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Effects of plant lectin from cobra lily, *Arisaema curvatum* Kunth on development of melon fruit fly, *Bactrocera cucurbitae* (Coq.)

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Abstract: *The lectin from tubers of cobra lily, Arisaema curvatum Kunth was purified by affinity chromatography using asialofetuin-linked amino activated porous silica beads. The concentration dependent effect of lectin was studied on second instar larvae (64-72 hr) of Bactrocera cucurbitae (Coq.). The treatment not only resulted in a significant reduction in the percentage pupation and emergence of the adults from treated larvae but it also prolonged the remaining larval development period. A very low LC_{50} value, 39 mgl^{-1} of lectin was obtained on the basis of adult emergence using probit analysis. The activity of three hydrolase enzymes (esterases, acid and alkaline phosphatases), one oxidoreductase (catalase) and one group transfer enzyme (GSTs: Glutathione S-transferases) was assayed in second instar larvae under the influence of the LC_{50} of lectin at increasing exposure intervals (0, 24, 48 and 72 hr). The Arisaema curvatum lectin significantly decreased the activity of all the enzymes except for esterases, where the activity increased as compared to control at all exposure intervals. The decrease in pupation and emergence as well as significant suppression in the activities of two hydrolases, one oxidoreductase and one GST enzyme in treated larvae of B. cucurbitae indicated that this lectin has anti-metabolic effect on the melon fruit fly larvae.*

Key words: *Arisaema curvatum, Bactrocera cucurbitae, Catalase, Esterases, Lectin, Phosphatase, Transferase*
PDF of full length paper is available with author ***(*singhk24@yahoo.co.in)***

Introduction

Advancement in genetic engineering and molecular biology has helped mankind to develop and manage novel crop genotypes towards a safe and sustainable agriculture in order to circumvent a plethora of environment related problems which had cropped up due to excessive and intensive use of organic pesticides. A large number of bio-molecules (proteinase inhibitors, α - amylase inhibitors, lectins *etc.*) which exercise insecticidal activity against economically important pests and are nontoxic to man, beneficial insects and other organisms have been attracting the attention of the researchers (Ranjekar *et al.,* 2003). Already some transgenic plants expressing these insecticidal proteins have been produced with promising results against some pest species (Gatehouse *et al.,* 1999; Wang *et al.,* 2005). Lectins are plant proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono or oligosaccharide thus conferring the molecule with the ability to agglutinate specific carbohydrate coated cell surfaces (Goldstein and Portez, 1986; Peumans and Van Damme, 1995). Most important function performed by lectin in the plant is the storage and in addition, they are also employed by the plants in their defensive arsenal. The lectins are present in higher concentration in the seeds of the plants but they are also widely distributed in other parts such as leaves, bulbs, tubers, barks *etc.* at lower concentrations. Many edible plants used as foodstuffs by human beings and animals contain lectins and the toxicity of these lectins to mammals has already been studied in considerable detail. Evidence about the toxicity of a number of plant lectins to phytophagous insects is available in literature (Peumans and Van Damme, 1995) and the potential role of some of these in insect control has been assessed mainly through insect feeding experiments utilizing artificial diets or seeds containing lectin preparations or by topical application. The majority of the artificial diet bioassays utilizing the plant lectins have been limited to the lepidopteran (Fitches and Gatehouse, 1998; Powell, 2001; Setamou *et al.,* 2003) and coleopteran pests (Bandyopadhyay *et al*., 2001; Macedo *et al.,* 2002). However, only a little work has been done that pertains to the study of anti-insect activities of plant lectins against dipteran pests which also comprises economically important group of insect pests with their own specific life cycle, morphology and behavior.

