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POTENTIALLY DEFENSIVE PROTEINS IN MATURE SEEDS OF 59 SPECIES OF TROPICAL LEGUMINOSAE[1]

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Abstract—A survey of 59 species of tropical legume seeds revealed high interspecific variation in proteinaceous capacity to inhibit bovine trypsin (a digestive enzyme) and to agglutinate human (type B, Rh positive) and laboratory rabbit red blood cells. The legume subfamily Mimosoideae was conspicuous for the absence of seeds with very weak trypsin inhibition. Congenerics sometimes differed strongly from each other with respect to both trypsin inhibition and phytohemagglutination. Half the species of seeds displayed no hemagglutinating capacity with one or the other kinds of red blood cells, and in only 27% of the 30 cases where there was some activity did the same species of seed actively agglutinate both species of red blood cells. A species of seed that had hemagglutinating capacity was almost invariably associated with moderate to high levels of trypsin inactivation. While it has been long known that a great diversity of small toxic and potentially defensive molecules occur in legume seeds and that one species of seed often contains several of them, we now feel that it is reasonable to consider legume seeds as also containing a high diversity of potentially toxic protein molecules. A single seed is likely to contain, at the least, three to four classes of defensive compounds, any or all of which, or some in combination, may be the cause of a seed being rejected by a potential seed predator.

Key Words—Seed predation, seed chemistry, lectins, protease inhibitors, phytohemagglutins, Costa Rica, seed defenses, Leguminosae.

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INTRODUCTION

A seed is a bag lunch for a seedling. However, there has been strong selection for seed traits that lower the probability that the seed will become lunch for a seed predator. Since seeds are rich in nutrients of value to a wide variety of animals, we expect that natural selection will have led to each species of seed having a variety of defenses, some of which function against certain potential seed predators, and others that function against others. Some seed proteins, e.g., lectins or phytohemagglutinins (Liener, 1979; Rudiger, 1984; Janzen, 1981) and proteinase inhibitors (Ryan, 1979), are among these potential defense traits (e.g., Janzen, 1977, 1978, 1981; Janzen et al., 1976; Lee, 1979; Olsnes and Pihl, 1973; Laskowski and Kato, 1980; Gatehouse et al., 1979; Adam 1974; Stripe et al., 1976; Warsy et al., 1974; Jayne-Williams and Burgess, 1974; Tannous and Ullah, 1969; Pearch et al., 1979).

One important step in understanding the defenses of a seed is identifying the potentially toxic compounds in that seed. This is less simple that it would appear for three major reasons. First, "toxicity" is a dosage- and animal-specific trait, although, of course, certain classes of compounds (e.g., alkaloids, cyanogenic glycosides) have a higher chance of being toxic than do others. This means that simply identifying a compound in a seed does not disclose the seed's toxicity to animals (although some quite educated guesses are possible). Second, a given phytochemist tends to be specialized with respect to identification of a certain class of compounds. It is therefore almost impossible for the ecologist to send a bag of seeds to a single phytochemist and ask for a profile of all the potentially defensive compounds in that seed. Third, there are two major groups of potentially defensive compounds in seeds.

There are relatively small molecules that are generally straightforward to describe, identify, and isolate. Their mode of action on animal systems in generally very specific, and these compounds have a long tradition of examination by pharmacologists; caffeine, strychnine, L-dopa, canavanine, and cyanogenic glycosides are some well-known examples. If seed defenses were constituted only of compounds such as these, we would undoubtedly understand seed relationships with seed predators much better than we do. However, seeds also contain large molecules made up of repeating patterns of sugars, phenols, and amino acids. These carbohydrates, tannins, lignins, phytohemagglutinins (lectins), and protease inhibitors (to name but a few) are difficult to describe, identify, and isolate. Their mode of action on animal systems is often generalized (e.g., one may be a "digestion inhibitor," another "inhibit uptake of amino acids by the intestine," etc.). These large molecules have been known for a very long time in a general sort of way; tanning leather with polyphenols so as to prevent access to collagen by bacterial enzymes, and boiling beans to denature toxic phytohemagglutinins may be just as ancient to humans as is chewing plants for alkaloid pain-killers and mashing rotenone-rich foliage into streams

to stupefy fish. However, these large molecules have been largely the despair of phytochemists. An additional complication in interpreting large molecules is that they are often major parts of the biomass of a seed and clearly serve also as sources of raw biosynthetic construction materials for the developing seedling. However, given their physiological activity against animals, they are additionally a potential part of the defense repertoire of a seed.

