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Poor Correlation between the Levels of Proteinase Inhibitors Found in Seeds of Different Cultivars of Cowpea (*Vigna unguiculata*) and the Resistance/Susceptibility to Predation by *Callosobruchus maculatus*

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Larvae of *Callosobruchus maculatus* have poor performance in seeds of the cowpea (*Vigna unguiculata*) cultivar TVu 2027, and of its progenies IT81D-1045 and IT81D-1064, when compared to cultivars CE-31, CE-11, and CE-524. We did not find any correlation between proteinase (trypsin, chymotrypsin, Subtilisin BPN′, papain) inhibitory activity and the resistance shown by cultivars TVu 2027, IT81D-1045, and IT81D-1064. Similarly, there was no obvious relationship between the levels of lectin (hemagglutination activity) or tannins and the resistance or susceptibility of the seeds to predation by *C. maculatus*. The *C. maculatus* resistant cowpea seeds (IT81D-1045, IT81D-1064) support the development of *Zabrotes subfasciatus*, a bruchid that infests seeds of both *Phaseolus vulgaris* and *Vigna unguiculata*.

Lectins, tannins, proteinase inhibitors, and trypsin inhibitors, in particular, and other compounds found in large amounts in seeds of many plants are thought to be part of an array of constitutive defenses that plants utilize against attacking microorganisms and insects (Janzen et al., 1986; Xavier-Filho and Campos, 1989).

There are reports in the literature suggesting that proteinase inhibitors may also be involved in the resistance that some seed varieties present to their common predators (Xavier-Filho and Campos, 1989). For example, Gatehouse et al. (1979) suggested that the resistance of seeds of a cowpea (*Vigna unguiculata*) cultivar (TVu 2027) toward its bruchid pest *Callosobruchus maculatus* was due to the high levels of trypsin inhibitors found in these seeds.

However, we have found other cowpea cultivars whose seeds have trypsin inhibitory activity as high as the seeds of the cultivar TVu 2027, which are susceptible to *C. maculatus* (Xavier-Filho and Campos, 1984).

In addition, several observations show that the larvae of *C. maculatus* utilize mainly cysteine proteinases for protein digestion (Gatehouse et al., 1985; Kitch and Murdock, 1986; Campos et al., 1989) rather than serine proteinases, like trypsin.

These observations and the availability of seeds of different cowpea cultivars led us to investigate the proteinase inhibitor levels in seeds of this legume in relationship to the resistance to *C. maculatus*. Two other groups of compounds possibly implicated with the resistance, namely, tannins and lectins, were also investigated.

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METHODS AND MATERIALS

Rearing of Insects. The *C. maculatus* used in this work were supplied by Dr. J. H. R. Santos, Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brazil. Permanent colonies of the beetles were established on *V. unguiculata* (cv. Pitiuba, CE-31) seeds in this department since 1978. The insects were reared at 29 ± 5 °C and relative humidity 65% ± 5%. A *Zabrotes subfasciatus* culture was also established in 1987 from insects supplied by Prof. F. M. Wiendl of the Centro de Energia Nuclear na Agricultura, Piracicaba, São Paulo, Brazil. The insects were reared on *V. unguiculata* (cv. Pitiuba, CE-31) under the same conditions as for *C. maculatus*.

Cowpea Seeds. Six *V. unguiculata* cultivars were utilized in this study. Seeds of the cultivars CE-11 (Quebra-cadeira), CE-31 (Pitiuba), and CE-524 were supplied by J. B. Paiva of the Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brazil. These were local cultivars. Seeds of the cultivars IT81D-1045, IT81D-1064, and TVu 2027 were obtained through the Centro Nacional de Pesquisa Arroz-Feijão (EMBRAPA), Goiânia, Brazil; the cultivars IT81D-1045 and IT81D-1064 were bred from TVu 2027 at IITA (International Institute for Tropical Agriculture, Ibadan, Nigeria).

