Chapter 25

Bioprospection Studies at El Edén: From Plants to Fungi

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INTRODUCTION

In 1997, a project was started to search for bioactive compounds from some of the plant communities at the El Edén Ecological Reserve, which is located in Quintana Roo, Mexico. As part of this project, fungi were isolated and identified from different insects at two plant communities within the reserve according to the work in progress by Torres-Barragán et al. The search for bioactive natural metabolites and potential microbial insecticides is motivated by the problems associated with the extensive use of chemical pesticides. These agrochemicals affect not only pest insects, but also beneficial species. Moreover, insects tend to acquire resistance to synthetic chemicals, which creates new pest problems; the presence of chemical residues also causes environmental hazards and health problems.

Evolved resistance to pesticides in insects has led to an exponential increase in the number of insect species resistant to them; some estimates place this number at more than 500 species (Mackenzie, Ball, and Virdee 1998). For example, the housefly *Musca domestica* has developed resistance to almost every chemical used against it. This problem points to the urgent need for alternatives to chemical pesticides (Mackenzie, Ball, and Virdee 1998).

In nature, mutual interactions among organisms include all direct as well as indirect effects. Some of these biotic interactions are regulated by secondary metabolites (i.e., infochemicals) that are produced and liberated by living beings. Each organism responds in a different way to infochemicals. The result is a vast communicative interplay, which is fundamental to the fabric of life. Organisms use chemicals to lure their mates, associate with symbionts, deter enemies, and fend off pathogens. Substances that transmit information between organisms are a fundamental part of the regulatory chemicals of nature.

Molecules that have a signal value in nature have sometimes been demonstrated to be useful to humans; the wide variety of medicinals underscores this point. Major recent additions to the therapeutic arsenal include ivermectin, cyclosporin, FK-506, and taxol—all compounds that can be expected to have evolved as signaling agents (Eisner & Meinwald 1995). Many diverse benefits can be expected from an ongoing search for natural products. Through laboratory experimentation, field research, and careful observation, species can be rated by "chemical promise"; this can aid in the important task of selecting species for chemical screening. This enables the assessment of some of the hidden value of nature. Most species remain to be discovered. These unknown species are potentially of immense value and deserving of protection, lest we be forever impoverished by their loss (Eisner and Meinwald 1995).

This project is focused on bioprospection and the search for the aggregated value of biotic resources, mainly from plants and microorganisms. Our aim is not only to assist in the biochemical exploration of dry tropical ecosystems in Mexico, but also to contribute to biodiversity conservation. Biological diversity reflects and underlies molecular diversity. The molecules found in nature may be regarded as solutions to challenges that organisms have confronted and overcome during molecular evolution. As the understanding of these solutions deepens, the efficiency with which new treatments can be discovered and/or designed for human disease and new natural agrochemicals also increases.

Nature assists discovery efforts in a variety of ways. Some compounds synthesized by plants and microorganisms are used directly as drugs, or for control of some pests. As their cultures disappear, the loss of the "shaman's" knowledge of the ethnobotanical uses of plants has been compared to the burning of the library at Alexandria. But for those of us who are beginning to understand how to read the molecules within living things, the loss of biological diversity itself is also the loss of a library—a library that contains answers to questions we have not yet learned to ask (Caporale 1995).

As part of this same project, a collection of two basic types of fungi is being made: entomopathogenic and plant-associated fungi. Both types of fungi constitute a rich germ plasm source to search for bioactive compounds and to determine their potential use as biocontrol agents in future studies.

EL EDÉN ECOLOGICAL RESERVE

El Edén Ecological Reserve is located in the Yalahau region in the northern portion of Quintana Roo, Mexico. This region contains the main ecosystems of the Yucatán Peninsula and the Caribbean. This zone was occupied by the ancient Maya culture and exhibits more biodiversity than anywhere else in the peninsula. Nowadays, this region also constitutes an important source of water and forest products for the future.

The tropical forests of the northern region of Quintana Roo are mainly dry ecosystems. These types of forests are in serious danger because of the inadequate development models adopted, the drastic changes in land uses, and the demographic growth. Dry tropical forests possess a great variety of species and biotypes and also are rich in endemisms, which make them a natural source to search not only for bioactive natural products, but also for biological insecticides that may hold the key to potential solutions of pest and disease problems (Gómez-Pompa 1998).