One of the more profitable ways of determining the defense repertoire of a seed is to test the seed contents against potentially susceptible biological systems. In fact, two of the large and potentially defensive types of proteinaceous molecules found in seeds are traditionally recognized by their physiological activity against animal systems rather than by chemical characterization. Here we report for 59 species of tropical legume seeds the results of challenging trypsin, a protease enzyme, with the finely ground contents of live seeds. We also report the results of asking if the same seed contents will agglutinate red blood cells of the human (type B, Rh positive) and the laboratory rabbit (*Oryctolagus cuniculus*).

As will be discussed below, we wish to emphasize that in such a general screening only positive results are useful; protease inhibitors and phytohemagglutinins display a wide variety of specificities, and a seed that neither inhibits trypsin nor agglutinates rabbit or human red blood cells may still contain a very potent proteinase inhibitor or phytohemagglutinin. For example, *Phaseolus lunatus* seeds showed no hemagglutination with type B blood (Table 1) but contain a powerful hemagglutinating factor for type A human blood (anonymous reviewer). The species of seeds to be screened were chosen in great part because they are the subjects of intensive feeding tests against seed predators in the Costa Rican habitats where they grow (e.g., Janzen, 1981; Janzen et al., 1986) and because they are subjects of an intensive long-term effort to characterize the entire defense repertoire of certain species of seeds (e.g., *Dioclea megacarpa*, Rosenthal et al., 1982; Rosenthal and Janzen, 1983, 1985; *Lonchocarpus* spp., Fellows et al., 1979; Janzen et al., 1986).

METHODS AND MATERIALS

Seed Sources. All the seeds listed in Table 1 were collected by D.H.J. between 1965 and 1980 in the lowlands of Guanacaste Province, Costa Rica except as itemized below. Exceptions: *Entada gigans* and *Oxyrhynchus trinervius* were beach drift seeds and *Mucuna mutisiana*, *Parkia pendula*, and *Pterocarpus officinalis* were rainforest seeds, from Corcovado National Park, southwestern Costa Rica; *Adenopodia polystachya* and *Canavalia bicarinata* came from mangrove forest margins near Puntarenas, Costa Rica; *Erythrina* spp. came from the area around San Jose, Costa Rica; *Mucuna andreana* came from rainforest edges at Finca La Selva, Heredia Province, Costa Rica; *Leucaena glauca* from St. John's Island, British Virgin Islands, Caribbean; *Ormosia* spp. were