Performance of *C. maculatus* and *Z. subfasciatus* on Cowpea Seeds. To measure the performance of *C. maculatus* and *Z. subsciatus* on cowpea seeds, three replicates of 20 seeds of each cultivar were used; two fertilized females (2–3 days old) were put in each glass bottle containing 20 cowpea seeds and left to oviposit for 24 h. After that period, the insects were removed and the eggs laid in excess of two were scrapped. The number of emerged adults was counted so that we could calculate the percentage survival to adult emergence (S); the days in which

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Table I. Performance of a Brazilian Strain of *C. maculatus* on Six Cowpea (*V. unguiculata*) Cultivars[a]

cultivar	% *S*	*T*, days	log (*S*/*T*)[b]
CE-31 (Pitiuba)	86.9 ± 3.3[e]	29.7 ± 0.5[d]	0.062
CE-11 (Quebra-cadeira)	90.0 ± 10.5[e]	28.4 ± 1.1[d]	0.069
CE-524	80.0 ± 2.5[e]	28.1 ± 0.3[d]	0.068
IT81D-1045	11.7 ± 1.4[d]	44.0 ± 1.3[e]	0.024
IT81D-1064	5.8 ± 3.8[d]	45.9 ± 5.6[e]	0.017
TVu 2027	(9.3)[c]	(39.5)[c]	0.024

[a] Values followed by the same letter in a column are not significantly different (5% level, Duncan's multiple-range test). The standard errors are indicated, and *n* (the number of replications) was 3. [b] Howe's index (Howe, 1971). [c] Data taken from Dick and Credland (1986) for a Brazilian (Campinas) strain of *C. maculatus*.

the adults emerged were also recorded so that we could estimate the mean development period (*T*).

Tannins. Tannin concentration was measured by the radial diffusion method developed by Hagerman (1987). Cowpea seed meal was extracted with 50% (v/v) aqueous methanol for 1 h at 25 °C with a meal to solvent ratio of 1:5 (w/v). For the diffusion assays, the extracts were applied to 4-mm-diameter wells cut in 1% agarose gels made in 0.05 M acetic acid and 0.06 μM ascorbic acid, pH 5.0, containing 0.1% bovine serum albumin. After 96 h, the diameters of the precipitation disks were measured. Tannic acid concentrations were estimated from a standard curve in which tannic acid content was plotted against the square diameter of the disks formed.

Hemagglutination Activity (Lectin). This was done essentially as described by Grant et al. (1983). Trypsin-treated rabbit blood cells (2% in 0.9% NaCl) were used to measure the hemagglutination activity (lectin) of both alkaline (0.05 M phosphate, 0.9% NaCl, pH 8.0) and acid (0.05 M glycine, 0.9% NaCl, pH 2.5) extracts of cowpea seed meal (meal to buffer ratios were 1:5 (w/v) and 1:10 (w/v), respectively). The extracts were serially diluted with 0.9% NaCl containing 5 mM Ca^{2+} and 5 mM Mn^{2+} on a Titertek medimixer (Flow Laboratories Ltd., Irvine, CA) and mixed with an equal volume of diluted erythrocyte suspension (final volume 0.1 mL). The amount of clumping was assessed by a microscope after standing 16 h at 25 °C. One unit of hemagglutination activity (HU) was defined as the amount of material per milliliter in the last dilution giving 50% agglutination.

Proteinase Inhibitor Assays. Trypsin and chymotrypsin assays were done essentially as described by Kunitz (1947); Subtilisin BPN′ activity was determined according to the method of Hagihara et al. (1958a,b); papain activity was measured as described by Arnon (1970). Inhibitory activities against the above proteinases were estimated in seed meal extracts (0.1 M phosphate buffer, pH 7.6) suitably diluted by measuring the difference in activity between solutions containing enzyme and solutions containing enzyme plus seed meal extract; inhibitory activity is expressed in inhibitor units as defined by Xavier-Filho (1974).

Chemicals. Trypsin (Type III), α-chymotrypsin, Subtilisin BPN′, and papain (Type III) were bought from Sigma Chemical Co. (St. Louis, MO). Tannic acid was from Nutritional Biochemicals Co. (Cleveland, OH). All other reagents and chemicals were of the best available grade. Casein (Hammarsten type) was bought from Merck (Darmstadt).