Already, we have screened anti-insect activity of lectins extracted from seeds of some plants through artificial diet bioassays and found that incorporation of lectin in artificial diet significantly affected percentage pupation and adult emergence of the melon fruit fly, *Bactrocera cucurbitae* (Coq.) (Singh *et al.,* 2006a; Kaur *et al.,* 2006a). These are the first reports about the use of lectins as an insecticide on diptern pest, *B. cucurbitae*. The melon fruit fly is one of the most important insect pest of cucurbits, which has defied the conventional control measures with organic pesticides and in some years its damage reaches upto 100% especially after the first shower of monsoon (Gupta *et al.,* 1978; Dhillon *et al*., 2005 a,b). The damage is mostly caused by the larvae of the female fly as they feed inside the fruits. After egg hatching, the larvae bore into the pulp tissue of fruit by making the feeding galleries. Young larvae usually leave the necrotic region and move to healthy tissue, where they often introduce various pathogens and hasten fruit decomposition and distortion (Dhillon *et al*., 2005b).

In the present study, the influence of lectin extracted from tubers of cobra lily, *Arisaema curvatum* Kunth on the growth and development of *B. cucurbitae* has been explored. *A. curvatum* lectin (ACL) has already been extracted, purified and characterized by Shangary *et al.* (1995) and methodology has been standardized. To our knowledge the anti-insect activity of lectin extracted from this plant has not been explored so far against any insect.

Materials and Methods

Plant material: Tubers of cobra lily (*A. curvatum*) plant used for extraction and purification of lectin were collected from the Kumaon Hills of Nainital situated in the Western range of Himalayas at altitude ascending upto 2300 metres. The collected tubers were identified with the help from Department of Botany, Kumaon University, Nainital, Uttarakhand State.

Extraction, isolation and purification of lectin from tubers of *A. curvatum*: The ACL was purified by the method of Shangary *et al.* (1995). Tubers were washed, peeled, chopped immediately after collection and soaked overnight in 0.01M phosphate buffered saline (PBS) pH 7.2 (1:5 w/v) at 4°C. After centrifugation at 20,000 rpm for 30 min, the clear supernatant obtained was dialyzed against 0.01 M PBS, pH 7.2 at 4°C to remove any low molecular weight substances, which may interfere in lectin activity. The dialyzed crude extract was applied to affinity column of asialofetuin-linked amino activated porous silica beads (pore size: 1000 A°, diameter: 100 µ) equilibrated with 0.01M PBS, pH 7.2. The bound lectin was eluted with 0.1 M Glycine–HCl buffer, pH 2.5 and the eluted fractions were neutralized immediately with 2 M Tris–HCl buffer, pH 8.8.

Hemagglutination assay: In order to check the activity of lectin rich fractions out of the total eluted fractions hemagglutination assay was done. Microtitre plate of polystyrene having U-shaped 96 wells was used to perform this assay. Thirty µl of 2% rabbit erythrocytes suspension was dispensed in each well containing the same amount of test lectin. The plate was incubated for 1hr at 37°C and the agglutination was observed with naked eye (Kaur *et al.,* 2002). The pooled active fractions were extensively dialyzed against 0.01 M PBS at 4°C in order to bring the purified lectin in physiological buffer and to remove Tris ions, which interfere in protein estimation.

SDS-PAGE: SDS-PAGE at pH 8.3 was prepared by the method of Laemmli (1970) to ascertain the purity of affinity purified lectin preparation.

Protein estimation: Protein concentration was determined in crude and purified lectin preparations by the method of Lowry *et al.* (1951) using bovine serum albumen as standard before preparing various test concentrations for performing experiments with eggs and larvae.

Insect culture: The standardized procedure described by Gupta *et al.* (1978) was followed for rearing the stock cultures of melon fruit flies and wire mesh cages (Rescholar equipment; L 45 x B 45 x H 50 cm) were used for confining the flies. The adult flies were provided with pieces of pumpkin fruit, *Cucurbitae moschata* Dusch for oviposition along with proteinex (Pfizer India) and 20% sugar solution as food. The stock culture of fruit flies were maintained in insect culture room with regulated temperature (25 ± 2°C), relative humidity (70-80%) and photophase (10:14 LD).

Experiments with larvae: About 100 gravid females were released in mesh cages having fresh pumpkin pieces for 8 hr and the egg charged pumpkin pieces were dissected in saline water after 64 hr of the removal of the fruit flies, for harvesting the larvae. The harvested larvae were shifted to culture vials (25 mm D x100 mm L) containing artificial treated diet (Srivastava, 1975) of various concentrations (0, 6.25, 12.50, 25, 50, 100 µg ml^{-1}) of ACL. The experimental vials were kept in culture room and observed daily for various parameters such as development period, pupation and adult emergence. There were six replications with 20 larvae in each replication for each concentration.The experiments were repeated twice and the mean of the two experiments was used for analysis.