TABLE 1. POTENTIALLY DEFENSIVE COMPOUNDS IN SEED CONTENTS OF 59 SPECIES OF TROPICAL LEGUMINOSAE

Plant	Dry weight (mg)		Protease inhibitor[c] (μg dry seed/μg trypsin at 50% inhibition)[d]	Hemagglutinin activity[e] (HU/mg dry weight)	
	Entire seed[a]	Seed contents[b]		Rabbit	Human[f]
Caesalpiniodeae					
Bauhinia glabra	101	72	11.9	12.0	0.0
Bauhinia pauletia	69	49	321	0.5	0.0
Bauhinia ungulata	30	15	393	1.0	0.0
Caesalpinia bonduc	4101	2297		0.0	0.0
Caesalpinia coriaria	30	15	476	0.0	0.0
Caesalpinia eriostachys	371	260	16.2	0.0	0.0
Caesalpinia pulcherrima	214	150	526	4.2	0.0
Cassia biflora	4	2.2	393	0.0	0.0
Cassia emarginata	28	17	1071	0.0	0.0
Cassia grandis	1152	772	19.0	1.8	7.0
Cassia leptocarpa	12	7	$\gg$2500	0.0	0.0
Delonix regia	758	440	395	trace	0.0
Schizolobium parahybum	1208	737	$\gg$2500	0.4	0.0
Mimosoideae					
Acacia angustissima	12	10	211	0.0	0.0
Acacia cornigera	236	130	71.4	trace	1.3
Acacia tenuifolia	42	21	57	0.0	0.0
Adenopodia polystachya	288	216	6.7	0.0	0.0
Albizzia adinocephala	16	10	55	0.0	0.0
Albizzia caribaea	33	20	38	trace	1.7
Albizzia longepedata	30	18	151	0.0	1.3
Entada gigans	23621	22440	27.6	0.0	0.0
Enterolobium cyclocarpum	1423	740	26.7	0.0	0.0
Leucaena glauca	48	27	147	0.0	0.0

Lysiloma divaricata	35	20	41	0.0	0.0
Mimosa quadrivalis	7	6	613	0.0	0.0
Parkia pendula	100	65	39	0.3	0.0
Pithecellobium mangense	35	18	41	1.1	0.0
Pithecellobium platylobum	203	152	28.6	trace	0.0
Faboideae					
Ateleia herbert-smithii	68	41	$\gg$2500	0.0	0.0
Canavalia bicarinata	233	189	54.3	81.0	0.0
Canavalia brasiliensis	568	449	33.3	106.0	trace
Canavalia maritima	1225	919	161.9	157.0	0.0
Centrosema plumieri	94	73	105	0.0	0.0
Centrosema pubescens	44	36	267	0.0	0.0
Crotalaria incana	6	5	264	0.0	0.0
Crotalaria pumilio	4	3	208	0.4	2.0
Dalbergia retusa	56	44	72	0.0	0.0
Dioclea megacarpa	12701	9907	57.1	75.0	1.8
Oxyrhynchus trinervius	2200	1716	17.1	0.0	0.0
Erythrina berteroana	282	186	5.7	0.0	8.7
Erythrina poeppigiana	326	261	148	0.0	2.1
Gliricidia sepium	140	119	>1000[g]	trace	0.0
Indigofera hirsuta	1	0.6	559	0.0	0.0
Lonchocarpus acuminatus	317	292	2200	0.0	0.0
Lonchocarpus costaricensis	309	250	1050	0.0	0.0
Lonchocarpus eriocarinalis	215	174	112.4	0.0	0.0
Lonchocarpus minimiflorus	68	58	1063	0.0	0.0
Lonchocarpus rugosus	51	41	648	trace	0.0
Lonchocarpus salvadorensis	110	105	>1500	0.0	0.0
Machaerium arboreum	321	305	17.1	0.5	0.0
Mucuna andreana	9205	8100	138.1	8.0	0.0
Mucuna mutisiana	9445	7367	85.7	27.0	0.0
Mucuna pruriens	379	315	235	0.0	0.0
Ormosia tovarensis	3419	2838	2480	0.2	0.0

TABLE 1. Continued

Plant	Dry weight (mg)		Protease inhibitor[c] (μg dry seed/μg trypsin at 50% inhibition)[d]	Hemagglutinin activity[e] (HU/mg dry weight)	
	Entire seed[a]	Seed contents[b]		Rabbit	Human[f]
Ormosia venezolana	1281	1089	933	0.0	0.0
Phaseolus lunatus	75	65	29.0	trace	0.0
Pterocarpus officinalis	792	752	10.9	0.2	70.1
Sesbania emerus	7	5	786	0.2	trace
Sophora macrocarpa	607	449	71.4	trace	0.0
Pure SBA[h]				28,000 ± 10%	540 ± 10%

