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Biochemical basis of insecticide resistance and determination of esterase enzyme patterns in field collected populations of *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) from India.

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ABSTRACT

Insecticide resistance in *Cotesia vestalis*, a braconid endolarval parasitoid of the Diamondback moth *Plutella xylostella*. Resistance was assessed in the parasitoid populations collected from Anand (Gujarat), Bangalore (Karnataka), Bhubaneswar (Odisha), Coimbatore (Tamilnadu), Delhi, Hyderabad (Andhra Pradesh), Pune (Maharashtra), Jorhat (Assam), Tirupathi (Andhra Pradesh) and Varanasi (Uttar Pradesh). Three insecticides viz., Spinosad 45% SC, Indoxacarb 14.5% SC and Novoluron 10% EC were evaluated for resistance through qualitative and quantitative bioassays. The parasitoid population from Hyderabad was more resistant to all the three insecticides than others, based on the LC_{50} values with a resistant factor of 79.76, 12.92 and 17.28, respectively, while the population from Delhi was susceptible to all the insecticides. Enhanced carboxyl esterase activity was observed in the resistant populations of the parasitoid collected from Hyderabad, Pune, Tirupathi and Varanasi. The enzyme activity was more pronounced with respect to Novoluron 10% EC (0.15-0.34 IU/mg/protein/min) than Indoxacarb 14.5% SC (0.13-0.31 IU/mg/protein/min) and Spinosad 45% SC (0.03-0.32 IU/mg/protein/min). Native PAGE and α -naphthyl acetate staining, revealed carboxylesterase bands in the various populations of the parasitoid. Variations in populations and degree of resistance accounted for the detoxifying enzyme activity. The use of potentially resistant parasitoids in biological control programmes is discussed.

Key Words Bioassay, *Cotesia vestalis*, Enzyme, Insecticide resistance,

INTRODUCTION

The endolarval parasitoid *Cotesia vedstalis* (Haliday) is one of the important biological control agents of the Diamondback moth, *Plutella xylostella* (Linnaeus), the most significant pests of brassicas [1-2]. Effective parasitism is influenced by the adoption of the parasitoid to prevalent biotic and abiotic stresses. Among the abiotic stresses, toxicity to insecticide is of significance. The Diamondback moth (DBM), had developed resistance to every class of insecticide used [3-5]. Insecticide resistance would cascade on to its natural enemy due to their co-existence in a habitat. Build up of resistance in the biocontrol agent would be more beneficial for their survival in areas of intensive spraying. A resistant bioagent can play an effective role in delaying the development of resistance in the host and can prove to be more potential in the integrated pest management strategies [6]. The use of naturally or artificially selected insecticide resistant strains of natural enemies has been advocated to enhance compatibility of biological and chemical control methods [7-8].

Documented cases of insecticide resistance in field population of natural enemies are relatively rare [9-11].

Increased metabolic detoxification of insecticides by enzymes is the most frequent type of resistance mechanism occurring in insects [12-13]. The cytochrome P450, esterases, and Glutathion –S-transferases that contribute to the degradation of insecticides and the correlation between total non specific esterase (EST) activity and insecticide resistance was reported earlier by [14-17] and others. Metabolic resistance to organophosphorous insecticides in many insect species associated with changes in the activity of carboxylesterases and their over expression was also reported by several researchers [18-20]. The occurrence of insecticide resistance in the parasitoid populations of *C. vestalis* obtained from DBM population from different geographic locations of India was studied through quantitative and qualitative enzymatic assays. Resistance of the host can confer resistance in parasitoids also due to their development within the body of the host.

MATERIALS AND METHODS

Collection and Maintenance of the different populations of the parasitoid

Larvae of DBM on cabbage and cauliflower crops were collected from different geographical areas of India viz., Varanasi (Uttar Pradesh), Tirupati (Andhra Pradesh), Hyderabad (Andhra Pradesh), Coimbatore (Tamil Nadu), Pune (Maharashtra), Hoskote (Karnataka), Anand (Gujarat), Bhubaneswar (Orissa), Jorhat (Assam) and Delhi. In the laboratory, the field collected larvae were transferred on to mustard seedlings raised in ice cream cups (2.2 x 2.7x 1.8") containing vermiculite. The individual cups were placed in cages (24 x 24 x 24") covered with Muslin cloth. Each cup could accommodate about 50 larvae of DBM. Parasitised adults of *C. vestalis* were collected and released on to fresh mustard seedlings containing DBM larvae.