Biochemical analysis: The second instar (64-72 hr) larvae were released on both the control and artificial treated diets (Srivastava, 1975) for periods of 24, 48 and 72 hr. The larvae were harvested after specified treatment period and were assayed for activity of five enzymes involved in digestion, development, growth, metamorphosis and detoxification. The estimations of various enzymes were done on the fresh weight basis by taking about 10 larvae for preparing the required concentration of homogenate. There were six replications for each experiment. The procedure of Katzenellenbogen and Kafatos (1971) was used for extraction and measuring esterase activity, using a 1% homogenate prepared in 0.1M sodium phosphate buffer (pH 6.5) and 1mM α-naphthyl acetate was used as substrate. Catalase activity was estimated according to the protocol given by Bergmeyer (1974), and 0.05M potassium phosphate buffer (pH 7.0) was used for preparing 5% homogenates and 0.05% solution of hydrogen peroxide (30%) was taken as substrate. Phosphatases (acid and alkaline) activity was determined by the method of Mc Intyre (1971) with 2% homogenates prepared in 0.05M sodium acetate buffer (pH 5.0) and 0.05M tris buffer (pH 8.0) for acid and alkaline phosphatases, respectively. Sodium α-naphthyl phosphate (0.5 mM) was used as substrate for both acid and alkaline phosphatses. The 2% homogenates were prepared in 0.1M sodium phosphate buffer (pH 7.6) for the estimation of glutathione S-transferases activity by following the methodology of Chein and Dauterman (1991). Distilled water was used for preparing 50 mM reduced glutathione (GSH) as substrate.

Statistical analysis: The data were analysed for analysis of variance (ANOVA), using SPSS statistical analysis program. The LC_{50} for adult emergence was calculated using probit analysis in SPSS statistical software.

Results and Discussion

A single band was obtained in SDS-PAGE at pH 8.3 which assured the purity of the affinity purified lectin preparation from cobra lily, *A. curvatum* (Fig. 1). The bioassay of cobra lily lectin with second

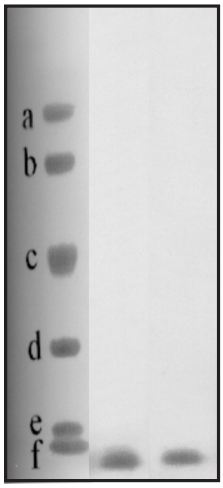

Fig. 1: SDS-PAGE of ACL (lane A) at pH 8.3 in the presence of 2% b-mercaptoethanol (running time 4 hr at a constant 150 V). 40 mg protein was loaded. Molecular mass markers (lane M) from top to bottom are: a - phosphorylase b (94 kDa),b- albumin bovine (67 kDa), c - ovalbumin (45 kDa), d - carbonic anhydrase (30 kDA), e - trypsin inhibitor (20.1 kDa) and f– α-lactalbumin (14.4 kDa)

instar larvae (64-72 hr old) resulted in significant reduction in percentage pupation, adult emergence (Table 1) and also significantly prolonged the total developmental duration (Table 2). There was significant and positive correlation between increase in concentration and decrease in pupation (r^2 = 0.9525 at p < 0.01) and adult emergence (r^2 = 0.9637 at p = 0.01). The pupation was reduced to 28.33% of control at 100 mg l^{-1} concentrations. The emergence of melon fruit fly adults was 43% lower over the control at 50 mg l^{-1} dose, the LC_{50} was 39 mg l^{-1} . The rate of growth of the treated larvae decreased, as a result the total development duration got prolonged significantly by 2.69 days in the 100 mg l^{-1} treatment as compared to control (Table 2).