STUDIES ON PLANTS

Some plants were selected to evaluate their content of bioactive or allelochemical compounds. Our studies were conducted in four ways:

1. One-square area of 25 m^2 within each of three plant communities of El Edén (tropical forest, secondary plant community, and savanna) was sampled, and the most abundant and/or conspicuous species were collected.
2. Some species were collected in the permanent marked-out transects in order to make systematic studies on flora, fauna, microorganisms, and soils.
3. Some species were studied because of their known ethnobotanical and medicinal uses that suggested the presence of bioactive compounds.
4. Some endemic species were collected because they are unique, and their importance for biodiversity conservation is very high.

Herbarium samples of each plant are kept at the Herbarium of the University of Yucatán, Mexico.

Biodirected fractionation studies of selected plants were obtained by using seeds, phytopathogenic fungi, and brine shrimp (*Artemia salina*) as test organisms. These studies were performed according to procedures previously described (Anaya 1996; Jiménez-Arellanes et al. 1996; Castañeda et al. 1996;

Anaya and Pelayo-Benavides 1997; Anaya and del Amo 1999). Some of the main results obtained from research are discussed as follows.

Bioassays with seeds

Table 25.1 shows the main results of the bioassays performed to test the effects of aqueous leachates from leaves and fruits of some Fabaceae species on the radicle growth and germination of three test species: (1) amaranth [*Amaranthus hypochondriacus* L. (Amaranthaceae)]; (2) barnyard grass [*Echinochloa crus-galli* (L.) P. Beauv. (Poaceae)]; and (3) tomato [*Lycopersicon esculentum* Mill. (Solanaceae)]. The aqueous leachates of all the Fabaceae species (except *Mucuna* sp. on barnyard grass) significantly inhibited the radicle growth of amaranth, barnyard grass, and tomato. These results suggest the presence of allelochemical compounds in the tested leachates. Radicle growth is more affected by allelochemical stress than germination. In general, tomato was the most sensitive species, as its radicle growth was inhibited by 53 percent to 84 percent by the Fabaceae aqueous leachates. Radicle growth of amaranth was inhibited by 36 percent to 58 percent, while that of barnyard grass was inhibited by 15 percent to 74 percent. Leachates of *Bauhinia jenningsii* P. Wilson, and *Mimosa pudica* L. (leaves and fruits), as well as leaves of *Lonchocarpus* sp. and flowers of *Mucuna* sp., were the most inhibitory on the radicle growth of amaranth. Leaves and flowers of *M. pudica* were the most inhibitory treatments on barnyard grass. *B. jenningsii*, *Mucuna* sp., *Lysiloma latisiliquum* (L.) Benth., and *M. pudica* were the most inhibitory on tomato .

Table 25.2 shows the effects of the aqueous leachates of leaves of other plant species from different families with a high phytotoxicity on the test plants. Once again, the radicle growth was the most affected process by allelochemical stress compared with germination. Radicle growth of amaranth and tomato were strongly inhibited by these treatments. The most active treatments on the radicle growth of the test species were leachates from *Hamelia patens* and *Eupatorium* sp. on amaranth, leachates of *H. patens* and *Lantana camara* on tomato, and that of *Jatropha gaumeri* Greenm. on barnyard grass. Leachates of *H. patens* also inhibited germination of tomato and amaranth by 72.5 percent and 32.5 percent, respectively.

Table 25.3 shows some preliminary results of the biodirected phytochemical fractionation studies of various plants with a strong allelochemical potential. In this study, plants were used as test organisms. The inhibitory effect of organic extracts was less significant compared with that of the aqueous leachates. The most inhibitory treatments on the test plants were chloroform extracts from *Callicarpa acuminata* and *Zuelania guidonia* on amaranth; methanol and hexane extracts from *C. acuminata*, methanol extract from *Thevetia gaumeri*, and chloroform extract from *Jatropha gaumeri*, on

TABLE 25.1. Effects of the aqueous leachates of leaves and fruits of some Fabaceae plants from El Edén on the radicle growth (percent) and germination (percent) of amaranth, barnyard grass, and tomato. Data are representative of four replicates and were analyzed by ANOVA test. Data are percentages compared to a control (100 percent).