[a] Dry weight of seed with seed coat. Derived from percent seed coat values in Janzen (1977).
[b] Dry weight of seed contents. Derived from percent seed coat values in Janzen (1977).
[c] Results from laboratory of C.A. Ryan, Department of Agricultural Chemistry, Washington State University, Pullman, Washington 99164 (1978). Chymotrypsin assays could not be run because seed carboxypeptidases split the chymotrypsin substrates.
[d] Large seeds had seed coats removed before grinding and testing; small seeds were ground entire and tested as such.
[e] Results from Laboratory of I.E. Liener, Department of Biochemistry, University of Minnesota, St. Paul, Minnesota, 55108.
[f] Human blood group: B, Rh positive.
[g] Precipitated out, could not get good results, very weak.
[h] Prepared as described in Uy and Wold (1977).

purchased in a native market in Merida, Venezuela; *Sophora macrocarpa* was collected to the north of Santiago, Chile.

These plant species are common, and the collections were unambiguously identified by D.H.J. Additionally, voucher specimens for all these species have been deposited in the herbarium of the Missouri Botanical Gardens, St. Louis, Missouri. All the Guanacaste plant names conform to Janzen and Liesner (1980) except that *Lysiloma seemannii* is now known as *Lysiloma divaricata*. D.H.J. identified all plants in the field and was certain that only mature and dormant seeds were collected for analysis.

Seed Treatment. Large seeds, those weighing more than 500 mg dry weight after removal of the seed coat, were ground and tested after removal of their seed coats. Smaller seeds were ground up entire for the tests. All seeds were living at the time of grinding and ranged between 5 and 10% water content (i.e., seemingly dry and hard). Seeds were finely ground in a Wiley mill before testing. This seed meal was not oven-dried before use or weighing for percentage determinations.

Proteinase Inhibitor Tests. Bovine trypsin was purchased from Worthington (Freehold, New Jersey) and was approximately 50% active as determined with the active site titrant *p*-nitrophenyl-*p'*-guanidinobenzoate (Chase and Shaw, 1967). Enzyme activity was determined by the method of Hummel (1959) using tosyl-L-arginine methyl ester as substrate. The powder from individual seeds was dissolved in 0.5 M KCl (50 mg powder per ml), soaked for 1 hr, and contrifuged at 10,000*g* for 10 min. The clear supernatants were employed for assays. Trypsin inhibitory activity was determined by the addition of increasing quantities of the extract to a standard quantity of trypsin and incubated for 5 min prior to assay. The data is reported as the quantity (dry wt) required to decrease the activity of 1 μg trypsin 50%.

Hemagglutinin tests. The finely ground seed was suspended in 10 volumes of physiological saline (0.9% NaCl) and vigorously shaken for 5 min. Any insoluble material was removed by centrifugation, and the hemagglutinating activity of the supernatant was determined by the photometric procedure of Liener (1955). In this method, serial dilutions of this extract (or a suitably diluted portion thereof) are added to an equal volume of a suspension of trypsinated rabbit or human red blood cells. The latter were prepared by prior treatment with glutaraldehyde, as described by Turner and Liener (1975), in order to increase their stability during storage. The mixture of each dilution with the red blood cell suspension was allowed to sit at room temperature for 2 $\frac{1}{2}$ hr, at which time the absorbance at 620 nm was measured in a Coleman Junior Spectrophotometer model 6A.

One hemagglutinating unit (HU) is arbitrarily defined as that amount of lectin which causes a 50% decrease in the absorbancy of the cell suspension under the conditions specified above. The reciprocal of the dilution (X) corre-

sponding to 1 HU may be calculated from the percentage of the cells remaining in suspension of those tubes having the closest values which are above (tube a) and below (tube b) the 50% endpoint. The following equation is employed:

$$\log x = \log c = (50 - b) \cdot \log 2$$

Where c = reciprocal of dilution in tube b, a = % of cells in suspension in tube a, and b = % of cells in suspension in tube b. The agglutinating activity of the seed extract is then calculated from the value of x and the weight of the seed material employed in making the original extract.