The activity of esterases enzymes which are usually involved in digestion and hydrolysis in metabolism, increased with the maturation of larvae from 72 hr age to 144 hr age during the normal course of development (Table 3). The increase in ACL activity against *B. cucurbitae* larvae was 1.5 times high (156.36 mM g^{-1}) over control (103.82 mM g^{-1}) after 72 hr of exposure (Table 3). The catalase enzyme showed a gradual increase in activity from 18.95 to 27.61 mM g^{-1} with the advancement in larval development in control wher as catalase activity in treated larvae showed a significant suppression (p<0.01) at all the three age groups assayed (Table 3). Among the phosphatases the activity of acid phosphatase continued to decrease from 72 hr to 144 hr of age during normal course of larval development. The treatment with ACL showed a similar trend of activity, but there was a significant suppression (p <0.01) compared to control at 120 hr and 144 hr time intervals (Table 3). The other phosphatase enzyme, alkaline phosphatase showed a slight but gradual decrease as the larval development progressed. The treatment with ACL showed a significant suppression (p <0.01) in the activity of alkaline phosphatase at 120 hr and 144 hr treatment durations as compared to their respective controls (Table3). The activity of GSTs showed a slow and gradual increase as the larvae matured during normal course of development in control but it was significantly suppressed (p <0.01) at all the three time intervals of lectin treatment compared to control. The suppression of GSTs activity was maximum after 24 hr of treatment (26.53 mM g^{-1}) as compared to that in control (34.27 mM g^{-1}).

The current study provides vital data regarding the deleterious effects of affinity purified monocot araceous lectin from *A. curvatum* delivered through artificial diet on growth, development and biochemical entities of melon fruit fly, *B. cucurbitae*. The mechanisms by means of which lectins exercise their toxic effects in insects are not clearly understood. In order to exert its deleterious

Table - 1: Survival of *B. cucurbitae* after treatment of second instar larvae with *Arisaema curvatum* lectin

Concentration (µg l^{-1})	Pupation (%) (mean ± S.E)	Adult emergence (%) (mean ± S.E)
Control	61.67±1.66	35.00±2.23
6.25	83.78±4.77	85.71±2.58
12.50	70.26±6.17	71.42±2.23
25	62.15±5.42	61.99±3.07
50	54.04±4.21	42.85±2.23
100	28.33±3.07	28.57±0.01
$LSD_{0.05}$	13.26**	7.16**
$r^2_{(df- 4)}$	0.9525**	0.9637**

** Significant at p<0.01 compared to control

Table - 2: Development period (days) of *B. cucurbitae* after treatment of second instar larvae with *Arisaema curvatum* lectin

Concentration (µg ml^{-1})	Larval period (mean ± S.E)	Pupal period (mean ± S.E)	Total development period (mean ± S.E)
Control	7.03 ± 0.24	7.05 ± 0.46	14.08 ± 0.56
6.25	7.42 ± 0.10	7.20 ± 0.11	14.62 ± 0.25
12.50	7.93 ± 0.10	7.25 ± 0.60	15.18 ± 0.37
25	8.35 ± 0.05	7.40 ± 0.70	15.75 ± 0.35
50	8.85 ± 0.07	7.54 ± 0.29	16.39 ± 0.29
100	9.33 ± 0.08	7.43 ± 0.25	16.77 ± 0.36
$LSD_{0.05}$	0.37**	0.20**	0.35**

** Significant at p<0.01

Table - 3: The influence of *Arisaema curvatum* lectin (39 µg ml^{-1}) on activity of various enzymes of second instar larvae (64-72 hr) of *Bactrocera cucurbitae*

