus vulgaris* under two different phosphorus levels and found yield differences among groups. For example, wild varieties had a lower mean shoot dry mass ($1.7 \text{ g} \pm 0.5 \text{ g}$) than cultivated varieties ($2.2 \text{ g} \pm 0.7 \text{ g}$) and the same was true for total leaf area (wild varieties mean leaf area = $278 \text{ cm}^2 \text{ plant}^{-1} \pm 93 \text{ cm}^2 \text{ plant}^{-1}$, mean cultivated varieties leaf area = $332 \text{ cm}^2 \text{ plant}^{-1} \pm 121 \text{ cm}^2 \text{ plant}^{-1}$). These differences are also clear in seed size; cultivated seeds are five to eight times larger than wild seeds (Tivy 1990). These reported differences in yield between wild and cultivated varieties and the gradient in resistance found in the present study are similar with those for maize and its wild relatives, in which the most primitive form was more resistant to herbivory and allocated less to yield (Rosenthal & Dirzo 1997). Our results with *Phaseolus vulgaris* are consistent with those for the other two species in the genus, as well as with resistance to *Colletotrichum sublineolum* in sorghum that is also positively correlated with phytoalexin diversity (Lo *et al.* 1999). The trends suggest that at least phytoalexin-induced defenses may be subjected to the same trade-offs between defense and yield and defense and growth that have been found for constitutive defenses.

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