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RESISTENCIA A HONGOS PATOGENOS Y FITOALEXINAS INDUCIDAS EN PLANTULAS DE FRIJOLES SILVESTRES Y CULTIVADOS DE TRES ESPECIES DEL GENERO *Phaseolus*

T E S I S

QUE PARA OBTENER EL GRADO DE MAESTRIA EN ECOLOGIA BASICA

PRESENTA

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A Sabina

Indice

Resumen

Se estudió la respuesta química inducida por hongos patógenos en plántulas silvestres y cultivadas de tres especies del género *Phaseolus (P. vulgaris, P. coccineus* y *P. lunatus*), así como la variación en la resistencia a hongos asociada a esta respuesta. Para cuantificar la resistencia a infecciones causadas por hongos patógenos se llevaron a cabo experimentos de campo y de laboratorio con plántulas de las tres especies. Los isoflavonoides (fitoalexinas) producidos en la respuesta inducida en plántulas se analizaron por medio de cromatografía de líquidos de alta eficiencia de manera indepenciente para las tres especies. La concentración total de las fitoalexinas, así como la diversidad química por plántula (calculada con el índice de Shannon), fueron los dos parámetros considerados en el presente estudio para la caracterización química de las especies. Finalmente, se estudiaron las propiedades inhibitorias del crecimiento de hongos *in vitro* de los extractos obtenidos de plántulas de las diferentes variedades de las tres especies.

Los resultados muestran un gradiente negativo entre la resistencia y el grado de domesticación (cultivar < criollo < silvestre) para las variedades de cada una de las tres especies estudiadas, y un gradiente en la misma dirección para la diversidad química de fitoalexinas y las propiedades inhibitorias del crecimiento. No se encontraron diferencias en la concentración total de las fitoalexinas entre plantas silvestres y cultivadas en ninguna especie.

Los resultados indican que las plántulas silvestres de las tres especies poseen una mayor resistencia a hongos patógenos y también una mayor diversidad química que las cultivadas. No se encontraron diferencias en las concentraciones totales de fitoalexinas entre variedades. Es posible que los patrones observados sean una consecuencia de una reasignación de recursos en las plantas cultivadas hacia mayores rendimientos, en detrimento de la asignación a la defensa.

Abstract

Seedling fungal resistance under field and laboratory conditions was assessed with a system of wild, landrace and cultivar varieties of three species of *Phaseolus* beans *(P. vulgaris, P. coccineus* and *P. lunatus*). In addition, phytoalexin isoflavonoids extracted from these varieties were separated and quantified; their chemical relative abundances were quantified (number and concentration of individual compounds) in order to estimate chemical diversities. The antifungal potential of phytoalexin mixtures from indiviual seedlings was tested *in vitro*.

We found a resistance gradient to fungal infections between wild, landrace and cultivar varieties for the three species. Wild seedlings were more resistant than landrace and cultivar varieties under field and laboratory conditions. Wild seedlings presented the highest isoflavonoid diversity, while those of the landrace presented an intermediate level and cultivar seedlings presented the lowest chemical diversity; a similar trend was obtained for the *in vitro* inhibitory properties. No differences were found in total isoflavonoid concentrations. These results are compatible with the interpretation that resource reallocation within plants have caused a reduction in chemical defense levels.

Introducción

El presente trabajo está formado por tres capítulos independientes que, sin embargo, forman parte integral del discurso de la tesis. Cada uno de los capítulos fue preparado para ser sometido en diferentes foros de difusión científica. El primer capítulo es una introducción al sistema experimental, y fue el texto base de una ponencia presentada en un Simposio Internacional sobre Ecología Química (Dirzo y Lindig, 1996). Los otros dos capitulos (2 y 3) son los manuscritos resultado del proyecto de investigación experimemtal, que han sido enviados a dos revistas internacionales para que sean considerados para su publicación. La literatura citada de las tres secciones se ha reunido al final del texto para facilitar la consulta de la misma.

El primer capítulo es una revisión de las consecuencias de la domesticación sobre las características de las plantas cultivadas, y de las diferencias de éstas con sus parientes silvestres. Como ejemplos se contrastan las diferencias entre las especies o variedades cultivadas con sus parientes silvestres de cinco géneros de plantas: *Zea, Oryza, Phaseolus, Cucurbita y Capsicum*. En las partes finales del capítulo se revisan los sistemas experimentales que se pueden formar con plantas silvestres y cultivadas emparentadas, así como algunos de los trabajos que se han llevado a cabo en la ecología evolutiva de este tipo de sistemas.

El segundo capítulo de la tesis corresponde al sistema formado por plantas cultivadas, criollas y silvestres de la especie de frijol común, *Phaseolus vulgaris*. Con dicho sistema experimental se cuantificaron las respuestas de las plántulas de las tres variedades ante infecciones de hongos patógenos, en términos de la morbilidad y las respuestas químicas específicas, para poner a prueba dos hipótesis básicas. La primera hipótesis establece que debe existir un gradiente, con una relación inversa entre nivel de domesticación y resistencia (cultivado < criollo < silvestre), entre las plántulas de las variedades estudiadas, como consecuencia de la reasignación de recursos para incrementar el

rendimiento en las plantas cultivadas (Rosenthal y Dirzo, 1996). La segunda hipótesis parte de que las plántulas de las especies del género *Phaseolus*, al igual que muchas otras especies, producen metabolitos secundarios específicos en las zonas cercanas a los puntos de infección fúngica en sus tejidos (Snyder y Nicholson, 1990). Este tipo de metabolitos secundarios se conoce genéricamente como fitoalexinas y son diferencialmente tóxicos para los hongos patógenos.

Las fitoalexinas producidas en la respuesta inducida por las plantas del género *Phaseolus* son compuestos de tipo isoflavonoide; más de 20 compuestos aislados de diferentes especies del género han sido estudiados exhaustivamente por diversos autores (vease Anderson, 1978; García-Arenal *et al.*, 1978; Bandara, 1979; Zavala *et al.*, 1989).

La segunda hipótesis es consecuencia por lo tanto del hecho de que los isoflavonoides de *Phaseolus* son una respuesta específica a infecciones y que son tóxicos para el agente infeccioso, y establece que debe existir un gradiente, similar al de la resistencia de la primera hipótesis, en la respuesta inducida entre las diferentes variedades estudiadas (cultivado < criollo < silvestre).

El tercer capítulo presenta los resultados obtenidos al repetir el trabajo experimental y confrontar las hipótesis anteriores con otras dos especies del género, *Phaseolus coccineus* y *Phaseolus lunatus*. En este capítulo, al igual que en el anterior, se discuten los resultados en términos de las teorías de defensa química existentes.

En la última sección, de conclusiones generales, se discuten solamente los resultados más relevantes y los patrones generales observados con todos los sistemas, ya que los resultados de cada sistema son discutidos individualmente en cada capítulo.

CAPITULO 1

Cultivated Plants and Their Wild Relatives. Model Systems for Chemical Ecology Studies

Origin of Agriculture and Cultivated Plants

The origin of agriculture, around 10,000 years ago, was a major turning point in the history of mankind. It occurred at the same time as the establishment of the first permanent settlements and was the beginning of all civilizations. Agricultural practices changed our relationship with nature and marked the onset of the major changes in the shape of ecosystems everywhere in the world where human communities developed. The landscape was, and still is, reshaped from natural ecosystems to simplified human-made agroecosystems in which plant assemblages (and biomass) consist of few cultivated species that are selected and harvested for our own use, both as food and raw materials. The development of agriculture would not have been possible without the appearance of cultivated plants, the descendants of wild relatives and whose development constitutes one of the most impressive cultural accomplishments of mankind.

The origin of cultivated plants, both in space and time, has been studied systematically over the last century. The geographic origin of cultivated plants was brilliantly studied and summarized for the first time by N. I. Vavilov, following the work of Alfonse de Candolle. Vavilov's "Centers of Origin of Cultivated Plants" published in 1926 (Vavilov, 1992), is an obligated reference in the study of this issue, not only because in his work he was able to clarify the origin of several crops in the old world (wheat, einkorn and emmer wheat, oats, etc.), but also because of the research methodology he established to determine such centers of origin.

Establishing the temporal origin of cultivated plants is a hard task because it is necessary to rely on sparse archeological data, and because of the difficulty of distinguishing between primitive cultivated varieties and wild types.

For rice, wheat, maize and potatoes, the four main staples of human diet, the oldest evidences of domestication range from 8,500 B.C., for some wheat varieties in the Near East, to 1 A.D. for the first hard evidence of consumption of *Solanum tuberosum,* the cultivated potato. Other crops were also domesticated in ancient times. For example remains of *Cucurbita pepo,* not belonging to a wild species, have been dated about 8,000 B. C. for the oldest archeological site in Oaxaca; cultivated lentils in Neolithic sites are dated to the 6th millennium. On the other hand, some species have a very recent history of domestication, and can be considered as incipient domesticated crops, such as *Vaccinium corymbosum* (a highbush blueberry). This species has been under domestication as recently as the beginning of this century (Sauer, 1993).

Nowadays, domestication of crop plants is not considered as an single event for each species in space or time, since for many species domesticated varieties have arisen in their center of origin from wild relatives several times during the history of agriculture. Others, such as the bean *Phaseolus vulgaris,* have been domesticated independently in geographically remote areas of South and Mesoamerica (Gepts, 1988).

The history of plant domestication can be divided in two stages. The first spans from the period between the diffuse historical origin of the first cultivated plants (and therefore of agriculture), to the beginning of this century. During the first stage, the domestication process took place under restricted local conditions and, as a result of such isolation, many varieties arise in response to human selection, and as a result of natural selection imposed by factors such as local climate, pests and soil conditions. The resulting varieties of these processes are now named landraces. The 1930's mark the origin of the second stage in plant breeding that is still under way. This stage began with the advent of the hybrid seed industry in the United States in the 1930's (Doyle, 1985). With this, new varieties known as cultivars

(from *culti*vated *var*iety) appeared. They were the result of more carefully bred populations that possess distinctive and homogeneous traits (Loomis and Connor, 1992). In the case of maize, farmers began buying commercial hybrid seeds in 1933, and 20 years later the open-pollinated landraces were totally replaced in the United States (Sauer, 1993). In 1943 a joint cereal breeding program between the Mexican Ministry of Agriculture and the Rockefeller Foundation gave rise to new varieties of maize and wheat and, subsequently to the CIMMYT (from the spanish for International Center for Maize and Wheat Improvment) global program. Thus, the introduction of wheat and maize cultivars all over the world began. The success of this program led to the foundation of the International Rice Research Institute (IRRI) for rice research in 1962, and other 11 international centers around the world also followed.

Cultivars represent a further step in the domestication process, because cultivated plants changed even more from their wild relatives than landraces did. In this second stage in the domestication of crops, cultivar yields were greatly increased compared with those of the landrace parent plants, and even more than those of the wild types.

Differences Between Cultivated Plants and Their Wild Relatives

During the process of domestication, plants have suffered a series of modifications due to the artificial selection of traits that are of human interest. Notable differences can be appreciated in those parts that are harvested. Of these, the most striking one is the gigantism of harvested parts in domesticated plants when compared to those of their wild relatives. The reduction in natural seed dissemination capabilities, for example indehiscent pods and fruits or non-shattering flower-stems in cereals (Tivy, 1990), are also important changes. Life history patterns have been modified as a result of the domestication process.

Particularly interesting are changes from perennial wild types to annual ones due to the agricultural annual cycles.

Differences between wild species and their cultivated relatives are a consequence of genetic changes as a consequence of artificial selection. For many species, this involves selection of polyploid descendants that can be reproductively segregated from their parent species. This is elegantly exemplified by the cabbage family (Brasicaceae). In *Brassica*, three diploid species exist with wild and cultivated representatives, they are; black mustard (*B. nigra* 2n = 16), cabbage (*B. oleracea* 2n = 18) and turnip (*B. campestris* 2n = 20). These three species can produce sterile interspecific hybrids. When the chromosomic numbers of these sterile hybrids are doubled, three new fertile species are produced. These new species are incapable of breeding with their parent species. The relationships between the species of *Brassica* are clearly summarized in the so-called triangle of U (fig. 1) (U, 1935).

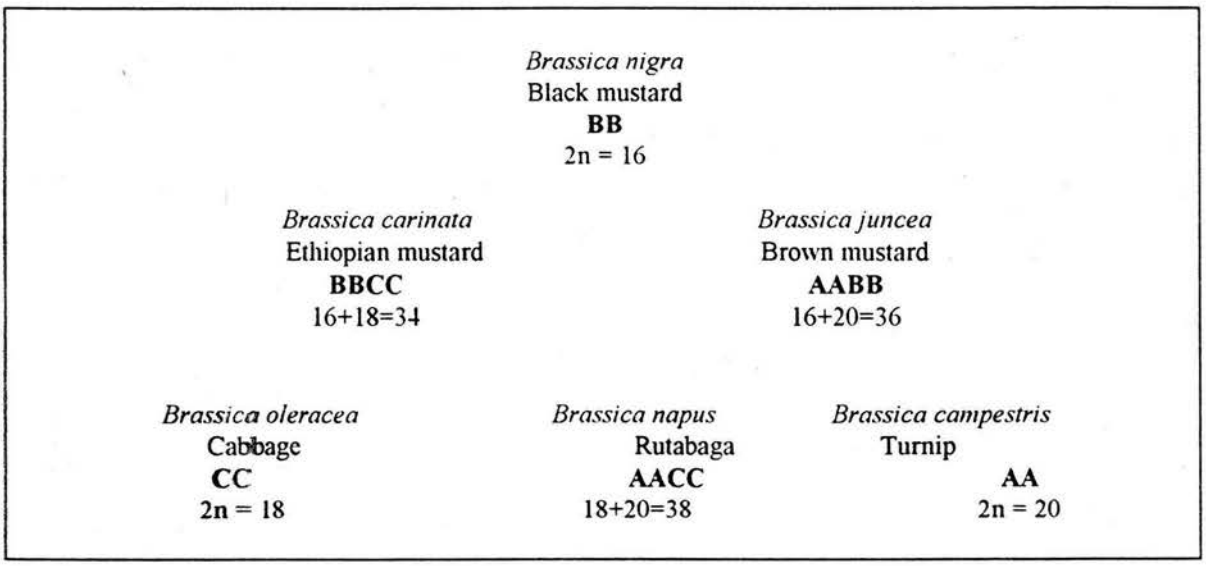


Figure 1. The triangle of U (U, 1935), that summarizes the chromosomal relationships between *Brassica* species. Diploid species have wild and cultivated representatives, polyploid species are strictly cultivated.