Enzymes	Status	Enzymatic activity (mM g^{-1}) at different time intervals (age of larvae) of treatment						
		0 hr (64-72 hr)	"t" value	24 hr (88-96 hr)	"t" value	48 hr (112-120 hr)	"t" value	72 hr (136-144 hr)
Esterase	Control	103.82 ±1.82	4.89**	123.40 ± 3.57	1.78$^{N.S.}$	134.54 ± 9.75	2.10$^{N.S.}$	156.36 ± 3.62
	"t"value			1.07$^{N.S.}$		0.72$^{N.S.}$		3.83**
	Treated	103.82 ± 1.82	2.74*	114.54 ± 3.47	6.09**	141.81 ± 2.84	16.55**	180.68 ± 5.21
Catalase	Control	18.95 ± 0.30	6.36**	21.46 ± 0.26	4.50**	23.28 ± 0.31	9.46**	27.61 ± 0.33
	"t"value			10.70**		8.48**		5.58**
	Treated	18.95 ± 0.30	4.68**	16.78 ± 0.35	4.92**	19.25 ± 0.36	5.76**	23.53 ± 0.65
Acid phosphatase	Control	56.77 ± 1.03	12.05**	40.16 ± 0.92	4.94**	32.71 ± 1.19	1.35$^{N.S}$	30.61 ± 1.01
	"t"value			2.08$^{N.S.}$		3.92**		6.62**
	Treated	56.77 ± 1.03	13.11**	37.22 ± 1.08	7.70**	27.27 ± 0.71	4.83**	22.27 ± 0.76
Alkaline phosphatase	Control	41.09 ± 0.60	4.37**	36.03 ± 0.99	3.46**	31.76 ± 0.74	1.92$^{N.S}$	29.93 ± 0.61
	"t"value			1.74$^{N.S.}$		2.92*		7.72**
	Treated	41.09 ± 0.60	6.85**	33.72 ± 0.89	4.44**	29.20 ± 0.49	7.63**	23.69 ± 0.53
GSTs	Control	30.66 ± 0.24	7.96**	34.27 ± 0.39	5.00**	36.60 ± 0.26	2.45*	37.40 ± 0.19
	"t"value			14.19**		7.45**		7.71**
	Treated	30.66 ± 0.24	9.11**	26.53 ± 0.39	6.83**	30.37 ± 0.41	4.50**	33.27 ± 0.50

**Significant at p<0.01, *Significant at p< 0.05, NS = Non significant

effects on insects, a protein must be resistant to proteolytic degradation of the insect digestive enzymes and second it must show binding to various insect gut structures (Zhu-Salzman *et al*., 1998; Bandyopadhyay *et al.,* 2001; Macedo *et al*., 2000; Vasconcelos and Oliveira, 2004). In insects belonging to orders coleoptera, lepidoptera and diptera the lectins may bind to peritrophic membrane in the midgut region and result in reduction of intake and uptake of food. Lectins with different sugar specificiteis may bind to different glycoproteins of this membrane and block the bidirectional movement of the nutrient or prevent the formation of the membrane itself (Czapla and Lang, 1990; Macedo *et al*., 2000; Machuka *et al.,* 1999; Eisemann *et al*., 1994). Alternately it is proposed that the lectin molecules might also enter the circulatory system of the insect to inflict systemic effects. For this they first need to bind to the receptors on the midgut epithelium to get transported to the haemolymph across the gut epithelium, resulting in subsequent systemic effects (Gatehouse and Gatchouse, 1998; Eisemann *et al.,*1994; Powell *et al*., 1998). The third proposed mode of action is that lectins may destabilize insect metabolism by interfering with gut enzymatic function by binding to glycosylated digestive enzymes in the gut. The activities of soluble and brush border membrane enzymes present in the midgut region of *Lacanobia oleracea* L. larvae were severely affected when they were chronically exposed to GNA (*Galanthus nivalis* agglutinin) and ConA (*Canavalia ensiformis* agglutinin). Thus, lectins upon binding to the gut may directly or indirectly affect the enzyme regulatory mechanisms of insects as a consequence of perturbation of the peritrophic matrix and/or brush border membrane environment (Fitches and Gatehouse, 1998). Among lectins which are having dimeric, trimeric or tetrameric structure only tetrameric lectins show more strong insecticidal effect due to their ability to bind strongly with complex glycoproteins (because of their multivalency) in the gut (Powell *et al.,* 1995, 1998). In the present study also, the asialofetuin binding tuber lectin extracted from *Arisaema curvatum* which is tetrameric in nature, having subunit molecular mass 12,900 daltons (Shangary *et al.,* 1995). Due to this it might have shown strong binding to the glycoproteins of the midgut region of the larvae and possibly had succeeded in blocking the bidirectional movement of the nutrients across the midgut membrane, leading to starvation and subsequently death of the larvae. In this way this lectin exercised antifeedant and toxic effect on larvae. The hypothesis gets evident support from the increase in the remaining development duration of the larvae (Table 2). Recently in our laboratory asialofetuin binding tetrameric lectins extracted from tubers of two other species of *Arisaema i.e. A. helleborifolium* Schot and *A. jacquemontii* Blume had shown high insecticidal effects against melon fruit fly by having 32 and 34 mg l^{-1} LC_{50} (Kaur *et al.,* 2006a, b).