TREATMENTS		**TEST SEEDS**					
		Amaranth		**Barnyard grass**		**Tomato**	
Plant species	**Plant part**	**Radicle growth**	**Germ.**	**Radicle growth**	**Germ.**	**Radicle growth**	**Germ.**
Bauhinia jenningsii	L	42.9*	97.5	45.1*	80.0	15.8*	80.0
Bauhinia jenningsii	F	41.8*	92.5	64.5*	85.0	23.3*	95.0
Mucuna sp.	L	58.4*	100.0	84.9	85.0	18.5*	82.5
Mucuna sp.	F	48.7*	90.0	54.0*	77.5	26.6*	97.5
Lysiloma latisiliquum	L	51.7*	92.5	38.6*	87.5	22.8*	97.5
Mimosa púdica	L	43.5*	100.0	32.3*	82.5	24.0*	87.5
Mimosa pudica	F	45.1*	92.5	26.3*	82.5	26.9*	90.0
Swartzia cubensis	L	59.1*	97.5	57.8*	80.0	36.5*	95.0
Lonchocarpus rugosus	L	48.7*	95.0	74.0*	87.5	43.0*	97.5
Lonchocarpus violaceus	L	58.3*	100.0	74.0*	95.0	40.7*	95.0
Lonchocarpus sp.	L	42.7*	100.0	45.2*	82.5	47.0*	90.0
Havardia (Pithecellobium) albicans	F	63.7*	95.0	50.2*	77.5	43.6*	90.0

L = leaves; F = fruits; *$p < .05$; Germ. = Germination

TABLE 25.2. Effects of the aqueous leachates of leaves of several plant species from El Edén on the radicle growth (percent) and germination (percent) of amaranth, barnyard grass, and tomato. Data are representative of four replicates and were analyzed by ANOVA test. Data are percentages compared to a control (100 percent).

TREATMENTS	**TEST SEEDS**					
	Amaranth		**Barnyard grass**		**Tomato**	
Plant species	**Radicle growth**	**Germ.**	**Radicle growth**	**Germ.**	**Radicle growth**	**Germ.**
Thevetia gaumeri	31.3*	72.5	65.7*	72.5	27.3*	87.5
Eupatorium sp.	29.2*	90.0	46.6*	75.0	25.7*	80.0
Ipomoea sp.	34.0*	92.5	53.0*	92.5	24.4*	95.0
Jatropha gaumeri	31.3*	100.0	31.4*	72.5	22.3*	97.5
Hampea trilobata	39.2*	97.5	40.0*	85.0	26.3*	97.5
Malvaviscus arboreus	40.7*	92.5	43.1*	90.0	20.7*	97.5
Allophylus cominia	48.0*	100.0	54.9*	95.0	41.2*	87.5
Lantana camara	32.4*	77.5	41.9*	95.0	17.8*	80.0
Hamelia patens	23.3*	67.5	56.7*	85.0	12.9*	27.5

*$p < .05$; Germ. = Germination

barnyard grass; and chloroform and hexane extracts from *C. acuminata* on tomato.

Bioassays with phytopathogenic fungi

Table 25.4 shows some preliminary results of the biodirected phytochemical fractionation studies of El Edén plants with a strong allelochemical potential. In this study, phytopathogenic fungi were used as test organisms. As the data indicate, *Alternaria solani* and *Fusarium oxysporum* were the most resistant fungi to the effects of chloroform-methanol extracts from bioactive plants. The fruits extract of *Pithecellobium albicans*

TABLE 25.3. Effects of the organic extracts of aerial parts of some plant species from El Edén on the radicle growth of amaranth, barnyard grass, and tomato. Data are representative of four replicates and were analyzed by ANOVA test. Radicle growth data are percentages of growth compared to a control (100 percent).

TREATMENTS	**TEST SEEDS**		
	Amaranth	**Barnyard grass**	**Tomato**
Plant species (solvent)	**percent of radicle growth**		
Acacia sedillense (methanol)	65.2*	68.2*	61*
Callicarpa acuminata (hexane)	62.5*	52.8*	51.5*
Callicarpa acuminata (chloroform)	50*	60*	48*
Callicarpa acuminata (methanol)	57*	50*	85
Jatropha gaumeri (chloroform)	69.7*	59.4*	81.6
Metopium brownie (hexane)	92.6	91.4	68*
Thevetia gaumeri (methanol)	62.6*	56.9*	72.8*
Zanthoxylum caribaeum (methanol)	71.9*	62.4*	69.4*
Zuelania guidonia (hexane)	62*	88.4	81.2
Zuelania guidonia (chloroform)	54.4*	77.3	81.2