Insecticides evaluated

The technical grade insecticides used in the study for bioassay were Indoxacarb (14.5% SC) (M/s Du Pont), Spinosad (45% SC) (Dow Agro chemicals) and Novaluron (10% EC) (Mekhteshim Agan). In the recent past, these insecticides were widely used by the farmers against DBM [21-24].

Bioassay for insecticide resistance

Residual assay was done by utilizing the adults of *C. vestalis*, obtained from the various geographical populations of DBM. The bioassay technique developed by IOBC/WPRS Working Group [25] was adopted. The commercial formulation of Spinosad (45% SC), Novoluron (10% EC) and Indoxacarb (14.5% SC) were used with field recommended dose i.e. 0.1% per litre of water. Each concentration was sprayed on to inner surface of the test tubes followed by air drying at room temperature. Controls were without treating with the insecticides. Twenty adults (12-24 h post emergence) were introduced in to each treated test tube and provided with honey. There were three replications for each assay.

Based on the mortality response to the highest concentration than the field recommended dose, corresponding serial dilutions were made as 180, 90, 45, 22.5, 11.25, 5.62, 2.81, 1.4 and 0.7 ppm for Spinosad, 40, 20, 10, 5, 2.5, 1.25, 0.62, 0.32, 0.15ppm for Novoluron and 56, 28, 14, 7, 3.5, 1.75, 0.87, 0.43, 0.21ppm for Indoxacarb to test the levels of toxicity in the different field populations. The treated test tubes with adult parasitoids were placed at 25°C, 14 L: 12 D BOD incubators at 65% RH. The mortality was recorded 24 h after treatment and the data was subjected to probit analysis by SPSS version 8.0, statisticareferencesl program. The data were transformed to log base 10 before probit analysis and antilog was calculated to have the LC₅₀. The fiducial limits and resistance ratio were calculated by the lethal ratio test. For each insecticide, the population with the lowest LC₅₀ (most susceptible population) was used as a reference population for comparison with the others.

Insecticide resistance level was determined by Resistance ratios (RRs) (calculated based on the LC₅₀ of the resistant population and the LC₅₀ of the susceptible population (reference population) and categorized on a 1-100 scale, as susceptible (RR=1), tolerance to low resistance (RR=2-10), moderate resistance (RR=11-30), high resistance (RR=31-100) and very high resistance (>100).

Enzymatic assay and estimation of protein

Field collected *C. vestalis* were homogenized in 0.1M sodium phosphate buffer (pH 7.0) containing 1% triton X-100 in a glass homogenizer. The homogenate was centrifuged at 10,000 rpm for 10 min in a microcentrifuge at 4°C and the supernatant was used for enzyme assay.

Total esterase activity was determined by microplate assay as prescribed by Brogdon and [26]. The aliquot

suspension (50µl) was used for each assay in triplicate and 200µl of reaction mixture containing 140µl α -naphthyl acetate and 60µl fast blue RR was added to the homogenate in ELISA plate and the plate was read with 450 nm filters. The plate was incubated at room temperature for 10 min and the T_{10} reading was noted using BIORAD enzyme immunoassay reader. The esterase enzyme activity in the parasitoids collected from different locations was plotted against absorbance at 450 nm/mg/protein/min and the frequencies of resistant population with elevated esterase activity was also recorded. The protein concentration of the supernatant was determined by Lowry's method using bovine serum albumin as standard.

Native Polyacrylamide Gel Electrophoresis (PAGE)

Esterase patterns in *C. vestalis* were determined in 8% polyacrylamide gel. The insecticide treated parasitoids (Indoxacarb, Novoluron and Spinosad) and those untreated (control), were homogenized using 1M sodium phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 10 min in a microcentrifuge at 4°C. The supernatant (20 µ) was loaded into each well with 5µl of bromophenol blue marker and the gels were run at 100V. Gels were stained with 0.4% α -naphthyl acetate and 0.1% Fast Blue B salt in 40mM Phosphate buffer (pH 7.4) after electrophoresis. Esterase activity was estimated based on the development of thick red colored gel bands observed after 20min at room temperature.

RESULTS AND DISCUSSION

Insecticide resistance

Field collected *C. vestalis* populations from different geographic locations were assayed for insecticide resistance, with insecticides (Indoxacarb, Spinosad and Novoluron), belonging to different groups. Indoxacarb an oxadiazine insecticide exhibits strong activity against lepidopteran pests, blocking the sodium gated channel of nerve cells. Spinosad, a biologically derived insecticide from the bacterium *Saccharopolyspora spinosa*, causes mutations in acetylcholine receptors by affecting central nervous system [27, 28], while Novaluron an insecticide of diflubenzoylurea group exhibits stomach and contact toxicity [29, 30].