RESULTS

The performance of a Brazilian strain of *C. maculatus* on seeds of the six cowpea cultivars used in this study is shown in Table I. Beetles developing on the susceptible cultivars CE-31, CE-11, and CE-524 showed high survival to adult emergence percentages (80.0–90.0%) and mean development periods between 28.1 and 29.7 days. The beetles developing on cultivars IT81D-1045 and IT81D-1064, both genetically bred from TVu 2027, showed lower *S* percentages ($P < 0.05$) (11.7% and 5.8%) and longer *T* values ($P < 0.05$) (44.0 and 45.9 days) than the values found for the susceptible varieties.

Examination of the performance of *Z. subfasciatus* on cowpea seeds shows that there is only a slight difference ($P < 0.05$) between the cultivars studied. This bruchid can feed in *C. maculatus* susceptible (CE-31, CE-11) and *C. maculatus* resistant (IT81D-1045, IT81D-1064) cultivars (Table II).

Table II. Performance of *Z. subfasciatus* on Seeds of Four Cowpea (*V. unguiculata*) Cultivars[a]

cultivar	% *S*	*T*, days	log (*S*/*T*)[b]
CE-31 (Pitiuba)	93.9 ± 3.2[e]	27.0 ± 0.3[c]	0.073
CE-11 (Quebra-cadeira)	95.0 ± 2.2[e]	27.6 ± 0.5[d]	0.072
IT81D-1045	89.5 ± 1.8[d]	27.7 ± 0.1[d]	0.070
IT81D-1064	87.5 ± 4.6[c]	27.9 ± 0.7[d]	0.069

[a] Values followed by the same letter in a column are not significantly different (5% level, Duncan's multiple-range test). The standard errors are indicated, and *n* (the number of replications) was 4. [b] Howe's index (Howe, 1971).

Table III. Tannin (Tannic Acid) Content of Seeds of Six Cowpea (*V. unguiculata*) Cultivars[a]

cultivar	tannic acid, %
CE-31 (Pitiuba)	0.41 ± 0.14[b]
CE-11 (Quebra-cadeira)	0.38 ± 0.08[b]
CE-524	0.34 ± 0.05[b]
ITB1D-1045	0.34 ± 0.06[b]
IT81D-1064	0.36 ± 0.10[b]
TVu 2027	0.46 ± 0.10[b]

[a] Values followed by the same letter in a column are not significantly different (5% level, Duncan's multiple-range test). The standard errors are indicated, and *n* (the number of replications) was 5.

Table IV. Hemagglutination Activity (Lectin) of Extracts of Seeds of Six Cowpea (*V. unguiculata*) Cultivars[a]

	hemagglutination activity, HU	
cultivar	alkaline extractn	acid extractn
CE-31 (Pitiuba)	390	98
CE-11 (Quebra-cadeira)	1560	24
CE-524	195	98
IT81D-1045	1560	390
IT81D-1064	390	98
TVu 2027	780	390

[a] One hemagglutination unit (HU) is defined as the amount of material in milligrams per milliliter in the last dilution of a series giving 50% agglutination of trypsinized rabbit blood cells (Grant et al., 1983). The data shown are representative of two different experiments.

The tannin contents of seeds of the six cowpea cultivars used in this work are shown in Table III. As can be seen, we did not find any significant difference in tannin content for the cowpea cultivars examined.

Hemagglutination activity (lectin) was measured by utilizing trypsin-treated rabbit erythrocytes in the presence of both calcium and manganese. Table IV shows that hemagglutination activity (lectin) could be detected in both alkaline- and acid-extracted meals of the six cultivars. The acidic extraction conditions seem to be the most efficient. The titers obtained do not suggest significant differences in rabbit blood agglutination between cultivars.

Extracts from meals of seeds of the six cultivars were assayed for proteinase inhibitor activity against the serine proteinases trypsin, chymotrypsin, and Subtilisin BPN′ and against the cysteine proteinase papain (Table V). In relation to the trypsin inhibitory activity, the cultivars can be grouped as low-inhibitor containing (CE-31, IT81D-1045, IT81D-1064) and high-inhibitor containing (CE-11, CE-524, TVu 2027). With regard to chymotrypsin inhibitor content, the cultivars can be classed as low-inhibitor containing (CE-31, IT81D-1045, IT81D-1064), medium-inhibitor containing (CE-11, TVu 2027), and high-inhibitor containing (CE-524). In relation to the Subtilisin BPN′

Table V. Proteinase Inhibitor Content of Seeds of Six Cowpea (*V. unguiculata*) Cultivars[a]

	proteinase inhibitors, units·g^{-1}			
	trypsin	chymotrypsin	subtilisin	papain
CE-31 (Pitiuba)	7060 ± 276^{b}	490 ± 49^{b}	180 ± 31^{b}	9.2 ± 0.9^{e}
CE-11 (Quebra-cadeira)	7900 ± 1153^{c}	1250 ± 184^{c,d}	260 ± 19^{d}	11.8 ± 0.7^{g}
CE-524	7830 ± 1065^{c}	1440 ± 258^{d}	290 ± 34^{d}	9.7 ± 1.0^{f}
ITB1D-1045	2830 ± 301^{b}	500 ± 39^{b}	260 ± 34^{c}	4.3 ± 0.4^{b}
ITB1D-1064	2840 ± 127^{b}	560 ± 31^{b}	231 ± 47^{c,d}	7.2 ± 1.1^{d}
TVu 2027	7840 ± 616^{c}	1100 ± 267^{c}	270 ± 50^{b}	6.3 ± 0.7^{c}

[a] One unit of proteinase inhibitor activity is defined as the amount of inhibitor that reduces by 50% the activity of a preparation of proteinase that produces an absorbance of 0.500 in the different assays (Xavier-Filho, 1974). The data were analyzed by Duncan's multiple-range test; values followed by the same letter in a column are not significantly different (5% level). The standard errors are indicated, and n (the number of replications) was 6.

inhibitor content, the cultivars can also be grouped into low-inhibitor containing (CE-31), medium-inhibitor containing (IT81-1045, IT81D-1064), and high-inhibitor (CE-11, CE-524, TVu 2027) containing. As for the papain inhibitor content, there is no possible grouping, as the activity detected in each cultivar differed significantly from all other cultivars.

DISCUSSION

Work by Gatehouse and collaborators (Gatehouse et al., 1979, 1985; Gatehouse and Boulter, 1983; Redden et al., 1983) led to the widely held assumption that trypsin inhibitors are totally or at least partially responsible for the resistance of seeds of the TVu 2027 cowpea cultivar to the bruchid *C. maculatus*. This view seemed to be strenghtened by a recent report from the same group that leaves of tobacco plants transformed with a cowpea trypsin inhibitor gene do not support the growth of the tobacco budworm, a lepidopterous pest (Hilder et al., 1987), which is perhaps not surprising since it is known that lepidopterous larvae rely on trypsin-like enzymes for protein digestion (Applebaum, 1985).

On the other hand, larvae of seed-eating bruchids utilize cysteine proteinases for protein digestion (Kitch and Murdock, 1986; Lemos et al., 1987; Wieman and Nielsen, 1988; Campos et al., 1989). Furthermore, we have previously observed that seeds from some cowpea cultivars were susceptible to *C. maculatus* despite the fact that they contained a high level of trypsin inhibitors (Xavier-Filho and Campos, 1984). In addition, Roy and Bhat (1975) have observed a low but positive correlation between trypsin inhibitor activity and susceptibility of *Lathyrus sativus* seeds to *Callosobruchus chinensis*, a bruchid related to *C. maculatus*.

Two of the cowpea cultivars utilized in this work (CE-31, CE-11) are normally grown by small farmers in the state of Ceará (Brazilian Northeast), and their seeds are subject to heavy damage by *C. maculatus* both in the field and in storage. Cultivar CE-524 is a recent introduction. Cultivars IT81D-1045 and IT81D-1064 were bred in IITA (International Institute for Tropical Agriculture, Ibadan, Nigeria) from cultivar TVu 2027 (CNPAF-EMBRAPA, 1987), which is one of the three bruchid-resistant cowpea varieties found in Nigeria (Singh et al., 1985).

The susceptibility of the three Brazilian cultivars (CE-31, CE-11, CE-524) is clearly shown in Table I. We observed a range of S and T values slightly lower and higher, respectively, than the ones found by Dick and Credland (1986) for a Brazilian (Campinas) *C. maculatus* strain on a Californian black-eyed pea sample. The resistance of IT81D-1045 and IT81D-1064, both bred from TVu 2027 (CNPAF-EMBRAPA, 1987), is also shown clearly in Table I. The values for S and T are slightly different from the ones reported by Dick and Credland (1986) for the Campinas strain of *C. maculatus* on TVu 2027 seeds.

Tannins are believed to be involved in the defenses that the plants developed against herbivores (Swain, 1979). Measurements of the tannin content of seeds of the six cowpea cultivars studied (Table III) showed a range of values (0.34–0.46%), which is below the value of 5% suggested by Janzen et al. (1977) and Boughdad et al. (1986), to have detrimental effects on *C. maculatus* development. The values found for the tannin content of the cowpea seeds examined did not show any significant correlation with either S or T.

Janzen et al. (1976, 1977) and Gatehouse et al. (1984) have shown that *Phaseolus vulgaris* lectins are toxic to *C. maculatus* larvae. Although lectins were not previously detected in *V. unguiculata* seeds (Janzen et al., 1976; Gatehouse et al., 1979), several recent reports on the presence of these substances in cowpea seeds can be found (Uhlenbruck et al., 1981; Oliveira et al., 1982; Roberson and Strength, 1983; Grant et al., 1983). We could detect hemagglutination activity utilizing trypsin-treated rabbit erythrocytes in both alkaline and acidic extracts of meals of seeds of the six cowpea cultivars studied (Table IV). No significant correlation could be found between hemagglutination activity (either alkaline or acid extracted) and S or T.

Measurements of the trypsin inhibitor content of seeds of the six cowpea cultivars studied in this work indicate that there is no correlation between trypsin inhibitor content (Table V) and resistance to *C. maculatus* (Table I). In fact, two of the resistant cultivars (IT81D-1045, IT81D-1064), genetically bred from TVu 2027, show trypsin inhibitor contents as low as that found for the susceptible cultivar CE-31. On the other hand, the high amount of trypsin inhibitor found in seeds of TVu 2027 is of the same order as the amount found in seeds of CE-11 and CE-524, which are susceptible cultivars.

It is interesting to note here that if the amount of trypsin inhibitor in the seeds is converted from enzyme units to a percentage of dry weight with the factor (6×10^{-5}%·g·unit^{-1}) derived from titration curves of active trypsin with purified cowpea trypsin/chymotrypsin inhibitor (Xavier-Filho, 1980), we find a value of 0.47% for the trypsin inhibitor content of the seeds of cultivar TVu 2027. This value is only half the amount found (0.92%) by Gatehouse et al. (1979). This possible overestimate of the physiological levels of trypsin inhibitor in seeds of TVu 2027 found by these authors could explain, at least in part, the results of their feeding experiments. They incorporated purified trypsin inhibitors into artificial seeds at the 0.8% level in their first report (Gatehouse et al., 1979) and at the 1.5–10% level in a subsequent work (Gatehouse and Boulter, 1983). Even at these high levels of incorporation, trypsin inhibitors did not completely inhibit the development of larvae to the adult stage (Gatehouse and Boulter, 1983). It is worth noting also that Shade et al. (1986) by utilizing an artificial seed system showed that an inhibitor

fraction from cowpea, when added at 5% concentration, caused nearly complete mortality but was ineffective at 1%.

The observation that sulfur amino acids, e.g., cysteine and methionine, can counteract the effect of trypsin inhibitors on growth and development of many types of animals, including insects, is well substantiated (Liener and Kakade, 1980; Gallaher and Schneeman, 1984; Broadway and Duffey, 1983). The rationale for the beneficial action of these amino acids is that they are needed for trypsin synthesis, which is much accelerated when the animals are fed trypsin inhibitors. Since larvae of *C. maculatus* rely mainly on cysteine proteinases for protein digestion (Campos et al., 1989), we did not feel the need to measure sulfur amino acid levels in the high-trypsin inhibitor containing susceptible cultivars (CE-11, CE-524).

Table V also shows the chymotrypsin inhibitor content of seeds of the six cowpea cultivars studied. There was also no correlation between chymotrypsin inhibitory activity and either S (r = +0.905) or T (r = −0.603).

As for the Subtilisin BPN′ and papain inhibitor contents, there was also no significant correlation between their amounts and S (r = −0.167, +0.905) or T (r = +0.013, −0.902).