There is hardly any report related to the influence of lectins on the enzyme system of the insects except for lectins from snowdrop, *Galanthus nivalis* L. and jackbean, *Canavalia ensiformis* DC. (Fitches and Gatehouse,1998) where it was observed that these lectins affected the activites of soluble and brush border membrane enzymes (α-glucosidase and alkaline phosphatase) in the midgut of *L. oleracea* larvae although the role of enzymes in development of resistance and in sequestration of xenobiotics has been well understood (Devorshak and Roe, 1999; David *et al.,* 2000). As there are a number of examples where the involvement of esterases in the metabolism of xenobiotics have been reported *e.g.* with Indole acetic acid (IAA) on banana fruit fly, *Zaprionus paravittiger* (Godbole and Vaidya) (Rup and Kaur, 1993); with 2, 4-Dichloro phenoxyacetic acid and atrazine on southern armyworm, *Spodoptera eridania* (Cram.) (Kao *et al.,* 1995); with *Lipaphis erysimi* (Kalt) with chlorogenic acid (Rup *et al.,* 1999); in wheat aphid, *Sitobion avenae* (Fab.) with hydroxamic acid (Loayza-Muro *et al.,* 2000); with 6

triazene herbicides on fall armyworm, *Spodoptera frugiperda* Smith (Yu, 2004) and in mosquito, *Aedes* species with polyphenols (David *et al.,* 2000; Tilquin *et al.,* 2004). In the present study, increase in the plateau of esterases activity in treated larvae after 48 and 72 hr suggests that esterases plays a significant role in detoxification of ACL and the increase in activity can be attributed to positive feedback response. In corroboration to the present finding, increase in esterase activity was also observed in second instar larvae of *B. cucurbitae* with lectin from *A. hellebolirfolium* Schott, *A. jacqumontii* (Kaur *et al.,* 2006 a,b). The catalase enzyme which is usually involved in decomposition of hydrogen peroxide and in the detoxification of xenobiotics rather got inhibited with the application of ACL indicating that some alternative enzymes might be involved in the detoxification of oxygen radicals generated by the application of lectin in *B. cucurbitae* and this lectin is having a toxic effect on the synthesis of this enzyme. Results analogous to the present findings were also perceived by Singh *et al*. (2006b) when they treated *B. cucurbitae* larvae with *Glycine max* lectin. The suppression in the activity of two hydrolases (acid and alkaline phosphatases) indicated that both acid and alkaline might not play any significant role in the detoxification of lectin from *A. curvatum* in *B. cucurbitae* and that ACL might be interfering in the feedback biomechanism of these enzymes during their synthesis. Similar results have also been observed by Kaur *et al.* (2006a, b) and Singh *et al*. (2006a, b). The group transferases (GSTs) generally play a central role in detoxification of endogenous and xenobiotic compounds in insects and are also involved in biosynthesis of hormones, intracellular transport and against oxidative stress (Enayati *et al.,* 2005). But this does not seem to be a universal phenomenon as in many insects the activity of GSTs gets suppressed under the influence of xenobiotics (Lee, 1991; Yu and Abo-Elghar, 2000). Also in the present study, GSTs activity showed a decreasing trend in treated larvae as compared to control. In congruent to the present finding, Singh *et al.* (2006b) also observed a decrease in GSTs activity under the influence of *G. max* lectin in second instar larvae of *B. cucurbitae*.

The present results showed great insecticidal potential in *A. curvatum* lectin, but the other related aspects such as its influence on the beneficial insects as well as on domestic animals and human beings in addition to localization of gene involved in synthesis of lectin needs to be explored thoroughly. The present study provides a very consolidated platform for going into the above mentioned studies.

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