The level of insecticide resistance varied among the different populations of the parasitoid. Based on the resistance factor, the population from Hyderabad recorded moderate level of resistance (12.92) to Indoxacarb followed by Delhi (4.18) and Anand (3.2). The populations from Bhubaneswar (2.5), Bangalore (2.4), Jorhat (2.02) and Pune (2.4) had a similar low level of resistance. Population from Coimbatore was the most susceptible to Indoxacarb (Table-1). Toxicity of Spinosad was highest in the parasitoid population from Bhubaneswar (LC₅₀ 5.09 ppm), while populations from Hyderabad and Jorhat had high resistance factor (79.76 and 32.45, respectively). Populations from Pune were highly resistance to Spinosad (LC 165.22 ppm) (Table-2). Resistance factor with respect to Novaluron was low (1.21 – 4.17) in the populations from Bhubaneswar, Coimbatore, Tirupathi, Varanasi, Bangalore, Jorhat and Anand. The resistance factor was 23.65 folds higher in the populations from Pune (Table 3). Earlier, Indoxacarb was reported to be toxic to *C. vestalis* [24], while spinosad residues on cotton leaves was toxic to the parasitoid [31-33].

Table 1. Dose mortality response of field populations of *C. vestalis* to Indoxacarb

Populations	LC (ppm)	95% Fiducial limits	Slope + SE	Chi. Square	Resistance factor
Anand (CvA)	3.56	2.05-6.1	5.575+3.635	5.219	3.2
Bangalore (CvH1)	2.68	1.23-5.48	4.761+2.351	1.288	2.4
Bhubaneswar (CvB)	1.76	1.11-2.7	5.498+ 3.849	6.421	2.5
Coimbatore (CvC) *	1.1	0.58-1.87	5.130+0.362	3.368	---
Delhi (CvD)	4.6	3.14-6.75	5.0594.356	1.339	4.18
Hyderabad (CvH)	14.22	8.79-25.83	4.895+4.931	2.562	12.92
Jorhat (CvJ)	2.23	1.37-3.58	5.797+2.940	2.856	2.02
Pune (CvP)	2.69	1.63-4.31	5.692+3.301	6.823	2.44
Tirupathi (CvT)	4.07	2.45-6.8	5.638+4.0049	4.468	3.7
Varanasi (CvV)	3.14	2.04-4.74	5.373+2.324	6.786	2.85

* Population with lowest LC₅₀ used as a reference.

The population from Hyderabad had a higher resistance factor with respect to all the three insecticides, while populations from Jorhat and Pune had high resistance factor for Spinosad and Novaluron, respectively, Variations in the insecticidal resistance among the populations of the parasitoid signify the intensity of usage of these insecticides

on the cabbage crop. The insecticidal application in India differs among the various states. The number of sprays are often determined by the intensity of the pests and its occurrence.. Ten of the active ingredients (16.4% of all pesticides) are reportedly sprayed on cotton [34]. On an average, cabbage, cauliflower and brinjal crops are given 15 applications of insecticides [35]. Such applications had resulted in the development of resistance of DBM to all the major groups on insecticides. The development of resistance of the insect pest had contributed to the buildup of resistance in the parasitoid also, since the population dynamics of the pest and parasitoids are density dependent.

Table 2. Dose mortality response of field populations of *C. vestalis* to Spinosad

Populations	LC (ppm)	95% limits	Fiducial Slope + SE	Chi. Square	Resistance factor
Anand (CvA)	16.79	9.38-31.44	5.310+4.873	3.636	3.29
Bangalore (CvH1)	10.56	5.9-18.62	5.460+4.547	3.468	2.07
Bhubaneswar (CvB) *	5.09	2.61-8.99	5.208+3.479	3.34	---
Coimbatore (CvC)	14.58	7.49-29.66	4.953+4.403	3.618	2.86
Delhi (CvD)	18.85	11.69-30.99	5.571+5.201	5.189	3.70
Hyderabad (CvH)	406.01	162.57-2445.97	4.188+3.23	4.429	79.76
Jorhat (CvJ)	6.47	3.38-11.51	5.247+3.810	4.252	32.45
Pune (CvP)	165.22	72.39-635.55	4.781+2.694	4.654	1.88
Tirupathi (CvT)	21.36	12.39-40.4	5.262+4.710	5.605	4.19
Varanasi (CvV)	19.04	11.73-31.45	5.458+5.340	7.434	3.74

* Population with lowest LC₅₀ used as a reference.

