Chapter 4
Resistance measurement

Assays for resistance
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A full manual of insecticide resistance laboratory and field techniques has been produced for the CFC/ICAC programme: K. R. Kranthi (2005) Insecticide Resistance: Monitoring, Mechanisms and Management Manual. Central Institute for Cotton Research pp155 *and is available from the editors of this book. The following sections cover only the most immediately relevant techniques and do not repeat the details presented in the manual.*

Assays for resistance

Bioassays enable evaluation of an organism's susceptibility to a toxicant. Various techniques are used to ensure that the toxicant reaches its site of action within or on the test organism, so that the expected toxic response can be evaluated on individuals and populations. The expected toxic response could be in terms of adverse effects on the biology of the test organism or mortality. An appropriate sample of insects is examined depending on the question to be answered. By using serial dilutions of the insecticides and measuring the proportion dying at each concentration, dose-response lines can be drawn and the insecticide concentrations producing particular levels of mortality can be calculated e.g. the Lethal Concentration for 95% of the test organism (LC_{95}). The Insecticide Resistance Action Committee (IRAC) maintains a list of standard and effective methods for insecticide resistance assays for a range of species on their website *www.irac-online.org*. The following techniques are used commonly to assess toxic effects on organisms.

Topical application: The method is very useful for contact poisons. Conventional techniques involving a Potter's tower and even the not-so-old method of Burkhard's microapplicators, have given way to the relatively recent hand-held Hamilton repeating dispenser (Fig. 4.1)

Fig 4.1 Hand held micro-applicator

The hand held dispenser technique has emerged as one of the most convenient methods of dispensing known amount of toxins accurately on insects. Technical grade insecticides
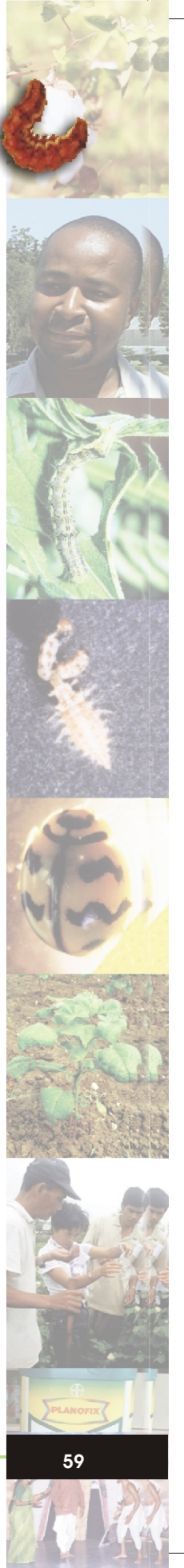
are dissolved in acetone and a pre-calibrated 1µl of solution is applied on the dorsal surface of the prothoracic region of third instar *H. armigera* larvae using a 50µl repetitive manual dispenser to deliver a specified amount of toxin.

Immersion method: Larval dip and immersion of small-bodied insects in insecticide solutions are convenient field based bioassays useful for extension and field workers. The methods are simple and are somewhat closer to field application of insecticides. The immersion technique, commonly known as the 'larval dip method' is another form of topical application using diluted solutions of formulated insecticides, that was specifically developed for simple toxicological evaluation of insecticides in field conditions or for extension and field workers. The methods appeared to be promising for lepidopteran larvae when first proposed in the early 80s, in terms of being rapid and practical for direct determination of resistance under field conditions by extension workers and farmers. However they are not used for routine resistance monitoring in any part of the world.

Insecticide surface coating assay: Commonly referred to as a residual test, the technique involves coating a thin film of diluted solutions of formulated insecticides on to leaf, paper, glass or plastic surfaces. Glass vials are coated with a thin film of insecticide solution in acetone, by evaporating the solvent through continuous rolling of the vials. Insects are released on to the treated surface and are thus exposed to the insecticide. The adult vial test has been used extensively to monitor insecticide resistance in *H. virescens*. It was used with *H. armigera* to show that endosulfan resistant alleles were sex linked (Daly & Fiske, 1998) and has had widespread use in Africa. In Asia the adult vial test has rarely been used to monitor resistance in *H. armigera,* the simpler topical bioassays being preferred.

Leaf-dip method (IRAC Method No 7): The leaf residue assays closely simulate field exposure conditions, and have been used to monitor insecticide resistance in *H. armigera*, whiteflies, aphids and mites. In *H. armigera* early second instar larvae are used in leaf-dip assays in Pakistan (Ahmad *et al.*, 1997). The method closely simulates field conditions, but tends to show variable results because of variation in the age of the leaf; stage of the plant; plant variety; environmental stress to plants and poor leaf feeding capability of *H. armigera*, in addition to the risk of avoidance of the treated surface.

Diet incorporation: Diet incorporation or diet surface-coating tests, were developed for oral toxicant bioassays. The tests are fairly simple, but depend on several factors that include the availability of large amounts of toxin, the thermal stability and consistent bioactivity under bioassay conditions. Diet incorporation or diet coating methods, are used to assess the effects of insect growth regulating compounds and oral toxicants on active feeding stages of insects. *In-planta* bioassays are used to evaluate the effects of systemic insecticides on sap sucking insects and to assess the efficacy of toxin-producing transgenic plants on target insects. The assays are most commonly used to assess the effects cry (crystal) Bt-toxins on larvae, but can be used with slight modifications for any oral toxicants such as insect growth regulators or insecticides, which act as stomach poisons.
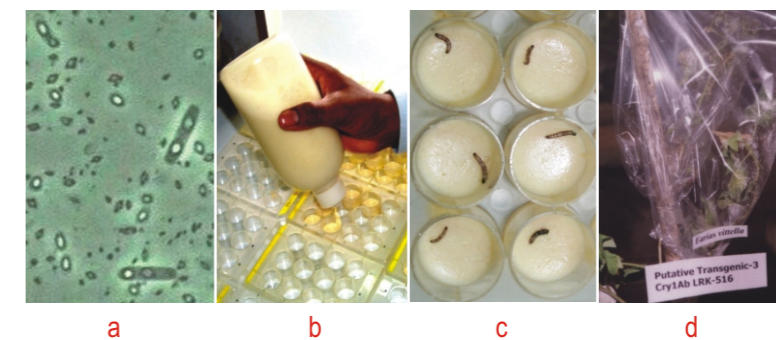

a   b   c   d

Fig 4.2a. *Bacillus thuringiensis* (spores & toxins); b,c. toxin incorporated diet; d. *in-planta* bioassays

Bioassays with transgenic plants: These bioassays help in evaluating efficacy of the plants on target pests, determining the expression levels of the Cry toxins and confirming resistance when it occurs in target pests. There

are a number of methods but the simplest way to estimate whole plant efficacy is to release 10 first instar *H. armigera* larvae on each branch (sympodium) and cover them with two layers of fine-perforated plastic bag, sealed to the braches. By bagging Bt and the isogenic non-Bt plants and making mortality observations daily, measures of % mortality can be obtained.

Discriminating/diagnostic dose assays

A diagnostic dose is expected to distinguish resistant from susceptible insect phenotypes. If a resistance diagnostic test is to be meaningful, the designated diagnostic dose should kill all susceptible insects and spare all resistant insects to correlate with field efficacy of the insecticide. Thus a diagnostic dose should be a discriminating dose that differentiates between the susceptible genotype SS and RS/RR, but not between RS and RR if the resistance gene is effectively dominant in the field situation (where R is the resistance conferring gene and S is the original, susceptible, gene).

Discriminating doses can be calibrated to differentiate between any two of the three genotypes RR, RS and SS, if the dose-mortality regression slopes of the three genotypes do not overlap, if resistance is monogenic, autosomal and non-recessive and if resistant and susceptible strains homozygous with respect to the resistant allele are available. Screening at these doses can then be used to monitor the changes in resistant allele frequencies in field populations. Such doses are determined by conducting toxicological assessments of genetic crosses. The LD_{50}, $LD_{99.9}$, $LD_{0.1}$ of the parents, F-1 progeny and progeny of reciprocal backcrosses are calculated.
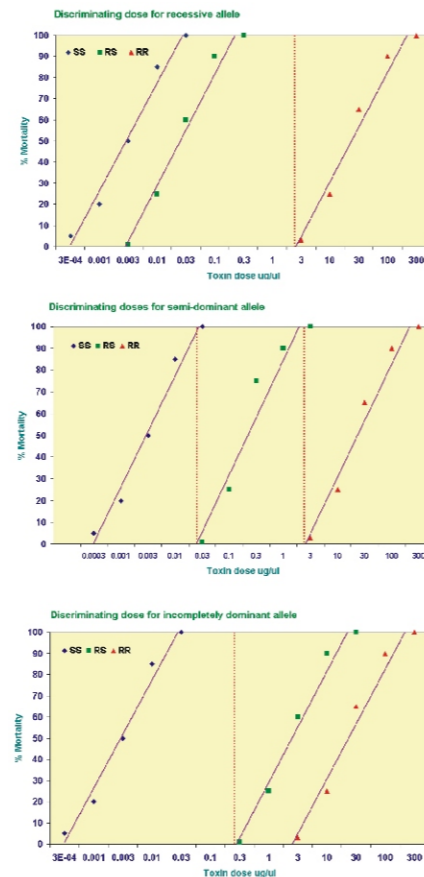

Fig 4.3 Dose mortality curves

If resistance is not inherited as a recessive trait, the discriminating dose would be equivalent to the $LD_{0.1}$ of the F-1 progeny, which could be almost equivalent to the LD_{50} of the backcross (SS x RS) progeny and would correspond to $\geq LD_{99.9}$ of the susceptible and $\leq LD_{0.1}$ of the resistant strains. The dose would discriminate RS genotypes from the SS and would be very useful in monitoring for the change in resistance frequencies and in assisting in calculating changes in resistant allele frequencies (provided that the treated population was at Hardy-Weinberg equilibrium). Similarly it is also possible to derive a dose that can distinguish between RR and RS genotypes by obtaining LD_{50} of the backcross (RR x RS) progeny. This should be $\geq LD_{99}$ of F1 hybrid progeny and $\leq LD_1$ of the RR homozygous parent strain.

In the absence of well defined resistant and susceptible homozygous strains, the discriminating dose should be deduced from the $LD_{99.9}$ of the baseline susceptibility data obtained from whatever laboratory susceptible strains are available and a wide range of strains collected from various geographical zones to fairly represent population variability in susceptible field strains. Note that field strain susceptibility is generally quite variable and one strain alone should not be used. Such an exercise can be carried out with field populations only before the insecticide has inflicted any selection pressure. Generally the most common and simplest method used to determine the discriminating dose has been through estimation of the LD_{99} of susceptible populations. This pre-supposes that resistant phenotypes do not get killed at this dose. But we do not always know that resistant alleles exist at a frequency of < 0.01 in the susceptible populations tested. It is possible that there may not have been any resistant alleles in the susceptible strain used for the assay but that these may exist in field populations, and therefore that the diagnostic dose thus derived may over-estimate resistance. Hence, one way of deriving a diagnostic dose is through several bioassays on large populations of field-collected insects so as to ensure that pre-existing resistant alleles are sampled.

Determining a diagnostic dose can be complicated if inheritance of resistance is recessive or incompletely recessive or polygenic. A recessively inherited resistant trait will have heterozygous genotypes, which show dose-mortality regression slopes that closely overlap with those of the homozygous susceptible genotypes. The diagnostic dose would thus depend on the magnitude of recessive inheritance. Completely recessive or incompletely recessive inheritance can lead to a diagnostic dose that may be grossly inadequate and can be several times less than the dose required to distinguish resistant homozygous genotypes. Similarly, dominant or incompletely dominant inheritance can shift the dose-mortality lines of the heterozygous genotypes closer to that of the resistant homozygous genotype and away from that of the susceptible genotype, thus the diagnostic dose derived based on susceptible strains may also be incapable of distinguishing truly resistant genotypes. The fact that laboratory selection processes generally select for many alleles, thus resulting in strains that are polygenic for resistance, compounds the problem. In most cases field selected strains have been found to be resistant to a particular toxin due to a single major allele, but laboratory selection for a few generations subsequently, appears to be selecting for genes with additive effects.

It is thus important to keep in mind the genetics of inheritance of the resistant allele while determining reliable diagnostic doses that are based on proper genetic and sturdy bioassay methods, which can reflect field efficacy of the toxins. It is possible to adjust slopes of dose-mortality regression curves using various bioassay techniques and then decide on the bioassay that gives slopes of the resistant and susceptible insects in a manner such that the LD_{99} of the susceptible phenotype just overlaps the $LD_{1.0}$ of the resistant phenotype. An appropriate method of fixing a reliable diagnostic dose would be to:

1. Determine the dose-mortality regression of resistant heterozygous and homozygous genotypes and the LD_{99} of the susceptible homozygous genotype;

2. Examine the predicted mortality of the resistant heterozygous or homozygous genotype at LD_{99} dose of the susceptible homozygous genotype.

The dose would not qualify for resistance diagnostic purposes if it kills more than 30% of the heterozygous genotype (ffrench-Constant and Roush, 1990) or worse if it also kills more than 30% of the resistant genotype. If the $LD_{0.1}$ of the heterozygous resistant genotype is greater than LD_{99} of the susceptible genotype, it should be preferred. Alternatively the $LD_{0.1}$ of the homozygous resistant genotype can be considered if it greater than LD_{99} of the heterozygous genotype if the slopes of heterozygous and susceptible genotypes overlapped extensively, as is the case with recessive or incompletely recessive traits. The experiments can be conducted by isolating the resistant homozygous genotypes from field strains using the F2 screen methods (Kranthi, 2005) and conducting bioassays on progeny of genetic crosses with resistant and susceptible strains.

Once the baseline is established, the entire data set can be subjected to log dose probit analysis to derive $LD_{99.9}$ values, which may be representative of the discriminating dose (see below). Ideally, the discriminating dose results will correlate with field levels of insect mortality, and will be a useful indicator from the resistance management perspective.

In many cases, it is difficult to usefully correlate the results of laboratory assays directly to the field efficacy of a pesticide (see below this chapter) as the exposure, life stages etc. are so different in the two situations. But, from the resistance management perspective, the discriminating dose is an important tool to monitor changes in resistance in field populations. Once the discriminating dose is finalized, the required sample size of the test population depends on the accuracy with which the dose is able to distinguish between the resistant and susceptible genotypes/phenotypes and the probable frequency of occurrence of the resistant allele in field populations. Higher frequencies

of the resistant allele will require a lower sample size for acceptable accuracy. At low resistant allele frequencies, the sample size required for accurate estimation may be prohibitively high.

The major advantages of the discriminating dose assays are:

a) The test insect numbers can be small ($\approx$ 100).

b) The assay can detect small increases in the frequency of the resistant insect genotypes.

c) It is simple to comprehend from a practical standpoint, provided that it correlates in some way with the probable % mortality under field conditions and hence is informative for pest management.

The major disadvantages are:

a) The assay becomes saturated at high levels of resistance and cannot distinguish between populations differing in variable degrees of resistance beyond the saturation point that shows at 95% to 100% resistance to the discriminating dose.

b) It does not indicate the magnitude of resistance

c) It may not diagnose resistance properly, if calibrated only from homozygous susceptible strains.

d) Obtaining valid correlations between lab mortality and field efficacy of the toxin is not easy.

Analyses of bioassay data

(For a full description of methods for insecticides and Bt toxins see Kranthi (2005) - available from the editors)

Data is required from both the putative resistant strain and a reference susceptible strain. Finding such a reference strain may not be simple for *H. armigera*, as most strains from most countries are resistant to a greater or lesser extent to most commonly used insecticidal materials. Even insects collected hundreds of kilometers from the nearest areas of regular insecticides use commonly harbour resistance, making the practice of using the least resistant strain of those in the worker's possession as the 'susceptible' strain,

very unwise. The 'Oxford' or 'Reading' strain of *H. armigera*, which was originally derived from W.Africa in the late 1980s, was used by many workers for a number of years. However, in 2001 it proved not to be fully susceptible to OPs and carbamates and the current authors are now using a strain which has been with Bayer Crop Science in Germany - the 'German strain'. It also derived originally from W.Africa in the late 1980s.

For the regression analysis, it is necessary to assess the biological response of the organism against a series of serially diluted concentrations. At least 5 concentrations of the toxicant are tested on each population. Once the bioassay results are found to confirm to a graded response depending on the concentration of the toxicant, they are then subjected to probit analysis through a series of manual calculations or on computer-aided programs such as POLO, MLP, MSTAT, GENSTAT etc. The dose/response data for the two strains is subject to a log dose probit analysis to obtain a regression equation that enables the calculation of the dose/concentration required for any particular % mortality that the insecticide causes in the test population. The analysis can also be done for biological responses other than mortality, such as weight reduction, moult inhibition etc. The dose response can be determined as LD_{10}, LD_{50}, LD_{90}, LD_{99} etc from the regression equation. The details of probit analysis are not being dealt with here. Generally the median lethal dose (commonly called the LD_{50} - a dose which kills 50% of the test population) is calculated to compare responses of test populations.

1. Use Abbott's formula to correct control mortality

$$\frac{\text{\% Test mortality - \% control mortality x 100}}{100 - \text{\% control mortality}}$$

If control mortality exceeds 5%, the replicate should be discarded.

2. Plot percentage mortality on a probit scale against log insecticide dose. Read the LD_{50} and LD_{90} values from the

graph. Alternatively software programs such as POLO-PC, MLP, MSTAT, GLIM or GENSTAT may be used for probit analysis. Resistance factors (RF) or resistance ratios can be calculated if data for the response of a susceptible strain is available (see Box 3.1 In Chapter 3).

$$RF = \frac{LD_{50} \text{ of test strain}}{LD_{50} \text{ of the susceptible strain}}$$

The comparison will be valid only if the regression lines of the susceptible and resistant strains are parallel. However, this is generally not the case and hence valid comparisons between populations where there are differing slopes of the regression lines due to genetic variability may not be justified. Robertson and Preisler (1992) proposed a method to derive resistance ratios, which includes LD_x and slope data of both the populations being compared. The method is explained in detail in the Insecticide Resistance Techniques Manual (Kranthi, 2005) mentioned above. Because simple lethal dose ratios do not provide any estimate of the error involved in the calculation, the most practical and least restrictive alternative is to estimate 95% confidence limits for each ratio. Based on estimates for the intercepts (α_i, $i = 1,2$) and the slopes (ε_i, $i = 1,2$) of two probit (or logit) lines and estimates of their variance-covariance matrices, (all of which are produced in the POLO-PC output) the confidence limits for the ratio can be calculated.

Field dose assays

A field dose in laboratory bioassays is used to distinguish between insects that are killed and those that survive at the field application rate. The method is based on conducting bioassays with a particular life stage of the insect that represents the most damaging stage, and which are killed at proportions equivalent to the overall species mortality in field with the recommended field dose.

Field application rates are generally guided by commercial and logistic considerations. If the chemical is relatively cheap, the recommended application rates may be several fold above the diagnostic dose (see below). In this case the field application rate continues to kill resistant individuals under field conditions, while the laboratory bioassays based on diagnostic dose show development of resistance to the molecule. On the other hand, if the technology is expensive, its use may be recommended at a dose that is affordable to the farmer yet provides effective management of the target pest. Such a dose may or may not be above the diagnostic dose. If it is less than the diagnostic dose, then the product may start losing field efficacy but the laboratory assays using an appropriate diagnostic dose may not show resistance as yet. Hence, a field dose assay, which is calibrated to give parallel mortality in the laboratory compared to that which a product gives under field conditions, especially on the most damaging stage of the pest, is useful from the resistance management perspective.

Diagnostic dose assays may or may not correlate to field efficacy of insecticides, because they are not calibrated to estimate field efficacy. The main purpose of a diagnostic dose is to distinguish between resistant and susceptible phenotypes. Field application rates of insecticides are determined by commercial considerations, including the field persistency of the chemical, propensity to be washed off the plant etc. and can be several times more or less than the equivalent of the diagnostic dose at the point of delivery to the insect. However, if a diagnostic dose can indicate efficacy of insecticides under field conditions, it can be used as a practical tool to recommend effective insecticides. To give a practical example, lab measured resistance to pyrethroids at reasonably high levels of 50-100 fold on third instar larvae, may not greatly impact on *H. armigera* control in the field, because although the pesticide may not control third instar and older stage larvae, it kills moths, neonates and younger larvae effectively due to contact action, which results in an acceptable level of pest control. Therefore an overall change in cumulative effects of the insecticide on all stages of the target pest would need to be

quantified and correlated in terms of the net effect on third instar larvae, before the mortality at discriminating dose would be used as an indicator of field efficacy of the chemical. Formulating such experiments to correlate laboratory measured resistance using a particular larval stage with cumulative effects of the insecticide on the target pest under field conditions can be demanding.

Laboratory measurement to predict field control

As described above, the strength of resistance to an insecticide is most easily measured by taking a sample of the pest from the field and measuring mortality of one particular standard life stage to a range of doses of the insecticide, thus generating a 'dose-response line' which tells us the quantity of insecticide required to kill any particular percentage of the population (LD_{50}, LD_{99} etc). Apart from being very labour intensive, the problem is that this procedure removes (deliberately) almost all the variables that affect insecticide efficacy in the field. This will include mixed life stages with differential exposure to, and susceptibility to, the chemical; concealment on or off the plant; temperature variations; rainfall; enhanced susceptibility to natural enemies etc. Much scepticism has therefore been expressed as to the value of such laboratory measurements for assessing the field efficacy of insecticides and thus for deciding on appropriate field rates for applications or for withdrawing highly resisted material. Measuring insecticide induced mortality directly in the field is, however, vastly more labour intensive and the results more variable.

The CFC/ICAC project set out to see whether laboratory-based resistance measurements to a range of rates to the common insecticides could be used to predict *H. armigera* mortality in the field. This involved a very large and labour intensive operation over two seasons at five sites (one in Pakistan, three in India and one in China) having differing resistance profiles to these chemicals. Large replicated field trails were laid out with 5 rates of representative pyrethroid,

organophosphate, carbamate and cyclodiene insecticides, centering on the field rate. Eggs from the field were reared to 2nd instar. Laboratory dose/response measurements were made on part of the population at 3rd instar. Others were placed in the field on 100 plants per plot, and the survivors counted and their place on the plant tagged 24 hrs later when they had settled on the plant. They were then sprayed as 3rd instars with the particular dose of insecticide and survivors counted 24 hrs later to calculate spray mortality. Survivors were collected and assayed for resistance

Figure 4.4 shows the results for just one chemical, quinalphos, and one site (Nanjing Agricultural University (NAU) in one season (2002), as an example. The laboratory
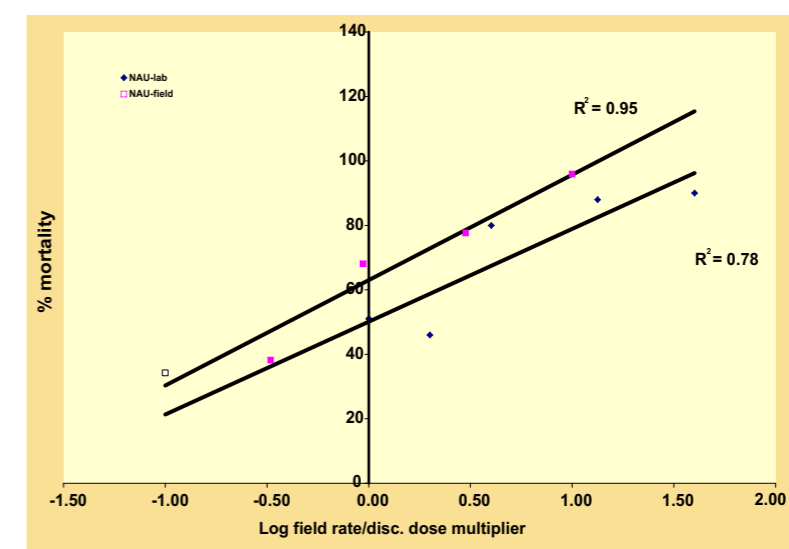

Figure 4.4: Log Dose/ mortality response lines for *H. armigera* with quinalphos near Nanjing, China 2002.

dose response line has its zero set at the discriminating dose (the level at which practically all the susceptible insects and none of the resistant insects would be killed). The field response line has its zero set at the normal recommended field rate for the chemical (notice that the kill at this rate is only 70% in this resistant population). As it happens the lines are parallel, but the value of the analysis is that it is possible to use the lab line to predict mortality at field rates.

Both lines will move downwards in more resistant populations and upwards in less resisted populations and the slopes may change, but field mortality can still be mapped onto laboratory mortality.

The overall analysis of all chemicals and sites showed:

• Field lines from areas with different resistance had different slopes (data not shown)

• The dose response in both the laboratory and the field appear to be log-linear

• The lab lines can be 'mapped' onto the field lines. It is therefore possible to use lab results to predict field failure at recommended rates; to recommend more appropriate rates or to suggest the withdrawal of the chemical.

Note: A single field selection event routinely enhances average resistance levels (as measured in surviving larvae) by 10-40%!

Calculating expected field mortality from laboratory data: Initially dose-mortality curves must be determined for the designated target stage of the susceptible strain under field conditions and the proportional relationship of the recommended dose with the LC_{99} is calculated. An assay can then be designed using the same proportional relationship with the discriminating dose. This will enable us to measure in the lab, the expected mortality that we will see in the field.

For example if the recommended concentration of an insecticide is 0.01% and the LC_{99} under field conditions for second instar *H. armigera* is 0.001%, then the proportional relationship would be 0.01/0.001 = 10. If the lab measured discriminating dose for the insecticide is 0.1 μg/μl per second instar *H. armigera*, the field dose the insects are receiving would be 0.1 x 10 = 1.0 μg/μl per larva. This quantity can be used in the lab to check expected field mortality at the recommended rate and to see if it is adequate. If over time, the laboratory mortality at this dose decreases, then we know that we have resistance increasing

and a developing problem in the field.

The method relies on the assumptions of dose-mortality in laboratory and field assays as discussed above. In setting field rates, due consideration has to be given to the distribution of the sprayed chemical on the plant, persistence under field conditions etc, to ensure adequate exposure of the insects to the chemical.

It must be emphasised that whatever method is used, if the measured resistance magnitude does not correlate with pest control levels under field conditions, the exercise of monitoring is of little practical value to direct pest management in the sense of deciding a dose of insecticide to apply. However, even if the laboratory measured resistance level correlates well to experimental pest control level in the field, it does not necessarily represent overall field efficacy, due to factors related to economic thresholds and density dependence. For example, if the initial *H. armigera* infestation levels were at 5 larvae per plant, low levels of resistance of 20-30%, resulting in 70-80 % mortality at the recommended field rate, can still leave pest numbers at or above to the economic threshold levels of one larva per plant, which will be construed as pest control failure. On the other hand, if the initial infestation levels averaged one or fewer larvae per plant, it is possible that even relatively high levels of resistance at 60-70% resulting only 30-40 % mortality could result in residual pest levels below the economic thresholds and may be perceived by farmers as satisfactory pest control. Hence, for pest management to be effective, it is important to adhere to the recommendations of pesticide applications at economic threshold levels on the basis of regular examination of fields in order to deal with populations before they reach the outbreak stage.

Insecticide Resistance Diagnostic Kits

As described above dtermining the presence of resistance to a particular insect in the field is normally done though laboriously bioassaying large numbers of larvae at a discriminating dose of the insecticide which will kill all the susceptible insects but allow the resistance individuals to

survive, as described above. This enables the assessment of the frequency of resistant phenotypes. This needs time, a laboratory and significant amounts of equipment. What would be very useful is to have ways of measuring whether particular insects were resistant to particular insecticides, right in the field itself. The simpler and cheaper the tests are, the more likely they will be to be utilized and the results used in deciding which insecticides are compromised by resistance and which are still useful. This requires some way of measuring a chemical or identifying a gene or some other factor which is always associated with resistance to a particular insecticide in the resistant insects and which is absent in the susceptible insects. Several types of assay are possible.

1. *Biochemical assays*:

Resistant strains may be characterized by the presence of a unique or over-expressed defense mechanism that is either absent or if present is expressed at lower levels in the susceptible strains compared to that in the resistant strains. Such strains can be characterized by biochemical assays that can detect and monitor insecticide resistance.

2. *Molecular assays*:

Molecular assays can be specifically designed based on observed mutations in the resistant allele itself or based on DNA fragments closely linked to the resistant allele.

3. *Immunological assays:*

Immunoassays are generally based on antibodies raised against a major biochemical molecule that confers insecticide resistance in insects. The assays either use ELISA or the 'dip-stick' format (see below) to detect the frequency of resistant insects in field populations.

As we have seen there are a number of major types of resistance mechanism to a range of different insecticides in *H. armigera.* Thus pyrethroids may be resisted by changes in the conformation of the sodium channel in the nerves, by changes in levels of detoxifying esterases, oxidases and glutathione S-transferases and by behavioural means. Organophosphates are resisted by genetic changes in the acetylcholine esterase molecule and by enhanced levels of particular detoxifying esterases. In developing a resistance detection method then, it is essential to be able to be clear what is being measured. Ideally there would be one major mechanisms of resistance in the insect which is always present in all resistant individuals no mater what other minor mechanisms might be there, and ideally this would operate in a dominant manner, so that finding any evidence of its presence would suffice to be sure that the individual was resistant to that chemical. One way to do this would be to be able to measure the presence (and perhaps quantity) of biochemical correlates of resistance. Consequently biochemical or molecular markers that co-segregate with resistance have been isolated and used to design diagnostic kits. In many cases the markers are resistance conferring molecules such as metabolic enzymes or genes that encode biomolecules that enable insects to survive insecticides.

Many research groups (Zhao *et al.,* 1996; Moores *et al.*, 1988; Raymond *et al.*, 1985; Hemingway *et al.,* 1986; Brogdon and Dickinson, 1983) have used classical colorimetric assays in microtitre plates to detect resistance associated with increased general esterase activity, or with insensitive acetylcholinesterase (AChE). Filter paper-spot tests were initially devised by Pasteur and Georghiou (1981), to detect esterase-B mediated resistance in mosquitoes. The test was later used for other insect species to detect resistance (Ozaki 1969, Rees *et al*., 1985). Devonshire *et al.* (1992) described immunological estimation of carboxylesterase activity in insecticide resistant *Myzus persicae* (Sulzer) and compared microplate esterase assays and immunological assays for identifying insecticide resistant variants.

There are thus several conventional, biochemical and molecular methods to detect and monitor resistance. The following are examples illustrating test methods for various metabolic resistance mechanisms such as enhanced cytochrome p450, esterase, acetylcholinesterase and

glutathione s-transferase mediated insecticide resistance. The first method was specifically designed within the CFC/ICAC project for which this book is an output, to detect insecticide resistance in cotton bollworm. The others
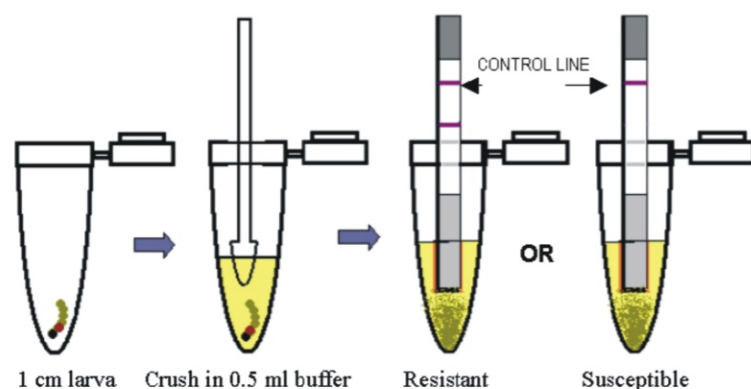

Fig 4.5 Immunochromatographic strip for instantaneous detection of insecticide resistant insects

could presumably be modified to do so.

1. Simple, instantaneous (10-min) immunochromatographic test kits to detect carbamate and organophosphate resistance in individual insects of Helicoverpa armigera. Kranthi (2005).

Two immunochromatographic dip-stick-format kits were developed at the Central Institute for Cotton Research, India, to detect resistance to carbamates (methomyl) and organophosphates (quinalphos, chlorpyriphos and profenophos). Enhanced esterase levels are the main resistance mechanisms to organophosphates in *H. armigera* and it turned out that the particular esterase isozymes involved, differed between the organophosphates and the carbamates. The strips are based on polyclonal antisera raised against the specific resistance associated esterase isozymes isolated from *H. armigera*. The tests take 10 minutes to complete, can be done in the field, does not require additional chemicals or facilities and is extremely cheap. The use of 20-40 strips would be adequate to determine the resistance frequencies in a region within a radius of about 20 km. The strips are simple to use and were

specifically designed for use of illiterate farmers. Each immunochromato-graphic strip is a 6x 0.4 cm containing an assembly of a nitrocellulose membrane on a plastic backing, overlaid by small filter pads and conjugate release pads, that enable the uptake of the test insecticide by capillary flow so that the nitrocellulose strip becomes saturated (Fig 4.5). The basic steps in the test procedure are outlined below.

Step 1. