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Biochemical Basis of Resistance in Rice Bean, *Vigna umbellata* Thunb. (Ohwi and Ohashi) Against *Callosobruchus maculatus* F.

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Abstract: Nineteen accessions of the wild plants including rice bean viz., *Vigna umbellata* Thunb. (Ohwi and Ohashi), a wild relative of the genus *Vigna* and other species belonging to *Vigna* were screened for their relative resistance to the bruchid, *Callosobruchus maculatus* F., a serious pest of stored pulses. The results showed that, accessions of *V. umbellata* were found to arrest the growth and development of *C. maculatus*, at grub stages in varying levels. Morphological characters like seed coat thickness and seed hardness were not found to be responsible for offering resistance to *C. maculatus*. The death of the grubs at the cotyledons was mainly due to the antinutritional factors in the cotyledons. Among the various biochemicals analyzed, the trypsin inhibitors in the resistant accessions were in the order of 3-5 times higher (1576.12 to 3120.08 TIU g^{-1}) than in the susceptible check (682.09 TIU g^{-1}). Similarly the cysteine protease inhibitors were 7 times higher in the resistant accessions (2061.78 to 4923.62 CPIU g^{-1}) compared to the susceptible CO 6 (686.77 CPIU g^{-1}). The protein profile also showed the presence of low molecular weight proteins in the range of 14-29 kDa in the resistant accessions. The correlation between different antinutritional factors with that of index of suitability also showed a negative relationship. The role of these antinutritionals in relation to the *C. maculatus* infestation is discussed.

Key words: *Vigna umbellata*, resistance, *Callosobruchus maculatus*, biochemical factors

INTRODUCTION

Pulses constitute a major source of protein for the people living in developing countries like India, where the per capita consumption of animal protein is relatively low. These pulses are referred to as poor man's meat and rich man's vegetable (Singh and Singh, 1992). Wide array of pests including *Helicoverpa armigera* Hubner, *Maruca vitrata* Geyer, *Clavigralla gibbosa* Spinola, *Melanagromyza obtusa* Malloch, *Mylabris* sp. etc. attack the crop during vegetative and reproductive stages (Durairaj, 1999). However, the bruchid, *Callosobruchus maculatus* Fab. which infest the seeds at storage assume special significance as it is a very serious pest causing up to 100% storage loss. Infestation of these insects starts in the field and continues in storage where sometimes it causes total destruction of the seeds within a period of 3-4 months (Dongre *et al*., 1996; Hall *et al*., 1997; Sarikarin *et al*., 1999). The estimated losses due to bruchids in various pulses ranged from 30-40% within a period of six months and the post harvest seed losses due to bruchids can reach even 100% during severe periods of infestation (Mahendran and Mohan, 2002).

The present management options of physical methods do not provide the expected levels of control. Insecticides though effective, cause other problems including residual effects and are carried

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through the food chain leading to disastrous consequences. Thus, enormous focus is being given to host plant resistance in recent years. The wild plants belonging to the genus *Vigna* are a potent source of resistance as evidenced by several authors. Marconi *et al*. (1997) observed several species of *Vigna* including, *V. vexillata*, *V. reticulata*, *V. luteola*, etc. as resistant to bruchids. The antinutritional factors including trypsin inhibitors, tannins and phytic acid were found to be higher in these species. Ignacimuthu *et al*. (2000) have also stressed the importance of protease inhibitors including trypsin and chymotrypsin inhibitors in conferring resistance against *C. maculatus*.

However, more thrust is yet to be given for the utilization of wild relatives of mungbean for the management of bruchids. Earlier report showed that, an accession TC 1966 of *Vigna radiata* var. *sublobata* was found to be completely resistant to *Callosobruchus chinensis* (Fujii and Miyazaki, 1987). Identification of resistant donors from non-edible leguminous tree seeds like and *Vigna bourneae* (Gamble) was attempted by Ignacimuthu *et al*. (2000). In view of the various reports available for the presence of resistant sources in the wild plants, an attempt has been made to assess the level of resistance in *Vigna umbellata* to *C. maculatus* and the possible biochemical factors responsible for the same.

MATERIALS AND METHODS

Rearing for Insects

The bruchid species, *C. maculatus* used for this work has been obtained from a culture maintained continuously at the Biocontrol Unit of Tamil Nadu Agricultural University, Coimbatore following the procedure of Strong *et al*. (1968). Permanent culture of beetles was established on *V. radiata* (cv. CO 6) and once in a month subculturing was done in order to maintain a continuous culture. The insects were maintained at a temperature of 30±5°C and 70±5% r.h. throughout the period of study.

Seeds

Seventeen accessions of *V. umbellata*, a wild *Vigna* species, *Vigna glabrescens* and a cross, VRM Gg1 × *V. sublobata* were obtained from TNAU, Coimbatore and raised for a season to obtain sufficient seed lot for the experiment. A cultivar, CO 6 was used as check for the entire study.

Bruchid Development on Different Accessions

To measure the performance of *C. maculatus* on different accessions of *V. umbellata*, three replicates of 25 seeds of each accession was used. Three pairs of freshly emerged adults of *C. maculatus* were released into a polypropylene envelope containing the test seeds. The insects were allowed for oviposition for 72 h under no choice conditions (Gibson and Raina, 1972). After that period, the insects were removed and the number of eggs laid on each accession was recorded. The seeds were left undisturbed for a period of about 60 days and the number of adults emerging during this period was recorded on a daily basis for calculating the per cent survival to adult emergence (S), mean developmental period (T) and index of suitability (I).

Biophysical Parameters

The biophysical parameters viz., hundred seed weight, seed coat thickness and seed hardness of seeds of different accessions were recorded (Table 1) for assessing the presence of antixenotic mechanisms, if any.

Biochemical Analysis

Trypsin and chymotrypsin inhibitory activity was measured following the procedure of Kakade *et al*. (1969) and the cysteine protease inhibitory activity was measured following the

Table 1: Biophysical characteristics of wild *Vigna* species

Wild *Vigna* species	100 seed weight (g)	Seed coat thickness (µm)	Seed hardness (kg)
V. umbellata-LRB 40-1	7.51	58.48	9.67
LRB 85	5.60	52.63	10.67
LRB 104	6.59	64.33	10.33
LRB 105	6.06	58.48	15.33
LRB 111	4.89	70.18	14.33
LRB 112	6.52	52.63	12.67
LRB 113	5.73	64.33	15.67
LRB 173	7.26	64.33	9.33
LRB 282	6.14	64.33	14.00
LRB 285	5.53	52.63	13.00
LRB 287	6.13	58.48	15.67
LRB 292	6.58	64.33	11.33
LRB 293	5.74	58.48	13.00
LRB 296	6.63	52.63[b]	12.67
LRB 297	7.18	46.78	15.33
TNAU-UMG	4.94	46.78	8.33
TNAU-UMY	8.14	52.63	16.67
V. glabrescens	2.36	64.33	4.33
VRM Gg1x *V. sublobata*	5.68	64.33	7.67
CO 6	3.21	40.94	5.67

procedure of Barrett (1981). They were expressed in terms of inhibitory units/g of seed materials. One unit of activity corresponds to that amount of trypsin/cysteine protease/chymotrypsin inhibitor which gives 50% inhibition of enzyme activity under experimental conditions. The inhibitory activity was expressed as trypsin/chymotrypsin/cysteine protease inhibitor units (TIU/CIU/CPIU) per g of sample.

Seed Protein Profile

To analyse proteins of promising *Vigna* species, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli *et al*. (1970). Proteins were extracted from the seeds with 0.1 M Tris-HCl buffer, pH 8.0 in a prechilled pestle and mortar for 15 min at 0-4°C. The extracts were centrifuged at 15000 g for 10 min at 4°C. The protein concentration in the extracts was adjusted in each well using five fold sample buffer and water in such a way that the same amount of protein was present per unit volume. Standard medium range molecular markers (Genei, Bangalore, India) were simultaneously run for comparison.

Statistical Analysis

All data were analyzed using the AGRES package and the level of significance was tested at $p = 0.05$. Correlation of different parameters was done using SPSS package.

RESULTS

The data on oviposition, adult emergence and developmental period showed significant differences. TNAU-UMY recorded the least number of eggs (11.67) and LRB 113 the maximum number (63.33) while the control CO 6 registered 33.33 eggs (Table 2). With regard to adult emergence on the 19 accessions tested, 15 accessions showed no adult emergence, the exceptions being LRB 113, LRB 296 and *V. glabrescens*. The percentage survival was also considerably lesser in these accessions when compared to CO 6 (28.41%). Further, those accessions which exhibited lesser per cent survival encountered a prolonged developmental period. The delay in developmental period was much pronounced in LRB 296 (38.67 days) as against CO 6 (30.90 days). Ultimately the index of suitability was lowest in LRB 113 (0.006) and was followed by LRB 282 (0.0097), LRB 296 (0.0181) and LRB 297 (0.0261) and highest in the check variety CO 6 (0.0469).

Table 2: Biological performance of *Callosobruchus maculatus* on different species of wild *Vigna* species

Wild *Vigna* species		Mean±Standard error				
		Eggs laid (No.)	Adults emerged (No.)	Survival (%)	Mean developmental period (days)	Suitability index
V. umbellata-	LRB 40-1	21.33±0.88 d	0	0.00	0.00	0
	LRB 85	13.67±0.88 a	0	0.00	0.00	0
	LRB 104	15.00±0.58 ab	0	0.00	0.00	0
	LRB 105	43.33±3.93 fg	0	0.00	0.00	0
	LRB 111	50.00±1.73 gh	0	0.00	0.00	0
	LRB 112	35.00±1.53 e	0	0.00	0.00	0
	LRB 113	63.33±4.06 I	1.00±0.00	1.59±0.07 a	33.33±0.33 c	0.0060
	LRB 173	21.33±2.03 d	0	0.00	0.00	0
	LRB 282	48.67±3.71 gh	1.00±0.00	2.08±0.06 a	32.33±0.33 d	0.0097
	LRB 285	13.33±2.33 a	0	0.00	0.00	0
	LRB 287	12.00±1.15 a	0	0.00	0.00	0
	LRB 292	57.00±3.61 hi	0	0.00	0.00	0
	LRB 293	15.00±3.21 a	0	0.00	0.00	0
	LRB 296	20.33±2.91 bcd	1.00±0.00	5.14±0.34 b	38.67±0.33 a	0.0181
	LRB 297	21.33±2.60 cd	0	0.00	0.00	0
	TNAU-UMG	37.00±1.53 ef	0	0.00	0.00	0
	TNAU-UMY	11.67±1.76 a	0	0.00	0.00	0
V. glabrescens		33.33±1.76 e	8.67±0.33	26.18±0.77 d	32.12±0.51 d	0.0441
VRM Gg1x *V. sublobata*		48.33±3.84 gh	0	0.00	0.00	0
CO 6		33.33±2.91 e	9.33±0.33	28.41±2.45 d	30.90±0.12 e	0.0469

In a column, means followed by a common letter(s) are not significantly different by LSD (p = 0.05)

Table 3: Quantity of various antinutritional factors in the accessions of *Vigna umbellata*

Wild *Vigna* species		Mean±Standard error		
		Protease inhibitors (Units/g)		
		Trypsin inhibitors	Cysteine protease inhibitors	Chymotrypsin inhibitors
V. umbellata-	LRB 40-1	1987.61±13.20 f	2205.42±05.85 hi	456.87±14.66 ghi
	LRB 85	2213.50±16.00 d	2677.69±12.66 cde	583.25±12.16 def
	LRB 104	2405.18±07.34 bc	2517.80±02.93 defg	481.86±03.52 gh
	LRB 105	3120.08±06.41 a	2494.37±17.27 defg	641.32±09.42 cde
	LRB 111	1930.79±13.70 fg	2061.78±09.11 I	431.32±03.07 hi
	LRB 112	1831.02±11.92 hi	2397.37±16.12 gh	557.00±06.04 f
	LRB 113	2349.52±09.45 c	2713.90±07.75 cd	487.61±18.98 g
	LRB 173	2421.58±11.56 bc	3069.61±19.92 b	735.53±14.70 b
	LRB 282	1803.82±10.28 hi	2197.56±02.39 I	451.02±06.79 ghi
	LRB 285	1792.18±16.06 hi	2437.92±13.44 fg	715.47±02.20 bc
	LRB 287	1853.75±05.51 gh	2581.45±20.63 defg	557.19±06.40 f
	LRB 292	1576.12±07.28 l	4923.62±07.72 a	883.35±13.67 a
	LRB 293	2194.76±04.48 de	2475.13±04.38 efg	380.17±03.54 j
	LRB 296	1760.71±14.59 ij	3028.86±11.36 b	659.71±15.68 cd
	LRB 297	1681.92±09.94 jk	2508.90±19.15 efg	574.54±05.57 ef
	TNAU-UMG	2130.47±03.66 de	2419.80±18.67 fg	408.98±01.24 ij
	TNAU-UMY	2513.20±10.05 b	2835.80±01.66 bc	467.89±01.51 gh
V. glabrescens		1648.07±17.48 kl	2152.64±13.91 I	591.67±15.20 def
VRM Gg1x *V. sublobata*		2090.73±10.20 e	2629.11±18.83 cdef	547.96±03.56 f
CO 6		682.09±14.18 m	686.77±07.24 j	314.56±13.04 k

In a column, means followed by a common letter(s) are not significantly different by LSD (p = 0.05), Values represent mean of three replications; NS = Non significant

Following these bioassay studies biochemical analyses were made to further study the reason underlying low rates of survival in the wild accessions when compared to CO 6. Analyses of biochemical constituents in the wild *Vigna* accessions revealed that the protease inhibitors viz., trypsin, cysteine protease and chymotrypsin inhibitors were relatively higher in the wild *Vigna* species (Table 3). Among the accessions, significantly higher levels of trypsin inhibitor was noticed in LRB 105 (3120.08 TIU g^{-1}) while a least quantity of 1648.07 TIU g^{-1} was noticed in *V. glabrescens*.

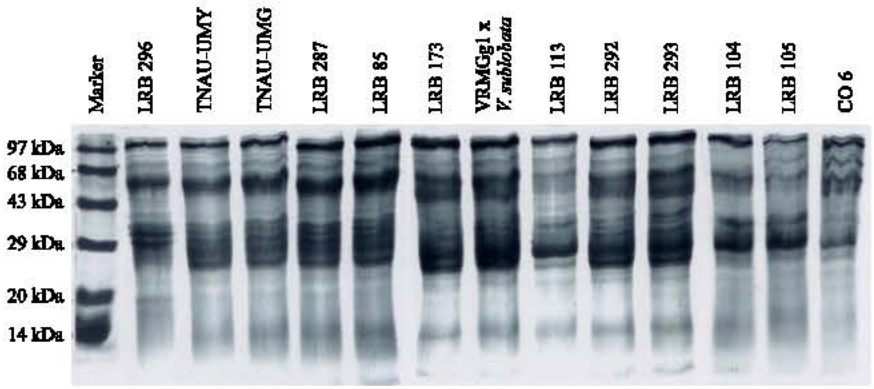

Fig. 1: SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) profile of promising accessions of wild *Vigna* species)

However the quantity of trypsin inhibitors was considerably lower in CO 6 (682.09 TIU g^{-1}). Similar was the case with respect to cysteine protease inhibitors. The accession LRB 292 registered the maximum quantity of cysteine protease inhibitors (4923.62 CPIU g^{-1}) which corresponds to a seven times higher quantity than CO 6 (686.77 CPIU g^{-1}). Even LRB 111 which registered the least quantity of cysteine protease inhibitor among the accessions (2061.78 CPIU g^{-1}) had three times higher quantity than CO 6. With respect to chymotrypsin inhibitors also, LRB 292 showed a maximum quantity of 883.35 CIU g^{-1} which was 2.8 times higher than CO 6 (314.56 CIU g^{-1}).

The SDS-PAGE protein profile of the wild *Vigna* accessions revealed a moderate banding pattern in the lower molecular weight region (Fig. 1). A polypeptide band corresponding to an apparent weight of 14 kDa could be noticed in all the wild *Vigna* species except the check CO 6. The region between 14 and 29 kDa exhibited similar banding patterns in all the wild accessions while CO 6 was devoid of such a pattern. Further an intense banding corresponding to a molecular weight of 29 kDa was observed in all the wild *Vigna* species while CO 6 exhibited a less intense banding in these regions.

DISCUSSION

All the wild *Vigna* species used in the present study were preferred for oviposition by *C. maculatus*. However except four accessions (LRB 113, LRB 282, LRB 296 and *V. glabrescens*) all the wild accessions failed to support the emergence of adults. Even these four accessions were found to delay the developmental period of *C. maculatus*. The developmental period of *C. maculatus* in LRB 113, LRB 282, LRB 296 and *V. glabrescens* was 33.33, 32.33, 38.67 and 32.12 days, respectively. On the other hand *C. maculatus* grubs completed their life cycle in a relatively shorter span of 30.90 days in CO 6.

The biophysical factors like 100 seed weight and seed hardness had a highly significant and negative relationship with that of index of suitability (r = -0.62 and -0.47, respectively) (Table 4). However, these factors might not have contributed to the resistance in these seeds. Because, the seeds of *V. umbellata* were heavier than the green gram seeds and were having sufficient resources for a growing grub. Moreover, if seed hardness had a role, the insects should have had difficulty in penetrating the cotyledons. Thus, the assumption by Southgate (1979) that size and hardness of the seeds influencing the adult emergence does not hold good. The seed coat thickness also cannot be considered as factors conferring resistance as the grubs penetrated and reached the cotyledons in all the cases. Thus, the death of the grubs in the cotyledons might be due to the presence of toxic substances

Table 4: Correlation of biophysical and biochemical parameters to index of suitability

Parameter	r	R^2	Regression line
Seed weight to index of suitability	-0.62**	0.39	Y = -57.304x + 6.3534
Seed hardness to index of suitability	-0.47**	0.22	Y = -111.46x + 12.624
Seed coat thickness to index of suitability	-0.35^{NS}	0.12	Y = -181.36x + 58.971
Trypsin inhibitor to index of suitability	-0.66**	0.44	Y = -0.00002x + 0.0481
Cysteine protease inhibitor to index of suitability	-0.48*	0.23	Y = -0.00001x + 0.0319
Chymotrypsin inhibitor to index of suitability	-0.19^{NS}	0.04	Y = -0.00002x + 0.0191

*Significant at p = 0.05, **Significant at p = 0.01, ^{NS}Non significant

present in the seeds. Earlier, Kashiwaba *et al*. (2003) opined that several accessions of *V. umbellata* completely inhibited the emergence of *C. maculatus* which support the present investigations. The cross, VRM Gg1 × *V. sublobata* showed complete resistance to *C. maculatus* though there was egg laying. An accession, TC 1966 of *V. radiata* var. *sublobata* was also found to be immune to *C. maculatus* (Fujii and Miyazaki, 1987).

Thus, the presence of antinutritional factors in the cotyledons of these resistant accessions is confirmed by the fact that all the wild *Vigna* seeds permitted penetration by the grubs, but failed to support a normal adult emergence Kashiwaba *et al*. (2003) witnessed mortality of first and second instar larva inside cotyledons of *V. umbellata*. Earlier Lale and Makoshi (2000) also observed larval mortality of *C. maculatus* in the seed coats of certain cowpea cultivars, when first instar grubs were trying to gain entry. Thus from the above discussions it is obvious that the only possible reason that could be attributed to the non-emergence of adults is the presence of toxins in the cotyledons. The findings of Kashiwaba *et al*. (2003) also reflected the above inference when they used artificial beans made of *V. umbellata* flour and found decreased adult emergence and prolonged mean developmental period of *C. maculatus*. Furthermore, the biochemical constituents having a negative correlation with that of index of suitability in the present studies supported the possible role of antinutritional factors for the non emergence of *C. maculatus* adults.

Significantly higher quantities of protease inhibitors was noticed in all the tested accessions when compared to CO 6. The role of protease inhibitors had been well documented by Shulke and Murdock (1983) who reasoned out that, protease inhibitors were capable of inhibiting the activity of digestive enzymes and reduced the quantity of proteins that can be digested. This leads to insufficient utilization of proteins by the insect and as a result the insect become weak with stunted growth and ultimately die.

Among the protease inhibitors, the trypsin inhibitor content in the wild *Vigna* species was considerably higher (1576.12 to 3120.08 TIU g^{-1}) than in the susceptible check, CO 6 (682.09 TIU g^{-1}). Moreover the trypsin inhibitor content had a highly significant and negative relationship at 1% level (r = -0.66**) stressing the importance of trypsin inhibitor as a potent antinutritional factor (Table 4). Saikia *et al*. (1999) reported a range of 2456 to 2534 TIU g^{-1} in different *Vigna umbellata* cultivars which backs the present findings. However, the role of trypsin inhibitor in bruchid resistance has been contradicted by Janzen *et al*. (1977) who observed that the diets incorporated with trypsin inhibitors were found to have little or no effect on *C. maculatus*. The accessions LRB 292 had higher amount of cysteine protease inhibitor and chymotrypsin inhibitor with low levels of trypsin inhibitor. The cysteine protease inhibitor also had a significant and negative relationship at 5% level with that of index of suitability (r = -0.48*). Hence, the resistance in this accession might have been governed by the trypsin and cysteine protease inhibitors alone. The role of cysteine protease inhibitor in conferring resistance to *C. maculatus* had been documented by many authors. Hines *et al*. (1991) observed that the soybean cysteine protease inhibitors inhibited the proteolytic activity of the gut extracts of *C. maculatus*. Koiwa *et al*. (1998) also observed that when soybean cysteine protease inhibitors were incorporated into a diet of *C. maculatus*, there was a prolonged developmental period and increased mortality in cowpea. Indepth investigations by Ryan

(1989) revealed that, the bruchids have cysteine protease as the predominant digestive enzymes and hence the inhibition of these enzymes would be of greater importance in conferring resistance against *C. maculatus*. Moreover the cysteine protease inhibitors had a significant and negative correlation with that of index of suitability, which affirms that cysteine protease inhibitors had an active role as an antinutritional factor. The amount of chymotrypsin inhibitors in wild *Vigna* species was found to be generally lower than that of trypsin inhibitors. A similar phenomenon was observed in non edible legumes by Ignacimuthu *et al*. (2000).

SDS-PAGE profile done for promising wild *Vigna* species also elucidated the role of proteinaceous inhibitors in imparting resistance to *C. maculatus*. Most of the workers attribute these inhibitors as proteins with a molecular weight of 14 to 29 kDa. In the present studies too, banding patterns were observed in the regions between 14 and 29 kDa which might be corresponding to these protease inhibitors. Oliveira *et al*. (1999) observed the presence of trypsin inhibitors with a molecular weight of 28 kDa that were responsible for bruchid resistance in *Canavalia ensiformis*. The bands corresponding to 14 kDa in the present findings may represent some form of cysteine protease inhibitors as reported by Brzin *et al*. (1998). They also observed a banding pattern for cysteine protease inhibitors at 27 kDa and attributed this subunit to a dimer of cysteine protease inhibitor, which was more stable than the monomer (14 kDa). Thus the intense banding at 27 kDa in the present study may correspond to cysteine protease inhibitor. This is further confirmed by the absence of the above-mentioned bands in CO 6.

Thus the present studies provide clear cut evidence on the role of trypsin and cysteine protease inhibitors to a greater extent along with chymotrypsin inhibitors in conferring resistance in *V umbellata* to *C. maculatus*. The search for genes from the wild is gaining momentum in the recent years as there is a possibility of incorporating such genes into cultivated varieties to make the latter resistant to crop pests. Thus, the present study emphasize the availability of resistant genes in the wild *Vigna* species which can be used in conventional breeding programmes as well as molecular approaches to evolve resistant cultivars of crop plants.

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