Table 3. Dose mortality response of field populations of *C. vestalis* to Novoluron

Populations	LC (ppm)	95% limits	Fiducial Slope + SE	Chi. Square	Resistance factor
Anand (CvA)	5.01	2.9-9.27	5.347+4.30	2.525	4.17
Bangalore (CvH1)	3.19	1.77-5.89	5.621+1.845	2.984	1.37
Bhubaneswar (CvB)	1.61	0.96-2.63	5.566+1.631	4.670	1.21
Coimbatore (CvC)	4.04	2.29-7.47	5.369+3.862	2.955	3.36
Delhi (CvD) *	1.2	0.61-2.16	5.154+0.612	3.485	---
Hyderabad (CvH)	20.74	12.53-49.54	4.535+3.981	2.101	17.28
Jorhat (CvJ)	2.72	1.79-4.11	5.463+3.676	4.226	2.26
Pune (CvP)	28.38	16.07-99.16	4.446+3.617	2.348	23.65
Tirupathi (CvT)	1.46	0.91-2.28	5.566+1.631	4.670	1.21
Varanasi (CvV)	4.51	2.8-7.44	5.631+4.386	3.070	3.75

* Population with lowest LC₅₀ used as a reference.

The degree of homozygosity of resistance and strain differences among the populations may have contributed to the variation in the level of resistance (Yu and Nguyen, 1996). Further, the degree of resistance towards a particular insecticide may be increased if a particular enzyme involved in its metabolism is induced by xenobiotics. The phenomenon of induction is reported as an adoptive response to a regulated process [37].

Enzyme Activity

The most common resistance mechanisms in insects are increased levels or activities of esterase detoxification enzymes that metabolize (hydrolyze ester linkages) a wide range of insecticides. Detoxification enzyme based resistance occurs when enhanced levels or modified activities of esterases and glutathione s-transferase prevent the insecticide from reaching its site of action.

Esterase activity in pesticide (Indoxacarb 14.5% SC, Spinosad 45% SC, Novoluron 10% EC) exposed populations are shown in (Fig-1). Among the parasitoids collected from different locations, the population from Pune (CvP) treated with Indoxacarb showed 2.4 fold higher esterase level than the lab population (population having the least esterase activity). Similarly, the populations from Tirupathi (CvT), Hyderabad (CvH), Anand (CvA), Delhi (CvD), Varanasi (CvV) and Jorhat (CvJ) populations showed 2.3, 2.15, 2.0 fold higher esterase level, respectively.

The parasitoid population from Hyderabad (CvH) exposed to novoluron showed 2.6 fold elevated esterase activity

where as population from Pune (CvP), Varanasi (CvV) and Anand (CvA) showed 2.2 fold esterase activity. Population from Pune (CvP) showed 10.32 fold higher esterase activity with respect to Spinosad, while it was 7.74 folds in the populations from Tirupathi (CvT) and Delhi (CvD) compared to the laboratory susceptible population (population having least esterase activity). (Fig.1) The enzyme activity was more pronounced with respect to Novoluron 10% EC (0.15-0.34 IU/mg/protein/min) than Indoxacarb 14.5% SC (0.13-0.31 IU/mg/protein/min) and Spinosad 45% SC (0.03-0.32 IU/mg/protein/min). The enzymatic activity was higher in the parasitoid population from Pune, for all the three insecticides tested (0.32 IU/mg/protein/min) indicating resistance across the different groups of insecticides and the intensity of usage of pesticides in the area.

Native page Electrophoresis

Native PAGE is commonly used to detect the elevated carboxylesterase isoenzymes using α and β naphthyl acetate as a substrate [38,39]. The ester hydrolyzing activity of the enzyme was proved by native page assay. Elevated activity of the enzyme was expressed with intense banding patterns. Populations from Hyderabad (CvH) and Pune (CvP) showed very high esterase levels with bright thick bands for all the three insecticides evaluated. A total of 5 α -esterase bands were detected in all the *C. vestalis* populations collected from different regions of India. Population from (CvH) showed greatest activity with three thick distinct bands, with respect to Novoluron while it was only two for the populations from Delhi (CvD) and Hoskote (CvH1) \ (Fig-2), Bhubaneswar (CvB) and Varanasi (CvV) (Fig -3). The population from Coimbatore (CvC), Pune (CvP), Anand (CvA) and Tirupathi (CvT) showed three bands with respect to all the insecticides and there was no expression of bands in the untreated control (Fig-3, 4 and 5).

Metabolic resistance is an important mechanism that contributes to insecticide resistance. The amplification of the esterase gene revealed elevated esterase activity that contributes to insect amplification of the esterase gene revealed elevated esterase activity [40,41]. Enhanced esterase activity was found in resistant population of diamondback moth to Malathion and Phenthoate [42], pyrethroids [43] and Neo nicotinoids [24, 44, 5]

Populations of the parasitoid from Hyderabad (CvH) and Pune (CvP) showed very high resistance with enhanced esterase activity. Very high resistance was observed for spinosad and moderate level resistance was expressed for Novoluron and Indoxocarb. Our studies reveal that enhanced esterase activity not only confers resistance to organophosphorous group of insecticides but even to other group of insecticides (viz., the biologically derived, diflubenzoylureases and oxidiazines groups). Very high LC50 values and elevated esterase levels conclusively establish the phenomenon of insecticide resistance in the different populations of the parasitoid.

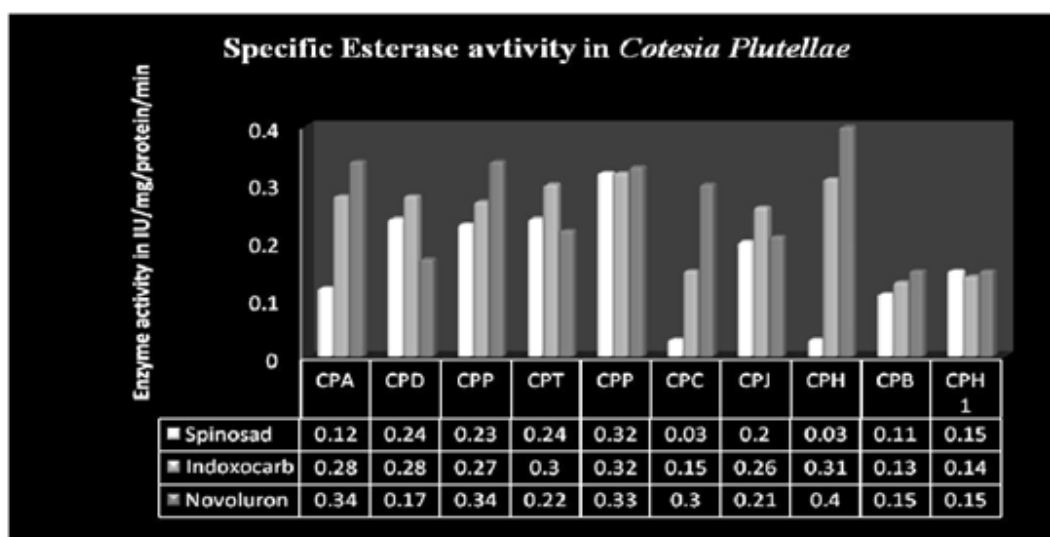


Fig1: Specific activity of esterase in field collected *Cotesia vestalis*

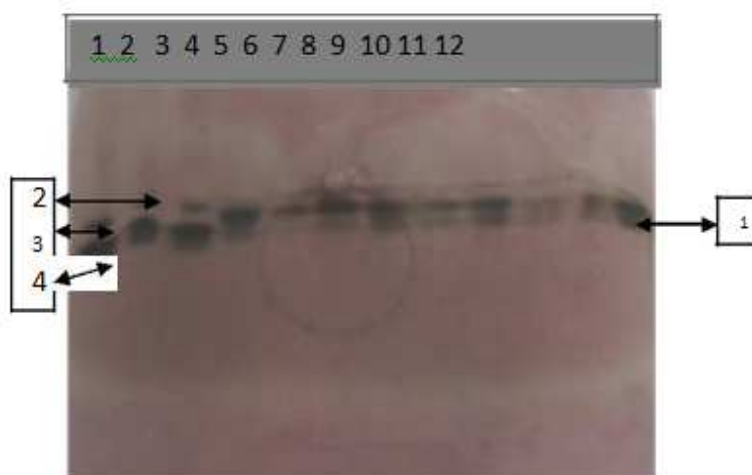


Fig2: Native PAGE of insect crude homogenates, stained for esterase activity with the substrate a-naphthyl acetate. 1-4 (Novoluron, Spinosad, Indoxacarb & Control) CvH population, 5-8 (Novoluron, Spinosad, Indoxacarb & Control) CvD population, 9-12 (Novoluron, Spinosad, Indoxacarb and Control) CvH1 population