Other species in which the cultivated plants are derived via polyploidy from wild species are alfalfa (*Medicago sativa* 4x = 32), derived from *M. coerulea* (2n = 16) that grows wild in the grasslands of southwestern Iran, the Caucasus and Eastern Anatolia, and the European plum (*Prunus domestica* 6x = 48) an allopolyploid hybrid between two wild species: *P. cerasifera* and *P. spinosa* (Sauer, 1993), just to cite two examples.

Phenotypic changes are more obvious than genotypic ones. Yield increments of the harvested plants have already been highlighted, but other phenotypic changes, less evident to the eye, but that can certainly be tasted, have also been a consequence of domestication. These differences are a result of the elimination of secondary metabolites or mechanical defenses, for example: less alkaloids in cultivated potato tubers or less lectins in cultivated *Phaseolus* beans (Sotelo *et al.*, 1995). In other crops, an increment in secondary metabolites is a direct and desired consequence of domestication, as in the case of nicotine in tobacco leaves or caffeine in coffee seeds.

In many cases domesticated plants are more vulnerable to herbivores and pathogens than their wild relatives. The most dramatic example of this trend was the famine suffered in Ireland in the first half of the 19th century due to potato crop failure caused by the potato blight. This trend has been exacerbated by the appearance of the modern cultivars. In some cases, and despite their impressive yields under controlled conditions, some cultivars had to be discarded because their great productivity declined after a few cultivation cycles under normal farming conditions due to pests (Salick and Merrick, 1990).

During the history of plant domestication several types of domesticated plants and relatives have appeared. Landraces and cultivars have already been defined, but also weedy derivatives are important byproducts of this process. All of the foregoing together with wild relatives, represent the genetic diversity of crops (Salick and Merrick, 1990).

Differences between cultivars and landraces are a consequence of the selection procedures followed for each one and also of the biotic and abiotic conditions in which the selection occurs. Landraces are selected and maintained under very heterogeneous (spatial

and temporal) field and cultural conditions, as already discussed, that promote local adaptations and diversity of uses. Many landraces can be recognized in the centers of origin of many crops, and for some species even hundreds of varieties can be collected in relatively small plots.

Cultivar varieties are selected under controlled conditions and with standardized selection criteria, and are threfore genetically and phenotipically homogeneous. In extreme cases, the cultivar can be a clonal line in which all the individuals have the same genotype (for a review on recognized types of cultivars depending on the propagation methods see Loomis and Connor, 1992).

Landraces and cultivars are different at the individual and population levels due to their particular selection conditions. As already stated, cultivars tend to be more genetically homogeneous than landraces and, as a consequence, the individuals of a particular line present very little phenotypic variation. The genetic frequencies of landraces is variable within and among them. For landraces gene flow between cultivated plants, weedy and wild forms is common. For cultivars this is rare or does not happen at all, especially when cultivated far from their origin centers. In any case, because seeds are acquired from seed companies every year, no genetic flow can naturally occur between cultivars and wild relatives; wild genes can only be acquired by cultivars as a result of breeding programs.

On the basis of the preceding discussion, for the purposes of this work, the differences between wild and cultivated plants of five crops are summarized as examples. The crops to be considered are: maize (*Zea mays*) and its wild relatives, *Phaseolus* beans, rice (*Oryza* spp) and *Capsicum* and *Cucurbita* species and their cultivated varieties. All of these crops have in common that they are cultivated for human consumption and, consequently, similar trends in domestication can be assumed.

Maize (*Zea* spp)

Maize was domesticated in Mesoamerica. The earliest remains identified as *Zea mays* were recovered from a cave in Tehuacán, Puebla. This maize belonged to an extremely primitive race, and is dated about 5,000 B.C. (Sauer, 1993). Other remains from the same valley of Tehuacán, dated between 3,500 to 2,300 B.C., show that agricultural practices had increased during this period and, in the archeological remains of this phase (called the Abejas phase), about half the maize remains were cultivated and included a tripsacoid variety (Bushnell, 1976). In the Gulf coastal plain of Mexico, the oldest evidences of maize cultivation at a preclassic Olmec site are dated ca. 2,250-1,750 B.C. (Rust *et al.*, 1994).

The genus *Zea* consists of four wild species in Mesoamerica; *Zea perennis* and *Z. diploperennis* which are perennials, and the annuals *Z. mexicana* and *Z. luxurians*. The now generally recognized progenitor of maize is *Z. mexicana* (Sauer, 1993). Evidence exists that the independent domestication of maize has occurred at least twice. Morphological data suggest that the Nal Tel-Chapalote system is derived from a Guerrero teosinte (*Z. mexicana*) and the Palomero Toluqueño system is derived from a Chalco teosinte (Galinat, 1987 *and* 1988). *Zea diploperennis*, a weedy perennial, is probably the most primitive species in the genus (Iltis *et al.*, 1979). Although significant morphological and developmental differences exist between maize and teosinte (Iltis and Doebley, 1980), the different growth patterns in both species are controlled by the same regions in corresponding genes. For example, the single stem and tassel of maize, and the branched architecture and multiple tassels of teosinte are largely controlled by a small region on chromosome arm 1L (Meyerowitz, 1994).

Several changes in *Zea mays* have occurred as a consequence of domestication. The time of flowering and its duration have been decreased and a tendency toward protandry has become established (Canales-de-Suárez and Miranda-Colín, 1984). Changes in the structure of the seed and in its nutritional content are the most important changes due to

domestication. Through modern selection techniques even more nutritious varieties have been developed.

By the time Columbus arrived to the Americas, *Z. mays* had already evolved into a very diversified array of races (Sauer, 1993), as a result of the great environmental heterogeneity under which this plant was cultivated. This diversity is still mantained by local farmers in its centers of domestication. Farmers of Chiapas mantain several landraces belonging to six races and four race mixtures, and despite the introduction of modern cultivars, farmers still keep landraces for particular soils and uses (Bellón and Brush, 1994).

Introgression between maize and teosinte could occur. In fact, it has been suggested that the indurated glume and rachis of modern maize races is associated in North America with introgression from wild plants (Wet *et al.*, 1978). Introgression with *Zea perennis* can also take place, and it has been proposed as a method to improve heterosys and variability in cultivated maize (Magoja and Pischedda, 1986).

Several secondary metabolites are produced by *Zea* species. Pollen phenylacetic acid content and phytotoxic properties have been studied in both cultivated *Z. mays* and teosinte (Anaya *et al.*, 1992). Aglutinins have been isolated from maize plants and their efficiency against bacteria has also been tested (Bradshaw-Rouse *et al.*, 1981).

The most studied secondary metabolites of these species are the hydroxamic acids, and particularly DIMBOA and DIBOA, both in their biochemistry and effect over herbivores. Wild forms present higher concentrations of these metabolites than cultivated ones (Corcuera *et al.*, 1982; Classen *et al.*, 1990).

Rice (*Oryza* spp)

The domestication of rice occurred independently in Asia and in Africa. Both domestication processes gave rise to two species of cultivated rice, *Oryza sativa* in Asia and *Oryza glaberrima* in Africa. The double origin of cultivated rice is supported by molecular marker evidence (Second, 1986). The wild relative of the African *O. glaberrima* is considered to be *O. brevilgulata* (for a revision on the origin and evolution of cultivated rice see Oka, 1974). *O. sativa* originated al least 10,000 years ago in Asia by domestication of *Oryza perennis*, and later diversified in two subspecies, *japonica* and *indica*. Remains 7,000 years old excavated at Hemedu, China, were not differentiated into any of these two types (Oka, 1994), suggesting this to be the center of domestication of this species. Abundant rice remains have been excavated from Neolithic settlements in China from 4050 to 5850 B.C (Tang, 1994).

Several changes associated to the domestication process in rice can be found. Comparative studies in photosynthesis and chlorophyll content between wild and cultivated rice show that wild rice has lower photosynthetic light saturation points than cultivated forms (25-35 Klx for the former and 35-45 Klx for the latter; Li *et al.*, 1984). The photosynthetic rates under saturation light conditions are also different, being lower for wild types. Although cultivated forms are sensitive to short days, changes in this trend due to domestication can be detected when large samples of cultivated and wild strains are compared. In general, the photoperiodic sensitivity has been reduced in cultivated forms. In one study of 227 cultivated strains, 76.7 % were photoperiodic sensitive, and for 390 wild strains, 89.7 % showed the trend (Katayama, 1977). Also changes in growth patterns between wild *O. perennis* and cultivated *O. sativa* are reported, consisting in differences in vegetative growth after heading (Morishima *et al.*, 1973).

Oryza species show a gradient in genetic diversity that range from the highest diversity values for perennial and allogamous *O. perennis* forms, followed by annual forms

of the species with intermediate diversities, and the cultivated *O. sativa* and *O. glaberrima* with the lowest, since they are mainly homozygous and therefore present lower genetic variabilities (Morishima *et al.*, 1970).

Wild species are more resistant to herbivores and pathogens than cultivated ones. This has been proved with *Oryza brachyantha* and IR3 1917-45-3-2 cultivar plants against the rice leaffolder *Cnaphalocrocis medinalis*. The differences in resistance are a consequence of larval preference for the cultivar, mediated by unidentified chemical constituents extractable with hexane and methylene chloride, and physical resistance of wild plants due to silica (Ramanchandran and Khan, 1991; Velusamy *et al.*, 1990).

Phaseolus Beans

Four *Phaseolus* species were domesticated by the American Indians because of the high protein content of the seeds. Of these, two are widely cultivated, *Phaseolus vulgaris* and *P. lunatus*. The cultivation of the other two species, *P. coccineus* and *P. acutifolius*, is restricted to their centers of origin (Sauer, 1993). *Phaseolus vulgaris* was independently domesticated in Mexico and the Andean region. The oldest remains range from 5,500 B.C. to 2,000 B.C. depending on the locality. The oldest bean record from Mexico was found in strata dated between 7,000 and 5,500 B.C., and the remains correspond to *P. coccineus* (Delgado-Salinas, 1988). The case of *P. lunatus* is more complex, because three branches of cultivated beans are recognized, the Hopi branch, extending northward to the United States, the Carib branch that includes the Caribbean islands and the Amazon basin, and the Andean branch. The oldest remains seem to indicate that this species was already cultivated in the central Andean region about 5,600 to 8,000 years B. C. (Baudoin, 1988). For all *Phaseolus* species, wild, landraces, cultivars and weedy types can be found, particularly in their centers of origin. The weedy types presumably are the result of outcrosses between wild and cultivated types.

Under domestication, *Phaseolus* beans have suffered a considerable increment in seed size. For example, seeds of *P. lunatus* cultivars are up to six times larger than wild ones. Changes from perennial life cycles in wild relatives to annual cycles are common in *P. vulgaris* and *P. lunatus* varieties, but *P. coccineus* varieties have retained the perennial caracter with only a few exceptions (Delgado-Salinas, 1988). Major changes due to domestication for these species are summarized in Table 1.

Table 1. Characters Considered as Consequence Changes of the Domestication Process of *Phaseolus* Beans.

Seed and Pod Characters	Plant Characters
Increment in size and weight.	Change for perennial life cycles to annual ones
Loss of seed hardness.	and loss of the tendency to produce rizomes.
Variation in seed shape and testa colors.	Dwarf forms.
Loss of seed dormancy.	Day neutral forms.
Pods generally indehiscent.	

Sources: Baudoin (1988); Delgado-Salinas (1988) and Sauer (1993).

Changes in nutritional value and secondary chemistry can also be found. When comparing wild and cultivated forms of these species, a tendency to increase the nutritional value of cultivated forms can be appreciated. For example, *P. vulgaris* wild beans contain more protein, ash, and crude fiber than cultivated ones, but the former contain less fat and carbohydrates. Cultivated beans of this species have a better aminoacid profile than wild ones resulting from the higher content of limiting aminoacids they have (Sotelo *et al.*, 1995).

Detailed studies have been carried out on the structure of the main storage protein in beans, phaseolin, because it is used as a molecular marker (Koenig *et al.*, 1990).

Regarding secondary chemistry, one common trend for all *Phaseolus* cultivated varieties is the reduction in the amounts of lectins that are present in seeds. Wild beans have a higher content of trypsin inhibitors and lectins. In one study of *P. vulgaris* seeds, the reported values are: 28 TUI per mg of tripsin inhibitors for wild seeds compared with 21 TUI per mg for cultivated forms (Sotelo *et al.*, 1995). In the case of *P. coccineus*, differences in lectin types present in wild and cultivated seeds are also reported (Morgan and Manen, 1985).

Saponin content of *Phaseolus coccineus, P. vulgaris* and *P. lunatus* seeds has been reported. Both *P. coccineus* and *P. vulgaris* seeds have similar levels of saponin, 3.4 and 3.5 respectively (expressed as percentage saponin content $g.kg^{-1}$); *P. lunatus* presented the lowest concentrations of the three species (Keith *et al.*, 1986). These saponin analyses were performed only on cultivated seeds of the three species and, therefore, comparisons with wild relatives still need to be made to assess the effect of domestication on the concentrations of these secondary metabolites.