RESULTS

The results in Table 1 show clearly that trypsin inhibition among a large array of legume seeds can range from negligible (e.g., *Cassia leptocarpa*, *C. emarginata*, *Schizolobium parahybum*, *Ateleia herbert-smithii*, *Gliricidia sepium*, *Lonchocarpus acuminatus*, *L. costaricensis*, *L. minimiflorus*, *L. salvadorensis*, *Ormosia tovarensis*) to very intense (e.g., *Bauhinia glabra*, *Adenopodia polystachya*, *Erythrina berteroana*, *Pterocarpus officinalis*). Very intense activity was found in all three legume subfamilies, but the Mimosoideae are conspicuous for the absence of seeds with very weak trypsin inhibition. Congenerics may differ strongly from each other (e.g., different species of *Cassia* seeds ranged from very intense inhibition to virtually no effect) as well as be quite similar. Except for the two species of *Ormosia* (which are traditionally viewed as very rich in alkaloids that are toxic to vertebrates), all really large seeds showed inhibitor activity; small seeds display such a variety of intensity that no generalization can be made about the direction of their intensity.

Half the species of seeds displayed no hemagglutination activity against laboratory rabbit or human blood cells. The same seed was active against both kinds of red blood cells in only 27% of the 30 cases where there was some activity. Overall, there was more agglutination response by the rabbit than by the human red blood cells; this conforms with the direction of the "control" results with a purified hemagglutinin SBA. However, in both cases the response is relatively weak when compared with SBA. A high level of hemagglutinating activity against one kind of red blood cell is apparently randomly associated with the level of activity against the other.

A species of seed that showed hemagglutinating activity was almost invariably associated with comparatively moderate to high levels of trypsin inactivation. However, there were at least two cases of some hemagglutination by seeds that displayed virtually no trypsin inhibition (e.g., *Schizolobium parahybum*, *Ormosia tovarensis*).

DISCUSSION

The positive results listed in Table 1 are only the tip of the iceberg. It is likely that every legume seed contains one or more protease inhibitor and/or one or more phytohemagglutinin. However, demonstrating this will require screening against a wide variety of enzymes (and perhaps screening under a variety of environmental conditions such as different but biologically realistic levels of pH and temperature). Likewise, given the strong specificity of different kinds of phytohemagglutinins for different species of red blood cells, a seed will have to be screened against many kinds of blood cells and perhaps other sugar-containing substrates before it can be certified as free of phytohemagglutinins (lectins). For example, as mentioned earlier, lectins in *Phaseolus lunatus* seeds do not hemagglutinate type B human blood, but do hemagglutinate type A human blood.

Since virtually every species of seed listed in Table 1 contains one or more kinds of uncommon amino acid, alkaloid, or cyanogenic glycoside, the results of this screening add robustness to the hypothesis that each species of legume seed is likely to have a variety of defenses. For example, *Dioclea megacarpa* contains canavanine and canaline, and now it is clear that it also contains a trypsin inhibitor protein and a hemagglutinating protein. Conversely, when an animal refuses to feed on a seed, it is clear that one cannot automatically attribute such a refusal to the first potentially defensive compound that is easily isolated from the seed. For example, *Pterocarpus officinalis* seeds contain the alkaloid hypaphorine, a potent feeding deterrent to at least one species of small seed-eating rodent (Janzen et al., 1982). However, it is obvious from the results presented in Table 1 that the refusal of wild rainforest animals to harvest the large and abundant *P. officinalis* seeds may also be due to their strong trypsin inhibitor ability, strong lectin activity, or the combination of these obnoxious proteins with the alkaloid (or even with some as yet undetected other defensive compound).