Fig3: Native PAGE of insect crude homogenates, stained for esterase activity with the substrate a-naphthyl acetate. 1-4 (Novoluron, Spinosad, Indoxacarb & Control) CvB population, 5-8 (Novoluron, Spinosad, Indoxacarb and Control) CvV population

Insecticidal resistance is a hereditary phenomenon related to the selection pressure and is governed by the developmental history frequency of pesticide use and the selection pressure [45]. The esterase activity was notably high in the populations from Pune, Hyderabad, Tirupathi where the climate is relatively warmer and the incidence of DBM and its parasitoid occurs round the year. [46] reported increasing esterase activity to be associated with decreasing latitude. In the present studies however, this was not congruent in the populations of the parasitoid from different locations.

Strain variations as well as degree of resistance may account for the differences, since enzymatic detoxification patterns are similar in pests and natural enemies. Parasitism by *C. vestalis* enhanced the detoxifying enzyme activity (GST) contributing to an increased level of resistance in the host [47]. Cytochrome P 450 was up regulated in DBM following parasitism by *Diadegma semiclausum* [48], *Tenebrio molitor* parasitized by *Scleroderma guami* [49]. Enhanced detoxifying enzyme activity in the host was reported to confer protection to the endolarval parasitoids *Diadegma semiclausum* and *C. plutellae* [50]. Therefore, exposure of parasitoids to selection pressure harbored by resistant hosts can promote the response to selection by the parasitoids and help produce potentially useful resistant strains of parasitoids for field applications [13, 51, 52, 2007), further helps for selection of resistant genes in the parasitoid, which provides for new insights to our understanding of the development of insecticide resistance.

Resistant population of *C. vestalis* would be more effective against the pest in areas of high insecticide usage. Our results facilitate understanding insecticide resistance in the parasitoid and its adaptive behavior for effective suppression of the pest.



Fig4: Native PAGE of insect crude homogenates, stained for esterase activity with the substrate a-naphthyl acetate. 1-4 (Novoluron, Spinosad, Indoxacarb& Control) CvA population, 5-8 (Novoluron, Spinosad, Indoxacarb& Control) CvC population, 9-12 (Novoluron, Spinosad, Indoxacarb& Control) CvJ population

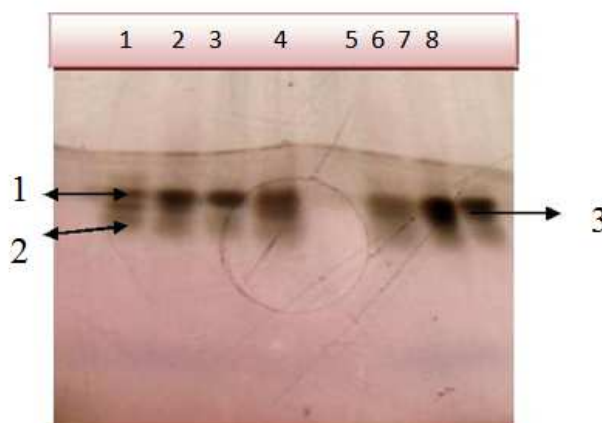


Fig5: Native PAGE of insect crude homogenates, stained for esterases activity with the substrate a-naphthyl acetate.1-4 (Novoluron, Spinosad, Control & Indoxocarb) CvP population, 5-8 (Control, Novoluron, Spinosad & Indoxocarb) CvT population

CONCLUSION

Insecticide resistance in *Cotesia vestalis*, populations collected from Anand (Gujarat), Bangalore (Karnataka), Bhubaneshwar (Odisha), Coimbatore (Tamilnadu), Delhi, Hyderabad (Andhra Pradesh), Pune (Maharashtra), Jorhat (Assam), Tirupathi (Andhra Pradesh) and Varanasi (Uttar Pradesh), was studied. Three insecticides viz., Spinosad 45% SC, Indoxacarb 14.5% SC and Novoluron 10% EC were evaluated for resistance through qualitative and quantitative bioassays.

The parasitoid population from Hyderabad was more resistant to all the three insecticides than others, based on the LC_{50} values with a resistant factor of 79.76, 12.92 and 17.28, respectively, while the population from Delhi was susceptible to all the insecticides. Enhanced carboxyl esterase activity was observed in the resistant populations.

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