Phaseolus lunatus is unique among these species because cyanogenic glucosides are present in its seeds. The chemical, biochemical and physiological aspects of the synthesis and accumulation of these compounds have been well studied (for a review see Conn, 1980). The time-course patterns of cyanide potential in *P. lunatus* have been reported as follows. Cyanide compounds begin to accumulate in seeds 5 days after flowering, the maximum is reached after 25 days and the final concentration of the mature seeds is approximately two thirds of the 25 days maximun value (Frehner *et al.*, 1990). Concentration of cyanogenic glucosides in wild *P. lunatus* seeds is higher than that of the cultivated varieties. In fact wild seeds can have almost 150 times more cyanogenic glucosides than improved cultivars (158 mg/100g of sample for wild beans and 1.06 mg/100g for the sample of INIA-503) (Lucas and Sotelo, 1984).

For *Phaseolus vulgaris* the volatile lipid breakdown products derived from the lipoxygenase pathway in response to infections of *Pseudomonas syringae* have been studied (Kevan *et al.*, 1993). It has been found that an important group of secondary metabolites resulting from microorganism infections in *Phaseolus* beans are isoflavonoid phytoalexins. Isoflavonoid phytoalexins can be elicited in *Phaseolus* plants by a series of agents that range from biotic ones -mainly fungi (Anderson, 1978; García Arenal, 1978)- to abiotic ones, like transition metal ions (Cu, Hg, Ag, etc.). The fungitoxicity of these compounds was demonstrated long ago under different experimental conditions (Skipp and Bailey, 1977). The importance of these compounds in the interactions between bean plants and pathogenic fungi is highlighted by the presence on fungi genes that encode phytoalexin hydratase enzymes that detoxify these compounds (Li *et al.*, 1995).

Several isoflavonoids have been isolated from different *Phaseolus* species. Some of the principal compounds of this type synthesized by *P. vulgaris, P. coccineus* and *P. lunatus* are genistein, phaseollin, phaseollinisoflavan and kievitone (O'Neill *et al.*, 1984; Stössel, 1985; Adesanya *et al.*, 1985; Goossens, 1983). Other flavonoids and flavonoid glucosides have been studied for taxonomic reasons in leaves, flowers and stems of several Phaseolinae genera, including several *Phaseolus* species (Williams, 1995). Also the pathway of isoflavonoid synthesis is one of the best known in terms of the precursors, intermediates and enzymes that intervene in the biosynthetic process (Dixon *et al.*, 1995).

Finally, the effect on the genetic diversity of the domestication of these species has been studied at different levels. For *Phaseolus vulgaris* and *P. acutifolius* the domestication process may have represented a major bottle neck, as phaseolin data indicates (Gepts, 1988). These results are supported by DNA fingerprinting studies for *P. vulgaris* with M13 related sequences (Sonnante *et al.*, 1994). Cultivated *P. lunatus* plants are also less diverse than wild ones but only for the small-seeded varieties (the Carib branch of these beans), but not for the large-seeded varieties (Gutiérrez *et al.*, 1995). In the case of *P. coccineus* the domestication process has not reduced the genetic diversity of the cultivated forms, at least

in the geographic range of origin of this species, in which high introgression rates between wild and cultivated forms occur (Escalante *et al.*, 1994).

Other Species

Other species with interesting possibilities because of their particular history and consequences of domestication are those of the genera *Cucurbita* and *Capsicum*. *Cucurbita* spp were domesticated independently from 5 species; but the progenitors of two domesticates remain unknown (Nee, 1990). The oldest remains of *Cucurbita pepo* are from the valley of Guila Naquitz in Oaxaca and are dated at 8,000 B.C. (Sauer, 1993). In other parts of the continent, *Cucurbita* remains are more recent. In the Viru Valley in Peru, remains are dated ca. 1,800 B.C. to 1,100 A.D. (West and Whitaker, 1979). Remains from North America of *C. argyrosperma*, are dated in the range from 1280 to 1490 A.D. (Fritz, 1994).

Several changes have occurred in *Cucurbita* species because of domestication. Wild populations produce significantly more female flowers than cultivated forms, which produce more male flowers (Reyes-Treviño and Miranda-Colín, 1979), and seed oil content is higher in cultivated plants. Several studies concerning resistance traits both to pathogens and herbivores exist for species of this genus (Provvidenti et al, 1978; Howe, et al, 1976).

Capsicum includes about 25 wild species which are perennials (Sauer, 1993). There are four cultivated species, *C. pubescens, C. baccatum, C. frutescens* and *C. annuum* (Sauer, 1993). For the first species, it has been suggested that domestication took place in Bolivia (Eshbaugh, 1974). With the exception of *C. annuum*, the other three species have their centers of origin in South America, and biochemical data support the idea that three independent clusters of wild and cultivated taxa exist (Mcleod *et al.*, 1983). What makes these species interesting is the great diversity of cultivated forms that can be found in their

distribution range, not only in fruit shapes and fruit concentrations of capsaicin but also in plant growth habits.

Wild and Cultivated Plants as Model Systems

Wild plants and their cultivated relatives can form model systems useful to prove several ecological and evolutionary hypothesis. As can be seen in the former species descriptions, cultivated species differ in many ways from their wild predecessors. The domestication process has changed plants in many ways and, by doing so, has provided several cultivated varieties with well defined characteristics. The characteristics that have been modified are of a physiological, architectural, chemical and life history nature. At the same time, cultivated plants maintain many of the basic characteristics of their wild relatives. This is particularly true for species in which the domestication process has not created reproductive barriers between wild and cultivated forms (for example, some *Brassica* species). Even in those cases in which the domesticated species are classified as different species than their wild relatives (*Zea*, *Oryza* and *Cucurbita* for example) several studies show that gene flow does occur under natural conditions.

The specialized literature is abundant in reports concerning cultivated plant traits, from nutritional value of the harvested parts to resistance traits against herbivores and pathogens. This can be easily appreciated after reviewing available databases such as the CAB database (Table 2). Also, reports of wild relatives of domesticated plants, and some comparisons with wild relatives can be found. This huge amount of information makes the wild and cultivated plant model one of the best described in terms of the characteristics of the involved parts.

Table 2. Number of references for the studied genera related to different biological aspects, as reported in CAB-Abstracts for the years 1993 through 1995.

	Host Resistance	Nutritional	Pathogen	Herbivory	Genetic	Taxonomy	Physiology
Zea	675	200	1337	7	1672	161	3521
Oryza	777	83	1302	1	1376	140	2347
Phaseolus	302	57	399	3	452	64	1024
Cucurbita	49	5	82	1	84	12	291
Capsicum	202	17	309	1	195	29	385

Source: CAB-Abstracts (1995).

The simplest model that can be arranged with a cultivated species is that of one wild and one cultivar variety, which also represents the model with the more contrasting differences in those traits that have been modified by domestication. Other models can be formed on the basis of domestication gradients by adding landraces or weedy relatives to the simpler model.

Models that highlight the effect of differences in architecture, secondary chemistry, growth habit, and many more traits can be set up to prove several hypotheses. Some of these differences have already been exploited as discussed below.

By selecting the ontogenic state of the wild and cultivated varieties under study, several confounding factors can be eliminated. For example, when studying resource allocation to defense between varieties, selecting seedling stages in those species in which the wild plants form a rhizome (for example in some *Phaseolus* species) is a way of avoiding different partitioning patterns between wild and cultivated forms because the rhizomes begins to form several weeks after germination.

Nevertheless, studies that compare wild and cultivated forms are scarce, and those that use such systems to prove ecological theory are practically unexistant. This can be clearly seen if a review of the nature of the articles published in specialized periodicals of the area is undertaken. In the particular case of the Journal of Chemical Ecology, a revision of 58 numbers of the Journal between the years of 1991 to 1996 showed that 58 articles out of a total of 951 deal with wild and their cultivated relatives, yet not a single one of them had as its main objetive proving basic ecological/evolutionary hypotheses.

The Study of Wild and Cultivated Plants in the Light of Chemical Ecology Theory

Chemical ecology theory provides a framework to explain some of the observed differences between wild and cultivated relatives. As stated by Herms and Mattson (1992), the allocation of resources by plants to chemical and structural defenses decreases growth by diverting those resources from this function. Thus, it is reasonable to conclude that, as a consequence of human selective pressure, a reallocation to increased yields causes a decrease in the allocation to other plant functions, including defense. This has been proved empirically both directly and indirectly. Directly, by measuring yields and susceptibility of different wild and cultivated related plants (Rosenthal and Welter 1995; Rosenthal and Dirzo, 1997) , and indirectly by demonstrating trade-offs between primary and secondary metabolism under cell culture laboratory conditions (Collin, 1987).

Hypotheses that deal with phenotypic variation in secondary metabolism, such as the carbon/nutrient balance hypothesis (Bryant *et al.*, 1983), or the growth-differentiation balance hypothesis (Tuomi *et al.*, 1990), are not useful in explaining differences in secondary chemistry among wild and cultivated relatives, because these hypotheses deal mainly with

observed differences in secondary chemistry between plants of the same species (or even the same population) growing with different resource limitations.

The growth rate and resource availability hypothesis (Coley *et al.*, 1985), and the growth-differentiation balance hypothesis (Herms and Mattson, 1992), both provide several elements to explain differences between wild and cultivated plants. These hypotheses predict that the optimal level of defense will vary with the intrinsic growth rate of the plant. This can explain why the defense levels of cultivated fast-growing plants are lower than those of wild slow-growing relatives.

When plants grow with plentiful resources they invest more in growth related functions than in differentiation ones, because this strategy makes the plant more competitive by enhancing further resource acquisition. Consequently, other plant functions such as defense, that divert resources from growth, are not favorably selected. In the case of domestication, where artificial selection also promotes higher growth rates (to increase yields), it is possible that natural and artificial selection pressures favor the same plant trends.

Not only can chemical defense hypotheses explain some of the differences between wild and cultivated plant systems. These hypotheses can also be tested with this kind of systems. As noted by Herms and Mattson (1992), "the hypotheses addressing the evolution of defense in plants have been developed and tested mainly with interspecific comparisons" - because more variation exists between species than within species. In this respect, wild-cultivated plant systems can be useful in providing evidence of patterns of resource allocation and life history strategies in plants. Also wild-cultivated plant systems can be useful for testing those chemical defense hypotheses dealing with phenotypic variation, as a result of the contrasting differences between wild and cultivated forms.

Recent Studies

In the following section, three recent studies comparing traits between wild and cultivated plants of three different genera, *Zea, Phaseolus* and *Brassica* are reviewed. These will to be discussed because they address some basic hypotheses in ecology.

The first study, by Rosenthal and Welter (1995), examines the tolerance in architecturally distinct maizes to damage inflicted by the herbivore *Diatraea grandiosella*. This work was carried out with *Zea diploperennis, Zea mays parviglumis*, a *Zea mays* landrace and one cultivar. The authors address three questions concerning the effect of architecture on herbivory tolerance, the effect of life history and domestication on this trait and the relationship between tolerance and plant defense.

This study exemplifies the kind of hypotheses that can be tackled with systems made out of related wild and cultivated plants; it also illustrates the kind of experimental precautions that have to be taken to overcome undesirable traits -such as differences in growth rates between species and varieties- and avoid interpretation errors due to these traits.

The results of this study showed that wild relatives were more tolerant than cultivated forms and that plant architecture plays an important role in this trait. For this particular system, tolerance and putative defense are both reduced with the change from perennial (wild maize) to annual (cultivated forms), and this tendency is contrary to the predictions of several optimization theories, as discussed by the authors.

Another study related to the one mentioned above was carried out by Rosenthal and Dirzo (1996). In this work the authors outline a conceptual resource allocation model that predicts a reduction in plant defense via artificial selection for yield.

For this study, the same model of *Zea* species described in the work by Rosenthal and Welter (1995) was used. Several parameters were measured, among them, level of leaf damage, growth rates and reproductive output.

The model proposed by Rosenthal and Dirzo is supported by their data with strong correlative results. In fact, in the four *Zea* taxa a gradient in investment to growth and reproduction is inversely associated with presumed relative defense against insects.

When these two studies are considered together they clearly exemplify the potential of wild and cultivated plant systems in proving ecological hypotheses under several experimental conditions.

Finally, in the work of Benrey *et al.* (1996), two systems are presented to study relationships between three trophic levels. They studied the consequences of plant domestication on some components of the interaction between phytophagous insects and their parasitoids, and hypothesized that cultivated plants provide higher quality resources than wild ones. The authors argue that: "Systems in which wild and cultivated species coexist provide ideal models to study interactions among three trophic levels" and provide plentiful evidence to support this by carefully describing the systems formed by wild and cultivated *Brassica olaracea* and *Phaseolus* species, as well as by the experimental results obtained with these studt systems.

Conclusions

The domestication process has produced several changes in the characteristics of cultivated plants, some of them common to all domesticates and others only in particular taxa, as discused in this review. Due to the great importance cultivated plants have for human societies, a great effort has been underway to study cultivated plant characteristics and to some extent also those of their wild relatives. This is particularly true for the staple foods of the human diet, the grains, tubers and legumes.

The experimental systems of cultivated varieties and their wild relatives represent well described models appropriate to prove ecological and evolutionary hypotheses, not only those related to the domestication process itself, but also more basic processes. Some

relevant hypothesis in ecology have already been tested with these systems. For example, those that involve hypotheses of resource allocation in plants, architectural traits and plant-herbivore interactions, and those of interactions between three thropic levels, as mentioned in this work (see Benrey *et al.*, 1996, Rosenthal and Welter, 1995; Rosenthal and Dirzo, 1997).

Several considerations are needed when working with wild-cultivated systems. First of all, the phylogenic relationships between the varieties under study should be clear enough to avoid interpretation errors. It is also important to know the domestication status of the cultivated varieties, because of the profound effects this can have on plant characteristics (genetic diversity, resistance to particular biotic and abiotic factors, etc.).

In many cases the development of modern cultivars involves the selection of very particular traits (for example, resistance to a single pest, or tolerance to deficiency of a given nutrient in the soil), while other cultivars are withdrawn from the market because of poor performances when compared with existing cultivars. As a result, it is important to know the technical characteristics of the chosen cultivars, in order to avoid interpretation errors and attribute observed differences to the overall domestication process and not to the very particular selection conditions for the chosen cultivar under study.

For chemical ecology studies, resorting to wild-cultivated systems can be useful in the study of allocation processes, costs of defense and trade offs between defense and other plant functions, also to understand the role of secondary metabolites in plant-herbivore and plant-pathogen interactions and the role of plant traits in three trophic level interactions. The following chapters present studies wich directly address this statements.

CAPITULO 2

Variation in Antifungal Induced Defenses In Relation to Domestication Status in *Phaseolus vulgaris* L.

ABSTRACT

As a consequence of human selection, cultivated plants present higher yields than their wild relatives. Commercial cultivars produce even higher yields than the landraces that have originated them, and much higher ones than their wild relatives. In many cases defenses against consumers in these varieties follow an inverse trend: lowest in commercial cultivars and highest in wild relatives, whereas those of landraces are intermediate. These trends can be explained if allocation to, or costs of, defense are high -a major assumption for several chemical defense hypotheses.

In this work a system of wild, landrace and cultivar varieties of *Phaseolus vulgaris* L., was studied to assess fungal resistance under field and laboratory conditions. In addition, phytoalexin isoflavonoids extracted from these varieties were also examined; their chemical relative abundances were quantified in order to estimate chemical diversities, and their antifungal potential was tested *in vitro*.

We found a gradient of resistance to fungal infections between wild, landrace and cultivar varieties. Wild seedlings were more resistant than landrace and cultivar varieties under field and laboratory conditions. Wild seedlings presented the highest isoflavonoid diversity, while those of the landrace presented an intermediate level and cultivar seedlings presented the lowest chemical diversity. No differences were found in total isoflavonoid concentrations. The *in vitro* inhibitory properties of the isoflavonoid mixtures produced by individual seedlings of each variety were also tested, and the extracts of wild seedlings were founf to be the most inhibitory.

INTRODUCTION

Chemical defense hypotheses in plants assume that chemical defenses are costly because they divert resources -photosyntates and nitrogenous compounds- from other plant functions, particularly from growth and reproduction (Herms and Mattson, 1992). This assumption is supported by strong evidence of inverse relationships between resource allocation to growth and other processes such as reproduction and defense (Bazzaz *et al.*, 1987, Mooney *et al.*, 1983), or between yield and resistance to consumers (Pimentel, 1977; Rosenthal and Dirzo, 1997). Also, trade-offs between primary and secondary metabolism are well documented for cell cultures (Collin, 1987).

Two major kinds of chemical defenses are known to occur in plants: constitutive defenses, present continuously in plant tissues which, in many cases, are stored in specialized structures within the plant, and induced defenses, those that are produced as a response to an environmental stimulus (stress, pathogens, herbivory, etc.) (Janzen, 1979). Plant defense theory predicts higher costs for constitutive defenses than those for induced ones because they continuously divert resources from primary metabolism to secondary metabolism. Furthermore, high metabolic costs have been calculated in terms of glucose investment for at least one type of secondary constitutive metabolites (Gershenzon, 1994).

Induced defenses have been proposed as an adaptation to minimize costs of defense because they incur costs as a function of actual attack and not probability of attack (Herms and Mattson, 1992). If this is correct, inducible defenses should be favored when costs of defense are high and the timing of attack is unpredictable (Clark and Harvell, 1992).

Under domestication, plants have increased their yields when compared to those of their wild relatives, particularly of those parts that are of agronomic interest (Tivy, 1990). It has also been observed that domesticated plants have lower levels of chemical defenses, mainly in those organs that are of interest for humans. For example, less alkaloids in potato tubers (Roddick, 1986), less cianogenic glucosides in cassavas, and less bitterness (due to

the presence of several secondary metabolites) in cucurbits and yams (Haekes, 1983), or low levels of lectins in *Phaseolus* beans (Sotelo *et al.*, 1995). Other protective structures have also been eliminated or substantially reduced, for example spines in several fruit trees and thorns on eggplants, like the African eggplant *Solanum dasyphyllum* (Haekes, 1983). This reduction in defensive chemicals and other defensive structures, which is almost certainly a consequence of selection for palatability and easiness of agricultural labor, cannot explain the reduction of defensive traits in non-harvested parts of the plants (Krischik and Denno, 1983).

Low levels of defenses in non-harvested parts of cultivated plants are attributed to the reallocation of limited resources within the plant to increase yields. This assertion is based on the fact that no net overall increment in photosynthetic rates as a result of domestication has been documented in several studies with cultivated plants (see Evans 1984; Rosenthal and Dirzo, 1997).

During most of the history of cultivation, human selection has been carried out under local conditions and has coexisted with natural selection pressures from herbivores and pathogens. This process gave rise to landraces. The history of modern cultivar selection began in the first half of this century. Because in many cases selection of modern cultivars has been carried out under conditions that practically eliminate pressure from herbivores and pathogens, the selection pressure for defenses is practically nonexistent. Consequently, a gradient of defense levels can be inferred 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. This model has been proposed and documented empirically with maize and their wild relatives by Rosenthal and Dirzo (1996).

Low costs have been attributed to the production of induced defenses (see Herms and Mattson, 1992) considering that these responses in many cases are restricted to some

plant parts. Following current hypotheses and models of chemical defense, it can be assumed that no sensible modification in the expression of induced defenses should be expected as a consequence of the reallocation of resources. However, these assumptions are conflicting with the observation that growth is interrupted after defensive induction because metabolites are diverted to secondary metabolism under culture conditions (daCunha, 1987). Therefore, in this work we hypothesize that high costs may be associated to the production and use of some induced defenses by plants.

Moreover, if induced defenses do represent a cost, which may be at least close to that of some constitutive defenses, it can be hyphotesized, following the model of Rosenthal and Dirzo (1996), that a decreasing gradient in resistance can be detectable between wild plants, landraces and modern cultivars, and these resistance differences should be related to similar trends in induced chemical defense parameters.

We tested the hypotheses stated above by i) studying the resistance against pathogenic fungi of seedlings of three varieties of the bean *Phaseolus vulgaris* in field and laboratory experiments; ii) measuring individual seedling chemical diversities of isoflavonoid phytoalexins; and iii) measuring the *in vitro* inhibitory properties of phytoalexins extracted from the same varieties.

METHODS

The system

In *Phaseolus* beans, an induced response is elicited when plant tissues are in contact with microorganisms, mainly fungal hiphae (for a review see Sequeira, 1983). This response consists in the production of phytoalexins, isoflavonoids with different chemical substitution patterns. In *P. vulgaris* about 15 of these compounds have been identified, although not all have antifungic activity and many are metabolic intermediates. Five major phytoalexins have been reported for this species: phaseolin, phaseollidin, phaseollinisoflavan, coumestrol and kievitone (Soriano and Medina, 1989). These compounds have been isolated from

experimentally infected plants with several pathogenic species (Anderson, 1978; Garcia-Arenal, 1978; Bandara, 1979; Zavala *et al.*, 1989). Antifungic properties of these compounds have been well established, although their effectiveness depends heavily on the susceptibility of the fungi species (Skipp and Bailey, 1977).

Resistance to fungal infections in green plants of *P. vulgaris* seems to rely on the induced response of isoflavonoids, since no other secondary metabolites with proven fungitoxic activity have been isolated from this species.

In Central Mexico, wild populations of *Phaseolus vulgaris* can be found growing on slopes, in secondary habitats, mostly on igneous soils (Delgado *et al.*, 1988). In the same region, landraces as well as modern cultivars are also grown. In some locations these varieties coexist in contiguous plots. *Phaseolus vulgaris* wild plants as well as many cultigens are generally annual, slender and branched climbers.

Phaseolus beans have suffered a series of modifications as a consequence of domestication. An increase in seed size (cultivated seeds are five to eight times larger than wild seeds), an increase in overall plant size, and the loss of dehiscence are the more obvious changes in domesticated plants (Tivy, 1990). Also a reduction in antinutritional factors such as trypsin and chymotrypsin inhibitors occurs in the cultivars of this species (Sotelo *et al.*, 1995).

For this study, a *P. vulgaris* system consisting of three levels of domestication was used, with seedlings derived from seeds from a wild population, seedlings from one landrace and seedlings from one commercial variety . The wild population chosen for this study is located about 3 km from the town of Tepoztlán (State of Morelos, Mexico) in a heavily disturbed oak forest. The landrace "amarillo", which is traditionally cultivated in the Mexican state of Puebla and the "Black Valent" cultivar which is regularly used in Central Mexico were obtained from Asgrow Co. in a local seed store. After collecting or purchasing, all seeds from which experimental seedlings were produced were stored at 5 °C until utilization.

Morbidity in Field and Environmental Chamber Experiments

A field experiment was set up in an agricultural field located in the Xochimilco region of the Valley of Mexico. During the first week of April 1996, the site was cleared after a five year resting period. This resting period allowed us to avoid the interference of pesticides and fertilizers that are normally used in this region.

Surface-sterilized seeds (previously immersed in a 1 % aqueous solution of sodium hipochlorite for 5 minutes) of wild and cultivar origin were randomly distributed in two 1.5 m^2 plots, each consisting of a 20 X 10 grid (100 seeds per variety in each plot). In order to eliminate possible epidemic effects, seeds were 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. The experimental grids were covered with a thin layer of soil and watered every day. Eight days after planting, seedlings were chosen to form cohorts if the radicle was clearly visible. For wild seedlings, all those that filled this criterion were chosen. Because considerable more cultivar seedlings with radicle were present, a number equal to that of the wild seedlings was randomly chosen from each grid. Cohorts were inspected every day until the first true leaves appeared in all non-infected seedlings, as these grew faster than infected ones.

For the growth chamber experiments, soil was collected from the wild population site and two agricultural fields, one from Xochimilco (the same place of the field experiment) and one from the Chapingo region of the State of Mexico. Three common planting and independent experiments were set up in each of the soil types collected.

Each soil sample was sieved through a number 10 mesh, and then stirred to homogenize it. Saturation water capacity (SWC) for each soil type was estimated by adding distilled water to a sample of 50 g of soil in a well drained container and weighing the wetted soil.

Homogenized soil was put in polyethylene cups up to approximately one fifth of the cup capacity. Sterilized distilled water was added to each cup to SWC, in order to have equal conditions in all the replicates.

Seeds of the three varieties were superficially sterilized with 1 % sodium hipochlorite solution for 5 minutes and rinsed with sterilized water. One seed of each variety was placed in each cup under sterile conditions and covered with a translucent plastic cover. Soil and water quantities for each experiment varied depending of soil type. In each experiment 50 seeds per variety were planted and cohorts of 35 seedlings selected.

The cups were placed in an environmental chamber (Coenviron, Model E15) with a photoperiod of 8 hours and a dark period of 16 hours. In order to obtain a constant temperature of 29° C inside the plastic cups the temperature during the photoperiod was set to 26 °C and during the dark period to 29° C. The high temperature and humidity prevalent during the experiments favored fungal growth, reducing the time of each experiment and reducing the possible contribution of other factors to the morbidity results.

Each cup was inspected daily in order 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 conservative criterion was followed to distinguish infected seedlings in all field and environmental chamber experiments. Only those which clearly showed fungal infection symptoms were counted, and those that were infected but also mechanically damaged (for example by herbivores) were not counted to avoid over-estimates attributed to opportunistic infections.

For all the above experiments the response variable was either of two states for each seedling: infected or non-infected. For this reason, the results were analyzed assuming that the result for each seedling is a consequence of a binomial trial with sample size 1 (Crawley, 1993), which was in fact the case considering that no interaction between seedlings was possible, neither in the field nor in the environmental chamber trials. The analysis was carried

out with GLIM 3.77 (Royal Statistical Society, 1985) using a logit link function and a binomial probability distribution. The 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, to identify the infectious agents.

Chemical Diversity and Total Concentration of Phytoalexins

Induction of the biosynthesis of phytoalexins can be achieved by methods which can be classified into two groups, biotic and abiotic. Biotic methods use as elicitors spore suspensions, fungi hiphae or other living microorganisms. A serious limitation of these methods is that they are difficult to standardize (Sequeira, 1983). Another limitation of using living organisms as elicitors, particularly fungi, is that several species have the ability of metabolizing plant phytoalexins into less toxic compounds that can be misidentified as plant metabolites (Vanetten *et al.*, 1989). One way to override this limitation is using abiotic elicitors such as metal cations (for example salts of Cu, Ag or Hg) that have the property of inducing the production of isoflavonoids but are unable to alter chemically the resulting phytoalexins (Adesanya *et al.*, 1985). For these reasons, in the present work $CuCl_2$ was used as the elicitor of the induced response.

After surface sterilization with sodium hipochlorite (as previously described) 150 seeds of each variety were imbibed in distilled and sterilized water for 8 hours. After this treatment, the seeds were germinated under sterile conditions at 25°C. At 48 hours after germination cohorts of 48 seedlings per variety were selected using the same criterion as that of the morbidity experiments. The selected seedlings were treated with aqueous 5mM $CuCl_2$ for two hours. Treated seedlings were then incubated for 48 hours. After incubation,

cotyledons were removed and all seedlings were individually extracted with 20 times their weight of chromatographic grade ethyl acetate.

For chemical diversity assessments, high performance liquid chromatography (HPLC) analysis of 20 extracts per variety was performed (Waters HPLC system Model 600 MS). Isoflavonoid separation was carried out in a Lichrosphere 10μ 250 X 3.2 mm column (Phenomenex), 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 to 1.2 ml/min (curve 6).

Isoflavonoid UV spectra were obtained for all the compounds present in the extracts using a photodiode-array detector (Waters, model 990).

Chromatograms of the different *P. vulgaris* varieties show between 2 and 4 peaks due to isoflavonoid compounds. Using the peak parameters of the identified isoflavonoids in the chromatograms, chemical diversity for each individual seedling was assessed with the Shannon Index (H'). Statistical analysis was carried out with Kruskal-Wallis ANOVA by ranks (assigned to the individual H' values) due to inequalities of variances.

For total concentration analysis, isoflavonoid extracts -16 per variety- were lyophilized and the remaining solid weighed; total concentration was then calculated as grams of isoflavonoid mixture per gram of fresh weight of seedling tissue. As with chemical diversity analysis, due to the inequality of variances, a Kruskal-Wallis ANOVA by ranks was applicable.

In vitro Assay of Phytoalexin Activity

With 12 extracts per variety, an *in vitro* assay was performed to asses the inhibitory properties of phytoalexin mixtures produced by each seedling. Although the fungitoxic activity of isolated isoflavonoids is a well established fact (Skipp and Bailey, 1977) we were interested in the overall effect of the mixtures.

For this assay we used an isolate of *Aspergillus* sp. previously obtained from an infected seedling. Three cultures were prepared by placing a little amount of inoculum at the center of each petri dish which had previously been filled with malt extract agar media.

The phytoalexin extracts 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 the phytoalexins. Sterilized capillary tubes (0.4 X 100 mm) were filled with 0.040 ml of each of the phytoalexin agar dissolutions. 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 fixed to the cover through the perforations in such a way that the media in the tubes was in contact with the growing hiphae in the cultures. By means of this arrangement each *Aspergillus* culture was a complete experiment with 4 replicates for each treatment (wild, landrace, cultivar and control). 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.

Growth of the hiphae 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. Only in two replicates the hiphae grew beyond the media in the

tubes. In both these cases, the total length of the mycelia was measured because mycelia grew over the sides of the tube.

In order to test the expectations that there should be detectable differences among treatments, three categorical variables were established: treatment (with four levels, one for each variety and the control), block (for each petri dish experiment) and replicates (four for each treatment) and growth as the dependent variable. Data were analyzed by means of ANOVA (GLM procedure, SAS Institute, 1988). The differences among individual treatments were analyzed with the Sidak T test (GLM procedure, SAS Institute, 1988).

RESULTS

Morbidity in Field and Environmental Chamber Experiments

In both field and laboratory experiments, the cultivar seedlings were more susceptible to fungal attack. Wild seedlings performed better in all the experiments, showing lower infection rates. Also, the severity of the infection was more acute in cultivar seedlings, although this was not statistically analyzed because the small number of replicates in each treatment did not allow a multi-category data analysis.

Field experiment results are summarized in Table 1. Because no significant differences were detected between the same treatments in different blocks (wild seedlings $P= 0.479$; cultivar seedlings $P= 0.522$, Table 1b) the entire data set was included in a single analysis. Significant differences were detected between infection rates of wild (0.13) and cultivated seedlings (0.29) of *P. vulgaris* under these conditions (Change in deviance due to treatment = 29.1, $P< 0.00001$).

TABLE 1. A) The number of expected seedlings of wild and cultivated varieties of *Phaseolus vulgaris* under experimental field conditions in Xochimilco, Mexico. Wild seedling numbers are lower because some seeds were consumed by predators. B) Deviance comparisons between blocks from the same variety. (GLIM binomial trial, for details see text) C) Deviance analysis for the complete data set (GLIM binomial trial, for details see text).

(A)

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

(B)

Wild seedlings		Cultivar seedlings	
Deviance (df=1)	*P*	Deviance (df =1)	*P*
0.5	0.47	0.41	0.52

(C)

	Non-infected seedlings	Deviance (df =1)	*P*
Wild seedlings	0.97	29.1	<0.00001
Cultivar seedlings	0.71		

Under environmental chamber experiments (Table 2) the infection rate of wild seedlings was the lowest in two of the three soils; infection was highest in the cultivar, regardless of soil type and significant differences with the cultivar seedlings were always detected (Table 2). The infection rate of the landrace seedlings was intermediate between those of the wild and cultivar seedlings. Interestingly, the infection rates of the landrace were, in two of the experiments, closer to those of the cultivar seedlings (with no significant differences), 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 seedlings.

TABLE 2. A) Proportions of non-infected seedlings of *Phaseolus vulgaris* in environmental chamber experiments carried out in soils of different origin and use (see text for details). B) Deviance (*P*) for among-varieties multiple comparisons in the three soils (GLIM binomial trial. for details see text).

(A)

	Non-infected seedling proportions		
Soil type	Wild	Landrace	Cultivar
Tepoztlán (wild population)	0.943	0.743	0.628
Chapingo	0.914	0.914	0.686
Xochimilco	0.937	0.750	0.7187

(B)

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)

From the 94 infected seedlings of the field and environmental chamber experiments we were able to obtain and identify fungi isolates, up to genera, with only three exceptions. Form the fungi genera isolated, some were pathogenic (*Pythium*, *Fusarium*), others were genera with pathogenic and nonpathogenic species (*Aspergillus*, *Sporotrichum*, *Cladosporium*) and others were opportunistic (*Geotricum* and *Sepedonium*). Infection trials were performed with the isolates identified as opportunistic genera, using seedlings of the cultivar (the most vulnerable variety according to our results), to test pathogen activity of these genera. Only *Sepedonium* sp. isolates were able to infect seedlings. These results

suggest that these seedlings were first infected or damaged by unidentified organisms and that fungal infection was a nonpathogenic process.

Phytoalexin Chemical Diversity, Total Concentration and In vitro Activity Assays

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 ($H_{(2,\ N\ =\ 60)} = 6.424$, $P = 0.0403$). These differences are due to the contrast between wild and cultivar chemical diversities. The intermediate chemical diversity of the landrace seedlings is in accordance with the results obtained from the morbidity experiments, even when comparisons with either wild or cultivar varieties were non significant.

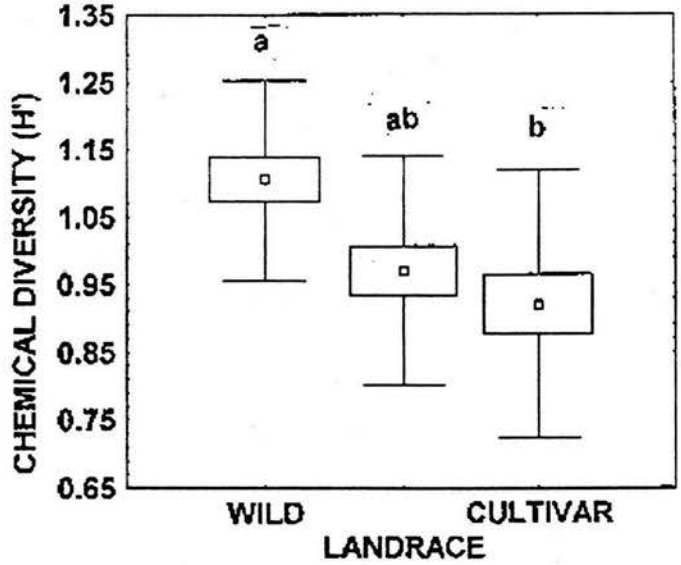


Figure 1. Mean (± SE, SD) chemical diversities of wild, landrace and cultivar seedlings of *Phaseolus vulgaris*. Means were calculated as the Shannon index for each individual seedling. Significant differences among varieties are shown by different letters (Mann-Whitney U-test: $P < 0.05$).

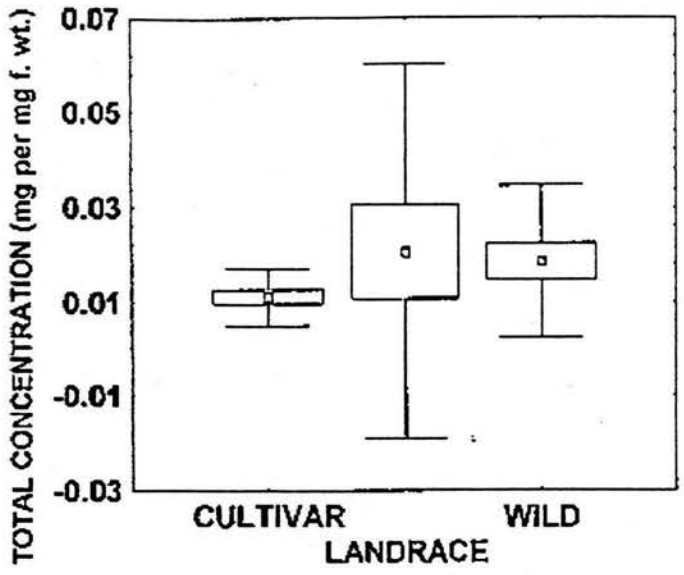


Figure 2. Mean (± SE, SD) total concentrations expressed as grams of isoflavonoids per grams of fresh seedling tissue of the three varieties studied. No differences between treatments were detected. (Kruskal-Wallis ANOVA by ranks ($H_{(2, N = 48)} = 2.16$, $P = 0.3396$).

When analyzing total phytoalexin concentration (Fig 2), there were no significant differences among varieties ($H_{(2, N = 48)} = 2.16$, $P = 0.3396$).

Growth of the hyphae inside the capillary tubes filled with agar of the different treatments was clearly different (Fig. 3). Table 3 shows the statistical analysis from this trial. There was no significant effect of either replicate or block, but a highly significant effect of treatment. There are significant differences (Alpha= 0.05, df= 39, MSE= 48.033, CVT= 2.77, MSD= 7.841) between two groups: control and cultivar samples in one and landrace and wild samples in the other. Within each of these groups, no statistical differences were detected (Fig. 3).

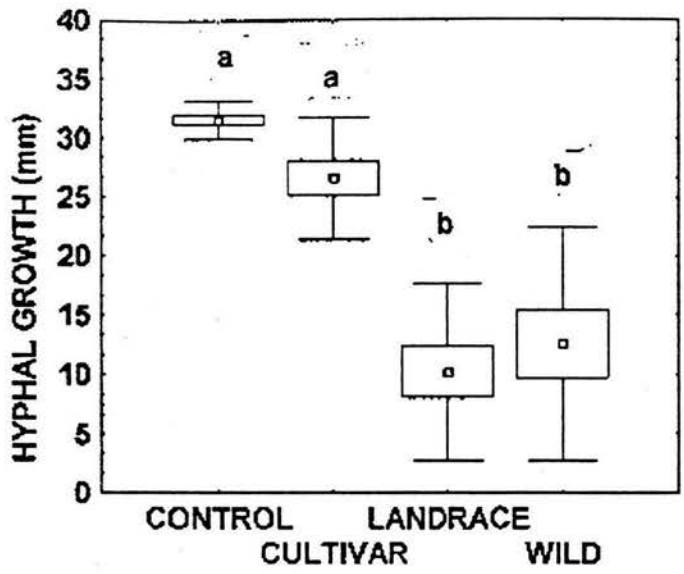


Figure 3. Hyphal growth (median ± SE, SD) as an indication of the inhibitory effects of the isoflavonoid mixtures produced by the three varieties studied. *Aspergillus* sp. mycelia growth inside the capillary tubes (see text) was inhibited in all seedling treatments. Significant differences are indicated by different letters ($P <= .01$; t-tests adjusted with Sidak's inequality).

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

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

DISCUSSION

Our results are consistent with a theoretical trade off between plant yield and defense and are compatible with those obtained from a comparison of maize and wild relatives, in which the most primitive form was more resistant to herbivory and allocated less to yield (Rosenthal and Dirzo, 1997). Our data reflect that wild types are more resistant than cultivated ones, and that modern forms are more susceptible.

Although opportunistic infections occurred in a few cases, the established criteria guaranteed that no gross overestimates were made. Furthermore, opportunistic infections can also reflect the susceptibility of infected seedlings for two main reasons: 1) because phytoalexin induction cannot be ruled out *a priori* in damaged tissues, and; 2) because some herbivores such as nematodes (Stadler *et al.*, 1994; for a revision see Mateille, 1994), caterpillars (Liu *et al.*, 1992) or phytophagous beetles (Fischer *et al.*, 1990) are susceptible to isoflavonoid phytoalexins.

The similarly inhibitory activities of control and cultivar treatments can be misleading because it would seem that cultivar seedlings have lost all resistance traits due to phytoalexin production. Most probably this is not the case, because in this trial the concentration of isoflavonoids in the agar was far below the concentration that fungi hyphae ordinarily confront in plant tissues (Snyder and Nicholson, 1990).

The fact that no differences were detected in total concentration of isoflavonoids among the varieties suggests that this trait is not a determinant of the resistance capability. Although differences in total concentration directly reflect different resource allocations, their lack does not necessarily reflect equal allocations among varieties.

The case of chemical diversity is different because wild seedlings have greater diversity than both cultivated varieties (Fig. 1). Furthermore, the mean chemical diversity of the landrace seedlings lies between those of the wild variety and the modern cultivar. Chemical diversities of landrace and cultivar seedlings were lower than those of the wild

seedlings because fewer compounds were detected, or one single compound represented almost the total peak area of the chromatogram, thus reducing the chemical diversity parameter. This reflects a lack or reduction in the biosynthesis of some of the isoflavonoids in these seedlings.

Results from the *in vitro* assay show the importance of phytoalexin diversity in the infection resistance in beans. Since the induced response appears to be non-specific because the same compounds are synthesized in plant tissues in response to a wide range of pathogens, the biosynthesis of a variety of phytoalexins can be beneficial to the plant because of the increased probability that the pathogen should be confronted with a toxic compound. Furthermore, because fungal pathogens are differentially susceptible to different isoflavonoids (Adesanya *et al.*, 1985) a seedling with high chemical diversity may be best defended against a wide range of pathogens. It is also possible that diversity itself endows plants with greater resistance capabilities due to synergistic effects of the secondary metabolites. In addition, variation in metabolites and or their relative representation may render the plant more difficult to be exploited by its consumers as has been suggested for Colorado lupines (Dolinger *et al.*, 1973). Such variation is more likely to be displayed with a diverse profile of defensive secondary compounds.

Pterocarpan phytoalexins synthesis, that includes isoflavonoids, is costly because they are synthesized from L-phenylalanine via a minimum of 11 enzymatic steps (Dixon *et al.*, 1995). Evidence has been presented that one of the steps is catalyzed by two independent enzymes (Guo *et al.*, 1994), and three reactions of flavonoid synthesis and the isoflavonoid branch pathway are involved. From L-phenylalanine to the simplest isoflavonoid, medicarpin, six reactions require NADPH that derives directly from the pentose phosphate pathway and also malonylCoA is an important precursor in some reactions (Dixon *et al.*, 1995). Other isoflavonoids derive from intermediates of the pathway to medicarpin, for example pisatin, which is synthesized in peas from one of the last intermediaries in the pathway by the catalyzing effect of an isoflavone reductase (Paiva *et*

al., 1994). Other phytoalexins seem to be synthesized by the enzymatic effect of isoflavonoid prenyltransferases that use as substrates the simpler isoflavonoids (Biggs *et al.*, 1987). The reduction in chemical diversity as a consequence of limited synthesis of a number of isoflavonoids, implies a reduction in the resources allocated to defense, because fewer precursors are diverted from primary metabolism to isoflavonoid final synthesis steps. In those cases in which certain compounds are not synthesized at all, the resources allocated to defense decrease even more because fewer resources are allocated to the production of isoflavonoid catalyzing enzymes and none of the precursors are diverted from primary metabolism. Thus, a reduction in chemical diversity implies also a reduction in defense allocation even in those cases in which all the phytoalexins are synthesized albeit, some, in minimal quantities.

The lack of expression of certain phytoalexins can be a consequence of genetic erosion in cultivated varieties, because pronounced reductions in genetic diversity have been documented particularly in commercial cultivars (Sonnante *et al.*, 1994).

Our results, which are consistent with the predictions of the model proposed by Rosenthal and Dirzo (1997) for the trade-off of defense and yield/growth, suggest that induced defenses can be, at least to some extent, costly, and may follow the same eco-evolutionary trends which constitutive defenses appear to follow under domestication.

CAPITULO 3

Phytoalexins, Resistance Traits and Domestication Status in *Phaseolus coccineus* L. and *Phaseolus lunatus* L. (Fabaceae).

Abstract

Resistance to pathogenic fungi and the isoflavonoids that confer such resistance capabilities were studied in two systems of wild and cultivated *Phaseolus* spp seedlings. Our results for *P. coccineus* show a gradient in chemical isoflavonoid diversity and pathogen resistance -high in wild varieties to low in cultivated ones.- *P. lunatus* varieties showed the same trend in resistance and cyanogenic capacity, but not in isoflavonoid chemical diversities. We suggest that the effectivity of the phytoalexin natural mixture against fungi depends more on its diversity than on its total levels in the seedlings.

INTRODUCTION

As a result of domestication, plants have higher yields, particularly of those parts that are of agronomic interest (Tivy, 1990). In many cases, domesticated plants also have lower levels of secondary metabolites in those same parts as compared to their wild relatives (Roddick, 1986, Sotelo *et al.*, 1995). Although this reduction in defensive chemicals in harvested parts can be explained as a consequence of selection for palatability, this mechanism cannot explain the reduction in defensive traits in non-harvested parts (Krischiik and Denno, 1983). Low levels of defenses in non-harvested parts of cultivated plants are considered to be a consequence of reallocation patterns within the plant. Because no net increments in photosynthetic rates have been detected for cultivated plants (Evans, 1984), increased yield is only possible if resources are diverted from other plant functions. These observations support the model proposed by Rosenthal and Dirzo (1997), which predicts a gradient of defense according to resource allocation. In this model, wild cultivars have the highest defense and lowest yield; whereas, modern cultivars have the lowest defense levels but the highest yield, because their selection has been carried out under conditions of very low pressures from herbivores and pathogens, thus allowing the greatest reallocation to yield. Because landraces have been selected under local conditions, in the presence of moderate pressure from herbivores and pathogens, they represent intermediate levels of defense and yield. Finally, wild relatives, as a consequence of high selective pressure, should allocate more to defense and less to yield.

Although the assumed costly constitutive defense is accounted for by the resource allocation model, induced defense is not readily explained by this model because this mode of defense has been considered to be either costly (da Cunha, 1987) or non-costly (Clark and Harvell, 1992; Herms and Mattson, 1992).

An induced response is elicited in *Phaseolus* beans when their tissues are challenged with fungal hyphae (for a review see Sequeira, 1983). This response is related to the

production of isoflavonoid phytoalexins, compounds that have been isolated from many artificially infected *Phaseolus* species (Adesanya et al,. 1985; O'Neill *et al.*, 1984; Stössel, 1985; Anderson, 1978; Garcia-Arenal *et al.*, 1978; Bandara, 1979; Zavala *et al.*, 1989). Furthermore, the fungitoxicity of several isoflavonoid phytoalexins have been established, although the degree of toxicity is species-specific (Skipp and Bailey, 1977).

We selected wild and cultivated populations of two species of *Phaseolus* beans, to explore the relationship between their induced defenses, their resistance, and their domestication status.

METHODS AND MATERIALS

Phaseolus coccineus and *P. lunatus* have been cultivated for several thousand years in Mesoamerica (Delgado-Salinas, 1988), although *P. coccineus* cultivation is now restricted to its centers of origin, mainly in the Mexican highlands (Sauer, 1993). From both species several isoflavonoid phytoalexins have been isolated, with genistein, phaseollin, phaseollinisoflavan and kievitone being the most frequent(O'Neill *et al.*, 1984; Stössel, 1985; Adesanya *et al.*, 1985).

P. lunatus is unique among *Phaseolus* species because its seeds contain cyanogenic glucosides. Chemical, biochemical, and physiological aspects of the synthesis and accumulation of these compounds have been well studied (Frehner *et al.*, 1990; for a revision see Conn, 1980). Concentration of cyanogenic glucosides in wild *P. lunatus* seeds is higher than that of the cultivated varieties. In fact wild seeds can have almost 150 times more cyanogenic glucosides than cultivars (Lucas and Sotelo, 1984).

Phaseolus coccineus grows wild in the highlands of central Mexico, Chiapas and Puebla, sometimes at the edge of cultivated bean plots (Delgado-Salinas, 1988). *P. lunatus*

can be found in the coastal zones of the Pacific ocean, particularly in the states of Guerrero, Michoacán and Jalisco (Baudoin, 1988). Cultivated forms (landraces) can be found in several Mexican locations and are mainly grown for local uses.

Wild seeds of *Phaseolus coccineus* were collected at two different populations, one located in the "Bosque de Tlalpan" National Park in Mexico City, and a second at Huitzilac (State of Morelos). Seeds from a landrace of this species ("ayocote" variety) were obtained from the state of Puebla. Two wild populations were included because the great variability this species shows.

Phaseolus lunatus wild seeds were collected in the Chamela region on the Pacific coast of the Mexican State of Jalisco, landrace seeds were obtained from the State of Guerrero, and seeds from a commercial cultivar were purchased from a NK Lawn and Garden Co. distributor.

Infection Rates in Field and Environmental Chamber Experiments

To assess seedling resistance to pathogenic fungi, we conducted two field and four environmental chamber experiments with *P. coccineus* seedlings and two environmental chamber experiments with *P. lunatus* seedlings.

One field experiment with *Phaseolus coccineus* took place in an agricultural field in the Xochimilco region of the Valley of Mexico. The site was cleared after a five year resting period, avoiding any possible effects of chemical pesticides and fertilizers normally used in this region. For this experiment, seeds from the Tlalpan population and the ayocote landrace were randomly chosen. These seeds were scarified by puncturing with a needle, superficially sterilized with 1 % sodium hipochlorite solution for 5 minutes and rinsed with sterilized water. The seeds were randomly distributed in two 15 X 10 grids (75 seeds per variety in each grid), spaced 10 cm for each other (to avoid epidemic effects), individually covered

with plastic meshes (to avoid animal predation and allow inspection with minimal disturbance), and covered with a thin layer of soil. The field plots were watered every day. Six days after planting, seedlings were chosen to form cohorts if the radicle was clearly visible. The experiment was ended when the non-infected seedlings presented the first true leaves.

In the population from the Bosque de Tlalpan three different zones in which wild plants of *P. coccineus* grow can be characterized. These vegetation zones correspond to a disturbed oak forest; a severely disturbed oak forest with planted eucalyptus trees, and the "pedregal" zone, characterized by plants that grow on old lava flows. To take into account this environmental heterogeneity, three experiments were conducted, one for each vegetation zone.

Seed treatment and cohort selection were carried out as in the previous experiment. In each experiment, 80 seeds of each variety were randomly distributed and covered with a mesh and litter added to cover the entire experiment. Water was supplied by natural rain.

For growth chamber experiments, soil was collected from locations where wild populations of both species grow and also from agricultural fields in the Chapingo region of the State of Mexico and from Xochimilco in Mexico, D.F. Independent experiments for each species were carried out in the different soil types. For each experiment the procedure described below was followed. The Xochimilco soil sample was used with *P. coccineus* to be able to compare field and laboratory results for this species.

Each soil sample was sieved trough a number 10 mesh and then stirred to homogenize it. Saturation water capacity (SWC) for each soil sample was estimated by adding distilled water to a sample of 50 g of soil in a well drained container and, after several minutes, weighing the wetted soil.

Homogenized soil was placed in polyethylene cups up to approximately one fifth of the cup capacity. Sterilized distilled water was added to each cup to SWC, in order to have equal conditions in all the replicates. Seeds were treated in the same manner as described for

the field experiments. One seed of each variety was placed in each cup under sterile conditions and covered with a translucent plastic cover. Soil and water quantities for each experiment varied with the nature of the different soils used. For all the experiment 65 seeds per variety were planted, and cohorts selected among those that germinated.

The experiments were carried out in an environmental chamber (Conviron, Model E15) with a light period of 11 hours and a dark period of 13 hours. In order to obtain a constant temperature of 29° C inside the vessels, it was necessary to set the temperature in the chamber at 26° C during the light period, and of 29° C during dark period.

Each cup was inspected daily in order to select the seedlings for the cohorts and later to follow the infection process (see later). For *P. coccineus*, cohorts were selected 48 hours after planting, and after 36 hours for *P lunatus*; seedlings showing only the radicle were chosen. Cohort sizes for all treatments were matched to the variety with the smaller cohort size, to avoid unbalanced experiments. Each experiment was considered completed when the first true leaves of the non-infected seedlings appeared.

The results of these experiments were statistically analyzed assuming that the result is a consequence of a binomial trial with sample size 1 (Crawley, 1993), as the response variable was either infected or non-infected, and no interaction between seedlings was possible. The analysis was carried out with GLIM 3.77 (Royal Statistical Society, 1985) using a logit link function and a binomial probability distribution. The probabilities were calculated with TAB 1.3 (Ezcurra, 1995).

Chemical Analysis

To calculate chemical diversity values and to obtain total concentration data for the varieties of the two species, several chemical analyses were performed.

After superficial sterilization with sodium hipochlorite (as previously described), 180 seeds of each variety of *P. coccineus* and 130 for the *P. lunatus* varieties were imbibed in distilled and sterilized water for 8 hours. Imbibed seeds were germinated under sterile conditions at 25°C. Cohorts were selected 48 hours after germination. The selected seedlings were treated with aqueous $CuCl_2$ 5mM for two hours and incubated for another 48 hours. After incubation, cotyledons were removed and all seedlings were individually extracted with 20 times their weight of chromatographic grade ethyl acetate.

For chemical diversity analysis, High Performance Liquid Chromatography (HPLC) analysis of 20 extracts per variety was performed (Waters HPLC system Model 600 MS). Isoflavonoid separation was carried out on a Lichrosphere 10μ 250 X 3.2 mm column (Phenomenex), 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. to 1.2 ml/min. (curve 6 of the Waters protocol). Isoflavonoid UV spectra were obtained for all the compounds present in the extracts, using a Photodiode-array detector (Waters, model 990).

Using the peak parameters of the identified isoflavonoids in the chromatograms, chemical diversity for each seedling was calculated using the Shannon Index. The diversity estimates were used to compare among varieties. Statistical analysis was carried out with Kruskal-Wallis ANOVA by ranks due to the inequalities of diversity variances.

Total concentration estimation of isoflavonoid mixtures produced by individual seedlings was carried out by lyophilizing extracts and weighing the remaining solids. Total concentration was then calculated as grams of isoflavonoid mixture per grams of fresh seedling tissue, and the results analyzed by means of a Kruskal-Wallis ANOVA by ranks (StatSoft Inc., 1993).

An analysis of cyanogenic capacity was performed with *P. lunatus* seeds. The standard curve was prepared with KCN as described by Lucas and Sotelo (1984), but without corn starch because we were only interested in quantifying the liberated HCN from the seeds. HCN release from the glucosides was elicited by acid hydrolysis (HCl, 0.5 N). The released HCN reacted with sodium picrate in paper strips (2 X .5 cm) and the reaction product, isopurpurin, was redisolved in distilled water (3 ml) and read at 510 nm with a spectrophotometer at 510 nm.

In vitro Assay of Phytoalexin Activity

To asses the inhibitory properties of phytoalexin mixtures, an *in vitro* randomized 2 block experiment with 8 seedling extracts per variety of *Phaseolus coccineus* was performed. An isolate of *Aspergillus* sp. previously obtained from an *P. coccineus* infected seedling was used for this assay. Two cultures were prepared on malt extract agar media.

The phytoalexin extracts were lyophilized, and the remaining solids were dissolved in two times the fresh weight of the extracted seedling of distilled sterilized water. Subsequently an equal amount of malt extract agar was added. Controls were prepared following the previous procedure, but without the phytoalexins. Sterilized capillary tubes (0.4 X 100 mm) were filled with 0.040 ml of each of the phytoalexin agar dissolutions. The open end of each tube was sealed with paraffin wax.

To prepare the assay, 2 petri dish covers were concentrically perforated (16 holes per cover at 1.0 cm from the edge). The covers were placed on top of the *Aspergillus* cultures and capillary filled tubes were randomly placed in the perforations in such way that the media in the tubes were in contact with the growing hiphae in the cultures. The spaces between the edge of the holes and the capillary tubes were sealed with epoxy resin to avoid contamination and loose tubes. Each one of two *Aspergillus* cultures was a complete

experiment with 4 replicates for treatment (wild, landrace, cultivar and control). The experiments were incubated at 25°C for 6 days.

Growth of the hiphae 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. For statistical analysis three categorical variables were established, treatment (with four levels, one for each variety and the control), block (2, one for each petri dish experiment) and replicates (four for each treatment) and growth as the dependent variable. Data were analyzed by means of an ANOVA (GLM procedure, SAS Institute, 1988). Post-hoc differences between individual treatments were established with a Sidak T test (GLM procedure, SAS Institute 1988).

RESULTS

The field and environmental chamber experiments all show that wild seedlings of *Phaseolus* spp. are more resistant to fungi than cultivated ones. For the field experiment that was conducted in the agricultural field, the data for the two blocks (grids) were combined because the results for each block were statistically undistinguishable (for wild seedlings; change in deviance = 0.9, d.f. = 1, P = 0.343, and for landrace seedlings; change in deviance = 0.08, d.f. = 1, P = 0.777). Analysis of the combined data showed a highly significant difference in infection rate (Table 1). The experiments conducted in the different vegetation areas of the Bosque de Tlalpan show a similar trend, although the differences for the oak forest were not quite significant (Table 2).

TABLE 1. *Phaseolus coccineus* seedlings infected after germination in the agricultural field experiment (A). Because there were no differences between blocks of the same variety (B). Blocks were pooled into one analysis (C). This analysis shows that wild seedlings are significantly more resistant than cultivated ones (GLIM binomial trial, for details see text).

(A)

	Wild seedlings		Cultivar seedlings	
	Total	infected	Total	infected
Block A	48	2	59	15
Block B	71	6	65	18

(B)

Wild seedlings		Cultivar seedlings	
Deviance (df=1)	*P*	Deviance (df =1)	*P*
0.9	0.34	0.08	0.78

(C)

	Non-infected seedlings	Deviance (df =1)	*P*
Wild seedlings	0.933	18.2	0.0002
Cultivar seedlings	0.733		

TABLE 2. Proportion of non-infected seedlings of *Phaseolus coccineus* wild and landrace seedlings after germination in three localities of the "Bosque de Tlalpan" (GLIM binomial trial, for details see text).

	Non-infected seedling (proportions)		Statistical Analisis Results	
	Wild	Landrace	Change in deviance	*P* (d.f. = 1)
Oak forest	0.904	0.770	2.7	0.100
Eucaliptus oak forest	0.914	0.718	6.1	0.013
Pedregal	.851	0.639	5.73	0.016

The environmental chamber experiments also show for both species that wild varieties are more resistant (Table 3). The resistance of the *P. coccineus* Huitzilac wild seedlings falls in between that of the Tlalpan and landrace seedlings, a trend that is also observed in chemical and *in vitro* assays. *Phaseolus lunatus* varieties present the expected resistance gradient, with the highest resistance in wild seedlings, followed by landrace and modern cultivar seedlings.

TABLE 3. Environmental chamber results from the experiments carried out in soils of different origin for both studd species. Both sets of results show gradients of resistance between the varieties under study, with *Phaseolus coccineus* (a) Tlalpan (wild) seedlings being more resistant, followed by Huitzilac (wild) and landrace being the least resistant. For *P. lunatus*, a similar gradient from wild to cultivar seedlings was found (GLIM binomial trial, for details see text).

(a) *Phaseolus coccineus*

		Non-infected seedlings proportion		
	Cohort size	Tlalpan	Hiutzilac	Landrace
Tlalpan*	40	0.975	0.800	0.650
Hutizilac*	38	0.974	0.868	0.658
Chapingo	31	0.840	0.700	0.600
Xochimilco	39	0.945	0.897	0.692

* Wild population soil samples

	Multiple comparisons for *P. coccineus* varieties (**deviance**, d.f. (*P*))		
	Tlalpan vs. Landrace	Tlalpan vs. Hutizilac	Huitzilac vs.Landrace
Tlalpan*	**2.885**, 78 (0.005)	**2.115**, 78 (0.037)	**1.487**, 78 (0.141)
Hutizilac*	**2.789**, 74 (0.007)	**1.549**, 74 (0.126)	**2.092**, 74 (0.040)
Chapingo	**2.606**, 68 (0.010)	**1.646**, 68 (0.103)	**1.048**, 68 (0.297)
Xochimilco	**2.662**, 77 (0.009)	**0.845**, 77 (0.401)	**2.194**, 77 (0.031)

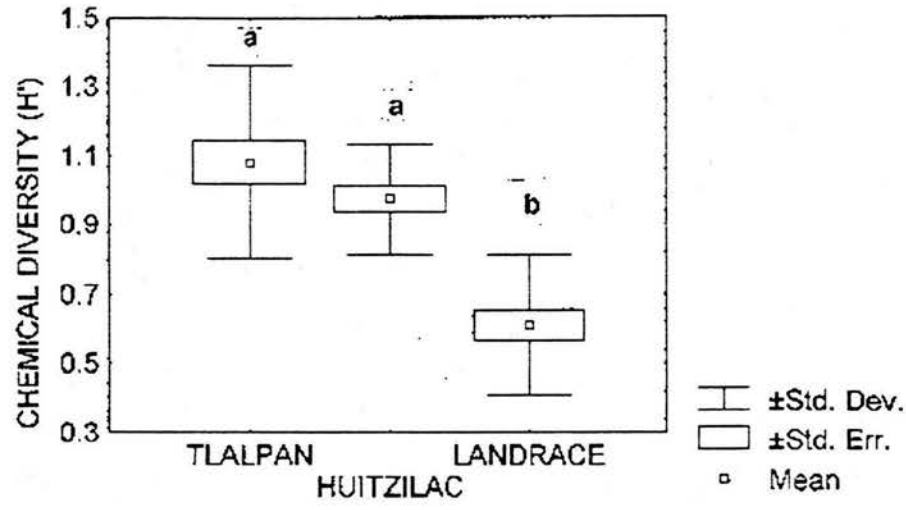
(b) ***Phaseolus lunatus***

		Non-infected seedlings proportion		
	Cohort size	Wild	Landrace	Cultivar
Chamela*	40	0.925	0.950	0.800
Chapingo	40	0.950	0.850	0.575

* Wild population soil samples

	Multiple comparisons for *P. lunatus* varieties (**deviance**. d.f. (*P*))		
	Wild vs. Landrace	Wild vs. Cultivar	Landrace vs.Cultivar
Chamela*	**1.917**. 68 (0.059)	**1.572**. 68 (0.126)	**0.471**. 68 (0.638)
Chapingo	**1.446**. 78 (0.152)	**3.395**. 78 (0.001)	**2.622**. 78 (0.010)

Isoflavonoid chemical diversity results for both species are shown in figure 1. For *Phaseolus coccineus* the differences between wild Tlalpan and landrace seedlings are statistically significant (Kruskal-Wallis ANOVA by ranks: $H_{(2,\ N = 56)} = 20.39$ $P = 0.001$). *Phaseolus lunatus* isoflavonoid chemical diversities show no trend between varieties (Kruskal-Wallis ANOVA by ranks: $H_{(2,\ N = 59)} = 1.491$ $P = 0.474$). The comparison between total concentrations for both species shows significant differences among some of the varieties (figure 2). Interestingly, in the case of *Phaseolus lunatus,* landrace seedlings have the lowest concentrations (Kruskal-Wallis ANOVA by ranks: $H_{(2,\ N = 30)} = 13.52$ $P = 0.0012$), whereas for the *P. coccineus* varieties the Huitzilac (wild) seedlings have the lowest total concentrations (Kruskal-Wallis ANOVA by ranks: $H_{(2,\ N = 41)} = 9.699$ $P = 0.0073$).



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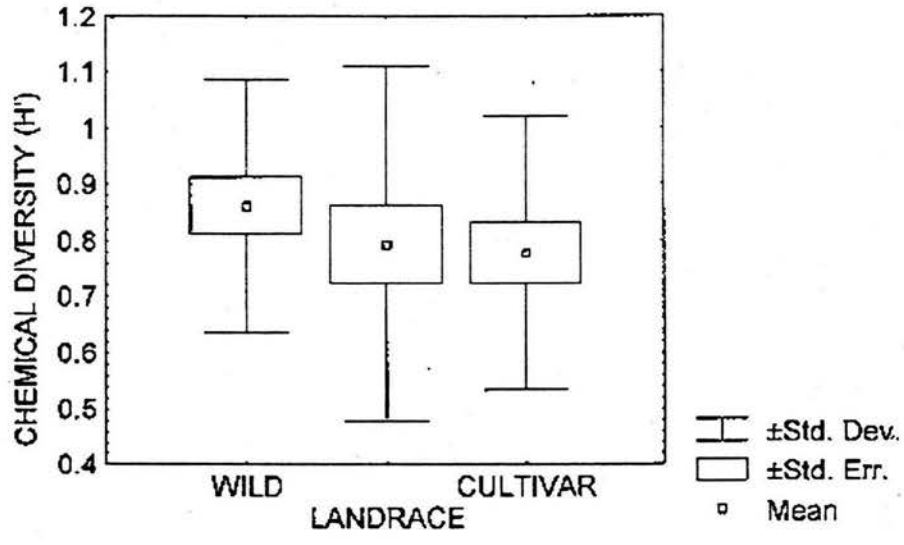
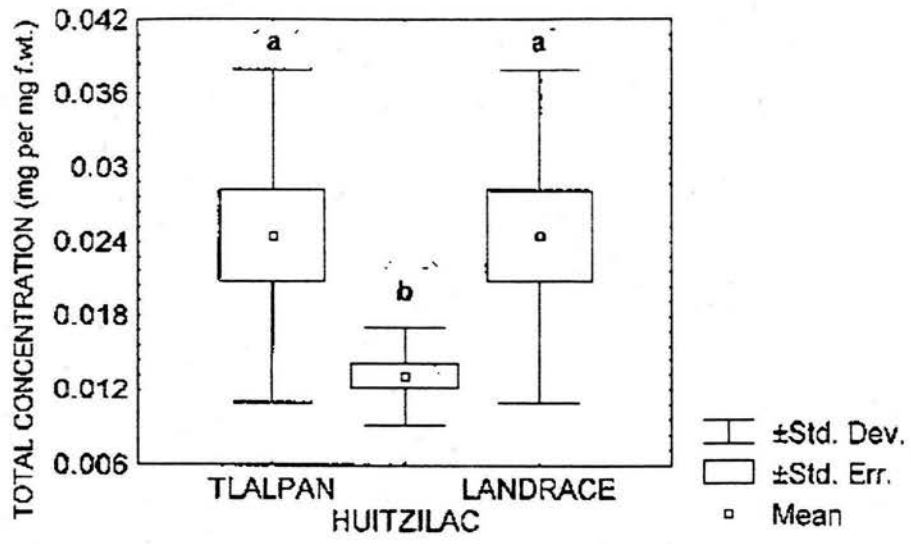


b

Figure 1. Chemical diversity of *Phaseolus coccineus* and *P. lunatus* calculated with the Shannon Index. *P. coccineus* varieties show a gradient in diversity (a), differences are statistically significant because of Tlalpan and Landrace values (n = 20 per variety $P = 0.001$). *Phaseolus lunatus* shows no differences for this same parameter (b) (n = 20 per variety $P = 0.474$). Means (±SD and SE) marked with different letters are singinificantly different (Mann-Whitney U-test: $P < 0.01$).



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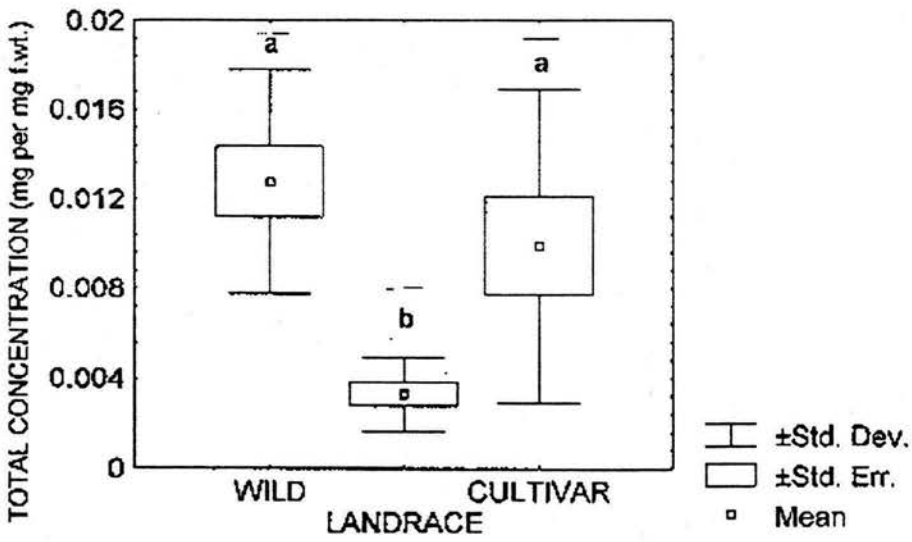

b

Figure 2. Total concentration of isflavonoids for the studied species. In both species one variety had lower concentrations than the others. *Phaseolus coccineus* variety results show lower concentrations for Huitzilac seedlings (a) and for *P. lunatus* the variety with lower concentrations is the landrace (b). Overall, *P. lunatus* concentrations tend to be lower than those of *P. coccineus*. Means (±SD and SE) marked with different letters are singinificantly different (Mann-Whitney U-test; $P < 0.05$).

Cyanogenic tests results for *Phaseolus lunatus* seedlings show the expected gradient for the three varieties (figure 3). Wild seedlings produced more HCN than the other varieties, although the value range is far lower than some reported values for this species (Lucas and Sotelo, 1984; Frehner *et al.*, 1990). Differences between varieties for this trend are highly significant ($F_{(2,12)} = 25.654$, $P < 0.0001$).

Results for the *in vitro* assay of isoflavonoid extract activity are shown in figure 4, significant effect on treatment was obtained ($F_{(3,16)} = 12.79$, $P < 0.0001$). All treatments differ from the control and, although no statistical differences were detected among them ($\alpha = 0.05$) a gradient in hyphae growth was observed.

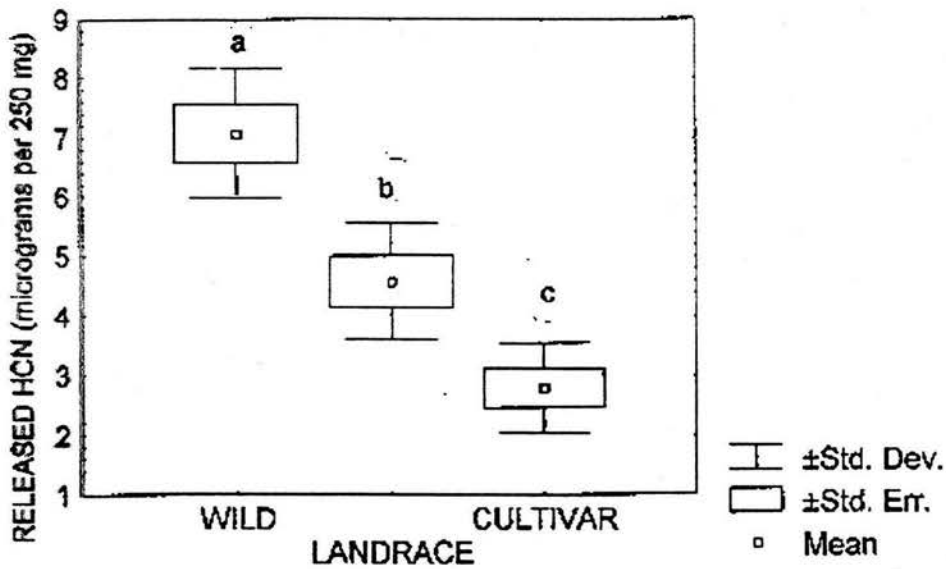

Figure 3. Released HCN (in micrograms) for *Phaseolus lunatus* varieties. Differences among all three varieties are significant (for details see text). Means (±SD and SE) marked with different letters are singinificantly different (Mann-Whitney U-test: $P < 0.05$).

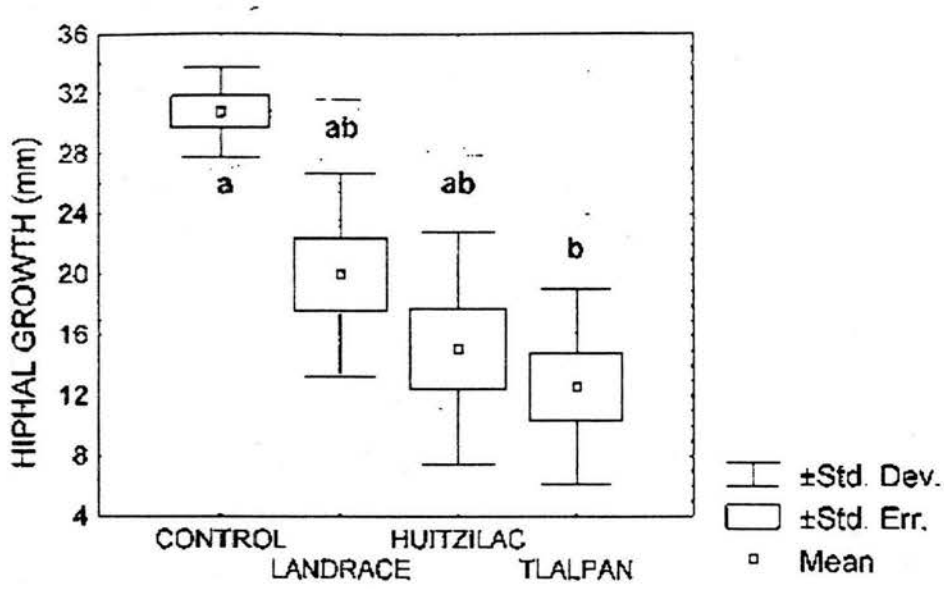

Figure 4.Inhibitory properties of the isoflavonoid mixtures produced by the three varieties of *Phaseolus coccineus*. *Aspergillus* sp. mycelia growth inside the capillary tubes (see text) was inhibited in all seedling treatments. Although no significant differences were detected between the varieties, there appears to be a trend from less inhibition in landrace seedlings to more inhibition in Tlalpan ones. Means (±SD and SE) marked with different letters are singinificantly different ($P <= .01$; t-tests adjusted with Sidak's inequality).

DISCUSSION

Overall, wild seedlings of *Phaseolus coccineus* and *P. lunatus* showed higher resistance to fungal infections than cultivated ones. The experiments carried out in the Bosque de Tlalpan wild population site also revealed that the proportions of non-infected seedlings for both varieties in forested sites were higher than the corresponding proportions in the pedregal site. This could be a consequence of grater stress imposed on the seedlings in the more open pedregal site which resulted in longer desiccation periods between rain events

and greater temperature changes during day and night than forested sites. This added stress may have resulted in a proportional decrement in healthy seedlings for both varieties.

The experiments conducted in the environmental chamber confirmed the trend observed in field experiments for *P. coccineus*. The Huitzilac seedlings (wild) presented an intermediate resistance in all the experiments, Tlalpan seedlings (wild) had the highest resistance and the landrace seedlings the lowest. This pattern was observed in the diversity of isoflavonoids chemical diversities and in the *in vitro* inhibitory properties of the extracts. *Phaseolus lunatus* environmental chamber experiments showed the highest susceptibility for the commercial cultivar although, interestingly, no differences were found between the landrace and wild seedling resistance.

It is possible that Hiutzilac seedlings were less resistant than Tlalpan ones because more introgression can occur in this population that grows near *P. coccineus* agricultural fields. High outcrossing rates in wild and in cultivated forms growing in Huitzilac have been observed for this species (Escalante *et al.* 1994).

Opportunistic infections cannot be ruled out in some cases. Nevertheless, cases of opportunistic infections can also reflect the susceptibility of infected seedlings, since mechanically damaged tissues can also produce isoflavonoids and because some herbivores such as nematodes (Stadler *et al.* 1994; for a revision see Mateille, 1994), loopers (Liu *et al.*, 1992) or phytophagous beetles (Fischer *et al.*, 1990) are susceptible to isoflavonoid phytoalexins.

For both bean species, the wild seedlings had greater chemical diversity than cultivated ones, but no differences in total levels were found. *P. coccineus* data show interesting chemical diversity trends because wild *Phaseolus coccineus* Tlalpan seedlings have greater diversity than the other varieties. Furthermore, the mean chemical diversity of the varieties shows a gradient that corresponds to that of the resistance trend (Tlalpan > Huitzilac > Landrace). Chemical diversity values for Huitzilac and landrace seedlings were lower than those of the wild Tlalpan seedlings. For several seedlings fewer compounds were

detected or one compound was the overwhelmingly predominant component- the latter was particularly frequent in the landrace seedlings- thus reducing the chemical diversity parameter. The situation for *P. lunatus* is more complicated. In this species, two kinds of defenses are effective against pathogens (cyanide and isoflavonoids). Loss of cyanogenic compounds is certainly the result of human selection for palatability and perhaps increased yields, and it is possible that selection pressure by pathogens prevents further losses of chemical isoflavonoid diversity, thus explaining the observed results.

In the case of total concentration of flavonoids for *Phaseolus coccineus*, the lowest values were obtained for the Huitzilac wild seedlings. Higher, and statistically similar ranges, between wild Tlalpan and landrace varieties were also obtained. These results suggest that this characteristic is not determinant of resistance to fungi, because the more resistant Tlalpan seedlings and the more susceptible landrace had high and equal total phytoalexin concentrations. The same trend can be appreciated in *P. lunatus*, with lower total levels for the more resistant landrace variety than those of the commercial cultivar, although for this species cyanogenic capacity should also be considered.

Results from the *Phaseolus coccineus in vitro* assay show the role of phytoalexins in the resistance of beans. Although the differences between varieties are not statistically significant, again a trend toward less infection for the extracts of wild plants was observed (figure 4). This, together with the results of the chemical analyses, show the importance of phytoalexin diversity in the resistance against fungal infection in beans.

The induced response appears to be non-specific because the same compounds are synthesized in plant tissues in response to a wide range of pathogens (Skipp and Bailey, 1977). This biosynthesis of a set of phytoalexins can be beneficial to the plant because of the increased probability that pathogens could be confronted with at least one or several toxic compounds. It is also possible that diversity itself provides plants with greater resistance capabilities due to synergistic effects in secondary metabolite mixtures.

The lack of differences in flavonoid levels does not necessarily reflect equal defense allocation among varieties. Differences in chemical diversity could also be the result of different defense allocations if the associated costs of producing groups of chemicals vary for certain secondary metabolites.

A reduction in chemical diversity may imply a reduction in defense allocation because fewer precursors are diverted from primary metabolism to the final steps of isoflavonoid synthesis. Moreover, resources allocated to defense will decrease even more if fewer resources are allocated to the production of isoflavonoid catalyzing enzymes and no precursor diversion occurs from primary metabolism to the absent isoflavonoid pathway branches.

Our results for *P. coccineus* are in accordance with the model by Rosenthal and Dirzo (1997), which predicts that induced defenses can be to some extent costly and can follow the same trends constitutive defenses undergo under domestication. By providing useful empirical evidence, these results also illustrate the usefulness of wild-cultivated plant systems to test ecological theory.

Conclusiones Generales

Dos aspectos son importantes de resaltar de la presente tesis. Por un lado que proporciona un ejemplo más que aporta datos empíricos que muestran la utilidad de los sistemas formados por plantas silvestres y cultivadas emparentadas para poner a prueba hipótesis ecológicas y, por el otro, la contribución específica al trabajo dentro del área de la ecología química.

Las especies domesticadas han sido importantes para la formulación de teorías e hipótesis evolutivas y ecológicas. Por ejemplo, Darwin dedicó todo el primer capítulo del Origen de las Especies al problema de la variación bajo domesticación. Es notable el pequeño número de trabajos que utilizan esta variabilidad, y los sistemas discutidos en el primer capítulo, para poner a prueba hipótesis de este tipo. Aunque no es este el lugar para aventurar explicaciones para este hecho, es indudable, dados los resultados de los trabajos revisados en el primer capítulo así como los de la presente tesis, que estos sistemas experimentales tienen un gran potencial para poner a prueba diversas hipótesis en ecología y evolución.

Los resultados obtenidos en este trabajo de investigación confirmaron nuestras expectativas sobre la existencia de un gradiente de la resistencia entre las variedades, silvestres y cultivadas. Así como la existencia de un gradiente de características similares para la diversidad química de las fitoalexinas producidas. Este segundo resultado, que fue sorpresivo inicialmente y que, como se puede apreciar en las discusiones de los dos capitulos finales dificulta la interpretación en términos de un modelo simple de asignación, aporta elementos a la discusión del papel de la diversidad química en la defensa de las plantas.

El hecho de que las fitoalexinas de las especies del género *Phaseolus* sean, hasta donde sabemos, una respuesta específica a las infecciones causadas por hongos, permite centrar la discusión en términos de la resistencia y la asignación de recursos a esta

resistencia pues otras posibles funciones atribuibles, como almacenamiento de nutrientes, pueden ser eliminadas con razonable seguridad.

Aunque el diseño experimental no permite realizar comparaciones directas entre las diferentes especies, algunos patrones interesantes saltan a la vista. El más evidente es que a pesar de tratarse de especies que crecen en diferentes ambientes y de variedades que han sido seleccionadas bajo diferentes condiciones, para todas ellas se puede apreciar el mismo patrón general de resistencia y química secundaria. Esto fortalece el argumento de que la diversidad química juega un papel importante en la resistencia de las plantas ante sus consumidores, al menos para este tipo de sistemas.

Una comparación interesante es cuando se contrastan los resultados obtenidos en los valores de diversidad química de las variedades de *Phaseolus vulgaris* y *P. coccineus*. Para la primera especie, las diferencias entre las plantas silvestres y las criollas no son significativas, a pesar de que las silvestres tienen una diversidad química promedio mayor que las segundas. En el caso de *P. coccineus*, las diferencias entre las criollas y cualquiera de las dos variedades silvestres sí son significativas. Esto puede ser una consecuencia de que las plantas silvestres de *P. coccineus* presentan valores más altos de diversidad química que las de *P. vulgaris*. Esto implicaría que la domesticación puede causar decrementos mayores, sin comprometer la viablilidad de las plántulas de *P. coccineus*. Pero, en el caso de *P. vulgaris* cuyas plantas silvestres presentan valores menores de diversidad química, la domesticación sólo puede disminuir esta diversidad hasta niveles similares a los de *P. coccineus* sin comprometer la viabilidad de los cultivos. Esto suguiere que existe una diversidad química mínima eficaz para la defensa vegetal bajo ciertas condiciones selectivas. Sin embargo, estos patrones requieren de ser confirmados con experimentos diseñados exprofeso y comparando un número mayor de poblaciones silvestres y variedades cultivadas.

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