When an animal bites into a seed, it gets the defensive compounds along with a large bulk of material that is either quite edible and digestible, or merely inert from the viewpoint of the animal. The material other than the defensive compounds plays two important roles in the context of this survey. First, when examining the defensive compound repertoire of a seed (or any other plant part), it is commonplace to think in terms of concentration (or activity) of the compounds in the seed contents. This quantitative measure is presumed to have some biological meaning. However, we must caution that concentrations (or intensity of activity) as measured by the biologist are extremely difficult to accurately interpret in terms that represent how the animal experiences them. Not only is there the problem that one beast's poison may be another beast's dinner, but additionally there may be strong threshold effects. For example, Gatehouse

et al. (1979) showed that the threshold level of trypsin inhibitor in *Callosobruchus*-resistant cowpeas (*Vigna unguiculata*) lies between 0.5 and 0.8%.

Furthermore, impact of the defense compound may simply be to lower the gain from the food being eaten, rather that do some direct damage to the animal. For example, if a trypsin inhibitor occurs at some low level, it may inactivate only part of the gut enzyme pool, leaving another portion of the gut's enzymes to function normally. If the seed happens to contain a large amount of the substrate, the remaining enzyme pool may be able to harvest so much resource that the trypsin inhibitory effect is inconsequential from the viewpoint of the potential seed predator; seed predators eat mouthfuls of seed content, rather than just the compounds being assayed. In the context of the present survey, the message is that high or low values of proteinase inhibitor or phytohemagglutinin activity may not translate well into the inedibility or toxicity of a meal of seeds, especially when one considers the highly positive gains that an animal may get from a bite of the seed. Of course, the greater the amounts and kinds of potential defense materials in the seed, and the lesser the amounts of traditional nutrients present, the greater the chance that a seed will be inedible or toxic to a seed predator that is not specialized on that species of seed. Equally, the larger the defense repertoire and the less the nutrient content, the more biochemically specialized is likely to be the specialist seed predator that regularly feeds on a seed that is toxic to other animals.

Second, the other portions of the seed affect the interpretation of our survey results. When a caterpillar takes a bite of a leaf, some of the defensive compounds in the leaf may function in making leaf material unavailable rather than hurting the caterpillar directly; likewise, the contents of the seeds surveyed here may affect the ability of the proteinase inhibitors and phytohemagglutinins to react with the test substrates. For example, when the seed is ground up and placed in a liquid medium, the trypsin inhibitors may well react with some of the other proteins in the seed rather than with the trypsin offered as an assay. Likewise, the phytohemagglutinins may cross-react with complex polysaccharides in the seed meal, and thus be prevented from participation in the bioassay. A number of such responses are to be expected if selection has operated to produce a seed whose nutrients become unavailable when the seed is eaten, rather than (or in addition to) to produce a seed that is actually toxic or otherwise inedible to the seed predator.

In other words, the positive results in Table 1 can be believed, but no result, including the zeros, can be taken to indicate the maximum amount of reactable proteinase inhibitors or phytohemagglutinins that may occur in the seed. As mentioned in the Introduction, it is even quite possible for there to be inhibitors present that do not react with bovine trypsin but will react with other enzymes. Likewise, the values obtained in Table 1 cannot be viewed as the maximum possible levels of reaction in the gut of an animal, since the gut adds

various other substrates and reactants to the mix, creating a reaction environment that is obviously different from that used in the screening for the data in Table 1 (see Becker, 1984, for a discussion of similar philosophies vis à vis tannins as digestion inhibitors for leaf-eating caterpillars).

It is tempting to expect an inverse relationship between the activities of the two quite different classes of proteins for which we assayed. However, the expectation disappears when one considers that these two classes of compounds are only two of at least three to five classes of potentially defensive compounds in a seed (and perhaps there are double that number of kinds of defensive compounds when one considers that it is commonplace for a single species of seed to contain several kinds of alkaloids or uncommon amino acids). Furthermore, the plant has other defenses such as seed hardness, seed number, phenology, dispersal mode, etc. (Janzen 1978). A seed that is short on any one these defenses may well be exceptionally well-endowed on some other defense axis (e.g., Janzen, 1969, 1977; Janzen and Higgins, 1979) rather than just increase one of the potential defenses that we happen to screen for here.

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