* $p < .05$

(Kunth) Benth significantly inhibited (25 percent) the radial growth of *A. solani* at three days of treatment. The roots extract of *Philodendron radiatum* Schott. and the stems extract of *Heliocarpus* sp. significantly inhibited *A. solani* at eight days of treatment (32 percent and 33 percent, respectively). The leaves extract of *Zuelania guidonia* inhibited the radial growth of *F. oxysporum* at three days and eight days by 35.8 percent and 32.8 percent, respectively. The leaves extract of *Croton glabellus* L. inhibited the growth of *F. oxysporum* at eight days by 25 percent. Of all fungi species tested, *Helminthosporium longirostratum* was the most sensitive to the treatments. This fungi was significantly inhibited by all chloroform-methanol extracts, except by that from *P. albicans* at three days of treatment, and *Jatropha gaumeri* leaves at eight days. The treatments that most inhibited the radial growth of *H. longirostratum* at eight days of growth were the leaves extracts of *Lantana camara* L. (66 percent), the roots extracts of *P. radiatum* (64 percent), and the leaves extracts of *Z. guidonia* (58.6 percent).

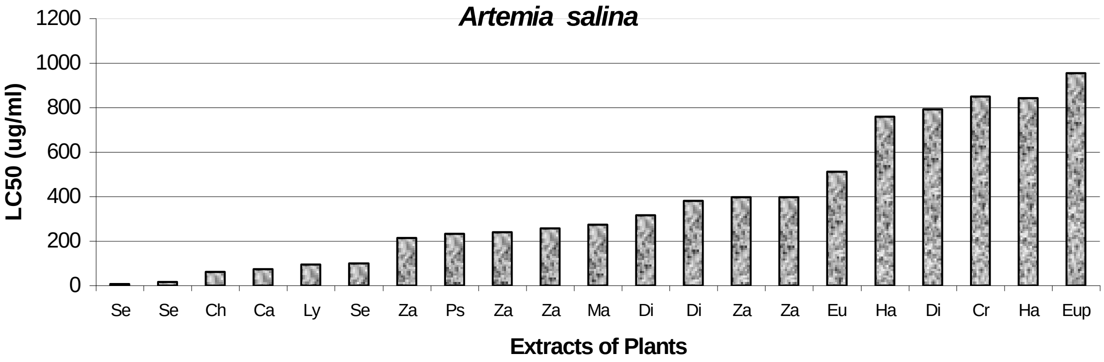
TABLE 25.4. Effects of chloroform ($CHCl_3$) and methanol (CH_3OH) extracts of some plants from El Edén on the radial growth of three phytopathogenic fungi. Data are percentages of radial growth compared to a control (100 percent).

PLANT SPECIES and parts used	**FUNGI TEST SPECIES**					
$CHCl_3$:CH_3OH (1:1) Extracts	***Alternaria solani***		***Fusarium oxysporum***		***Helminthosporium longirostratum***	
200 μg/ml	percent of radial growth					
	3 days	8 days	3 days	8 days	3 days	8 days
Philodendron radiatum –roots	81.8	68*	108.9	100	60.4*	36*
Jatropha gaumeri; leaves	90.9	80.9	98.4	98.4	76.4*	81.2
Sebastiania adenophora; stems	113.6	102.4	105.4	107.4	73.5*	61.5*
Croton glabellus; leaves	79.6	2.3	82	75*	55.5*	53.7*
Zuelania guidonia; leaves	113	100	64.2*	67.2*	52.9*	41.4*
Pithecellobium albicans; fruits	75*	9.4	107.4	120	108.3	56*
Byrsonima bucidaefolia; leaves	99.3	114.3	103.7	108.2	68.2*	57.3*
Heliocarpus sp.; stems	77	67*	100	100	74.2*	57.6*
Lantana cámara; leaves	96.3	88.6	86.3	94	53.6*	34*

* $p \leq .05$

Bioassays with Artemia salina (brine shrimp lethality test)

Figure 25.1 shows the principal results of the biodirected phytochemical fractionation studies of some plants of El Edén with a strong allelochemical potential. In this study, we used brine shrimp (*Artemia salina*) as the test organisms. Leaves of *Sebastiania adenophora*, roots of *Chamaecrista glandulosa* (L.) Greene (Fabaceae), leaves of *C. acuminata*, and leaves of *Lysiloma latisiliquum* (Fabaceae) constituted the group of plants with the highest toxic effect on *A. salina* (LC_{50} less than 200 μg/ml). *Zanthoxylum*



Keys of plant species:

Se = *Sebastiania adenophora* (Euphorbiaceae) – from left to right: hexane, chloroform, and methanol leaves extracts