The bruchid *Z. subfasciatus*, a pest infesting seeds of both *P. vulgaris* (McFarlane and Wearing, 1967) and *V. unguiculata* (Meik and Dobie, 1986), develops almost as well in *C. maculatus* resistant cowpea seeds (IT81D-1045, IT81D-1064) as in *C. maculatus* susceptible seeds (CE-31, CE-11) (Table II). This finding suggests that the factor or factors present in the cowpea seeds of cultivars IT81D-1045 and IT81D-1064 (bred from TVu 2027), which are detrimental to *C. maculatus* development, are not detrimental to *Z. subfasciatus*.

We are at present carrying out fractionation experiments on the meal of the resistant cowpea seeds and testing the resulting fractions in an artificial seed system in order to separate and characterize the toxic factor or factors that seem to be mostly distributed in the globulin fraction.

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Registry No. Proteinase inhibitor, 37205-61-1; trypsin inhibitor, 9035-81-8; chymotrypsin, 9004-07-3; subtilisin BPN′, 9014-01-1; papain, 9001-73-4.

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Isolation and Identification of 5-Hydroxyindole-3-acetic Acid and 5-Hydroxytryptophan, Major Allelopathic Aglycons in Quackgrass (*Agropyron repens* L. Beauv.)

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The compounds in quackgrass (*Agropyron repens* L. Beauv.) responsible for inhibiting growth of other plants have been identified as 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophan (5-HTP). Identification was accomplished by use of TLC, HPLC, MS, UV, IR, and C,H,N analysis. These compounds accumulate to high levels, throughout the plant, as glucosides attached to the 5-*O*-indolyl moiety in β linkage. 5-HTP is further protected as an N-glucoside on the primary amine. Molecular weights of the glucosides range from 353 [5-(β-D-glucopyranosyloxy)indole-3-acetic acid] to at least 4159. 5-HIAA serves as a growth hormone, and 5-HTP serves as a growth inhibitor in quackgrass. Examples of seedling growth inhibition of corn (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.) are given.

Quackgrass (*Agropyron repens* L. Beauv.), a highly competitive perennial grass weed, has been shown by a number of workers to contain compounds that act as germination and growth inhibitors toward other plants (Ohman and Kommedahl, 1964; Toai and Linscott, 1979; Weston and Putnam, 1986). Most workers have suggested that these compounds are released only from dead quackgrass. They also indicate that these compounds are not phytotoxic unless subjected to some further breakdown, particularly anaerobic degradation in soils (Ohman and Kommedahl, 1964; Toai and Linscott, 1979).

It has been suspected for a long time that plants excrete substances from their roots that are inhibitory to other plant species and may be autotoxic under continuous monoculture. Bonner (1950) reviewed progress in this field of study up through the 1940s. Since that time, a great deal of work has been done and the science of allelopathy has become established. Rice (1984) covered work up to the early 1980s and in his book adopted Molisch's definition of allelopathy, "to refer to biochemical interactions between all types of plants including microorganisms" (Rice, 1984, p 1). Allelopathy refers to stimulatory as well as detrimental effects. This definition of allelopathy will be used in this paper.

Prior to 1987, a number of attempts were made to identify the specific chemicals responsible for the allelopathic effects observed for quackgrass. Although the compounds were not identified, a number of important properties of the compounds were deduced. Notable among these efforts were those of LeFevre and Clagett (1960) and, more recently, Gabor and Veatch (1981). Sikkema and Dekker (1987) noted that although a number of researchers have extracted allelopathic substances from quackgrass, identification of specific compounds and determination of their possible relevance to field observations have not yet been accomplished. They also suggested that there were at least two compounds active in quackgrass, one present throughout the plant suppressing seedling growth (Ohman and Kommedahl, 1960; Gabor and Veatch, 1981) and a second compound, present only in quackgrass leaves, acting as a seed germination inhibitor (Ohman and Kommedahl, 1960). Weston et al. (1987) isolated two flavonoid compounds from dried quackgrass leaves and rhizomes that inhibited radicle elongation in cress *Lepidium sativum* L. Burpee curly. They identified one of these flavonoids as the flavone tricin.

Harborne and Hall (1964) indicated that, while of limited distribution in the plant kingdom, "tricin, in combined form, is a characteristic grass flavone". They also con-

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