Ch = *Chamaecrista glandulosa (*Fabaceae) – $CHCl_3$-CH_3OH -1:1 roots extract

Ca = *Callicarpa acuminata* (Verbenaceae) – hexane leaves extract

Ly = *Lysiloma latisiliquum* (Fabaceae) – methanol leaves extract

Za = *Zanthoxylum caribaeum (*Rutaceae) – from left to right: hexane and methanol leaves extracts; hexane, chloroform, and methanol stem extracts

Ps = *Psychotria sp.* (Rubiaceae) – $CHCl_3$-CH_3OH -1:1 leaves extract

Ma = *Manilkara sapota* (Sapotaceae) – $CHCl_3$-CH_3OH -1:1 stems extract

Di = *Diospyros verae-crucis (*Ebenaceae) – from right to left: $CHCl_3$-CH_3OH -1:1 leaves and roots extracts

Eu = *Eugenia sp.* (Myrtaceae) – $CHCl_3$-CH_3OH -1:1 leaves extract

Ha = *Hammelia patens* (Rubiaceae) – $CHCl_3$-CH_3OH -1:1 leaves extract

Cr = *Croton sp.* (Euphorbiaceae) – $CHCl_3$-CH_3OH -1:1 roots extract

Eup = *Eupatorium sp.* (Asteraceae) – $CHCl_3$-CH_3OH -1:1 stems extract

FIGURE 25.1. Effects of chloroform ($CHCl_3$), hexane, and methanol (CH_3OH) extracts of some plants from El Edén on the survival of Artemia salina (brine shrimp). The effects of the extracts are expressed in LC_{50} (μg/ml). Only those extracts with a LC_{50} value below 1,000 μg/ml were considered.

caribaeum Lam. (Rutaceae), *Psychotria* sp. (Rubiaceae), *Manilkara sapota* (L.) P. Royen (Sapotaceae), and *Diospyros verae-crucis* Standl. (Ebenaceae) constitute the group that caused a LC_{50} less than 400 μg/ml. All other tested plant species have a LC_{50} over 400 μg/ml, but less than 1,000 μg/ml. Due to their bioactivity on brine shrimp, all these plant species could have a potential effect as insecticides and/or cytotoxics.

STUDIES ON FUNGI

Torres-Barragán et al. (in progress) made three insect collections in two zones within El Edén: the tropical forest zone and the agricultural zone in the surrounding area. Collections were performed during the rainy season (November to February) as well as during the dry season (March to October). All insect collections were transported to the laboratory at the Instituto de Ecología, Universidad Nacional Autónoma de Mexico (UNAM), for the isolation and cultivation of all fungi found inside the insects.

A total of approximately 3,400 insects, comprising 18 insect species, were collected from the two areas. Four types of insects were from the tropical forest: houseflies, ants, bees, and termites. The main genus of fungi isolated from these four types of insects were *Penicillium* sp., *Aspergillus* sp., *Paecilomyces marquandii,* and *Verticillium* sp. Thirteen types of insects were collected from the agricultural zone: treehoppers, leafhoppers, Chili sap beetles, the coleoptera *Acalina trivitata* and *Conotelus stenoides*, whiteflies, aphids, bean grubs, fall armyworms, leaf-cutting ants, Mexican fruit flies, sap beetles, and citrus leaf miners. From these types of insects, the main fungi isolated were *Aspergillus parasiticus*, *Fusarium moniliforme, F. oxysporum,* and *Aschersonia* sp.

Tropical forests and other complex communities are considered stable, as the impact of a sudden population change in one species will be cushioned by the large number of interacting species and will not produce drastic effects in the community as a whole. It has been suggested that such buffer mechanisms operate in tropical forests where insect outbreaks are unknown. Coley and Kursar (2001) suggest that insect herbivores are rare in tropical forests because they are highly regulated by the third trophic level. This fact may explain the lower number of insects found in the tropical forest of El Edén. This situation contrasts with cultivated forests, where pest outbreaks are common (Mackenzie, Ball, and Virdee 1998). It was possible to confirm this in the two plant communities of El Edén where the insect collections were made (Torres-Barragán et al., in progress).

On the other hand, in the agricultural zone where pest insects are abundant, the environmental conditions are ideal for insect pest proliferation—that is, a low biodiversity of crops coupled with low amounts of natural enemies that would otherwise control these pests. In this area, the whitefly can cause a total loss of production for both tomato crops and chili crops, mainly because of the viruses that the pests transmit to these plants (Urías-Morales, Rodríguez-Montesoro, and Silva 1995). This fact is one of the main reasons why croplands were abandoned in tropical agricultural zones, and underscores the importance of finding new alternatives for more natural pest control.

CONCLUSION

Microorganisms are essential to the health and functioning of ecosystems through mineralization and recycling of organic matter. They also play a significant role in bioproductivity, either directly via synthesis of food, medicines, and chemicals, or indirectly by making nutrients available for other primary producers. On the other hand, diverse studies on microbes from pest insects in natural protected areas have identified potential biological-control microorganisms (Charnley 1997). Detailed studies of mycoparasites population dynamics and their hosts are necessary in order to determine their potential use as biocontrol agents (Jeffries 1997).

The use of specific microbes in integrated pest management could also reduce dependence on chemical pesticides. The aim of the current investigation in this particular field is to identify those fungi from El Edén with a potential as microbial insecticides. For example, the genus *Fusarium* was one of the most remarkable fungi found in the insects of El Edén. *Fusarium* was isolated from 98.8 percent of the collected insects that showed fungal infection, with *Fusarium oxysporum* the most abundant species because it was isolated from 70 percent of the collected insects (Torres-Barragán et al., in progress) This species has received considerable attention from plant pathologists over the past 80 years because of its ability to cause vascular wilt or root rot diseases in a wide range of plants (Kistler 1997). In the 1970s, *F. oxysporum* f. sp. *orobanche* was developed in the former Soviet Union as a weed killer (Franz & Krieg 1976). One isolate of *F. oxysporum* has been evaluated as a *Striga* killer in the dryland zones of Africa where this parasitic plant causes losses of 70 percent in sorghum and maize production. In 1995, the results were dramatic: 85 percent of the *Striga* were wiped out at the seedling stage by this *Fusarium* isolate with the added advantage that it is not toxic to humans and causes no harm to cereal crops (Ciotola, Watson, & Hallett 1995).

On the other hand, a large number of *Fusarium* spp. are entomopathogenic. Highly pathogenic species are reported primarily from Homoptera and Diptera (Teetor-Barsh and Roberts 1983). *Fusarium oxysporum* is highly virulent to larvae of the mosquito, *Aedes detritus* Edw.;

to larvae of the rice green-horned caterpillar, *Melanitis leda*; and to eggs of the European corn borer, *Ostrinia nubilalis*. All these data indicate the importance of the current studies on the potential pathogenicity of the fungi isolated from El Edén insects.

Different sources of new natural products with a biocide potential have been found in some plant species and fungi of El Edén that could be used to control weeds and other pests, as well as to solve some disease problems. This project constitutes a long-term survey of the biotic chemical diversity not only of this ecological reserve, but also in the surrounding areas. In this project, methodology and research protocols were tested that could be applied to other tropical areas.

The aqueous leachates of some selected plants showed a strong phytotoxic effect. In general, the leaves are the part of the plants where this effect is more evident. Some of the most bioactive plants tested include all the Fabaceae species, *T. gaumeri*, *Eupatorium* sp., *Ipomoea* sp., *J. gaumeri*, *M. arboreus*, *H. trilobata*, *H. patens*, *A. cominia*, *L. camara*, *Z. guidonia*, *S. adenophora*, and *C. acuminata*. Families with a major phytotoxicity are Fabaceae, Apocynaceae, Asteraceae, Rubiaceae, Sapindaceae, and Verbenaceae.

In regard to defensive allelochemical compounds, Coley and Barone (1996) observe that leaves of tropical forests have both higher overall levels of defense and a greater diversity of defense compared to their temperate counterparts. This greater commitment to defense is an evolutionary response to elevated pressure from herbivores. In the tropics, mature leaves are long-lived and must therefore be resistant to both abiotic and biotic damages. Nowadays, the knowledge of the allelopathic/allelochemical potential of many plants allows them to be consider them as part of the defense mechanism.

Currently, the advanced steps of the biodirected fractionation of three plant species that have compounds with a strong bioactivity on seeds, phytopathogenic fungi, and insects are being performed: *Z. guidonia, C. acuminata*, and *S. adenophora* (results not shown). With the collaboration of Dr. Rocio Cruz-Ortega of Instituto de Ecología, UNAM, a study is being performed of the mode of action of bioactive aqueous leachates of selected plants on protein synthesis, genetic expression, and oxidative enzymes on crop plants.

This project contributes to chemical exploration of dry tropical ecosystems in Mexico and biodiversity conservation. The scientific, economical, and historical fields of research that the long-term project at El Edén has opened are immense and promising. Each plant, animal, and microorganism within this ecological reserve constitutes a very valuable source of natural products.

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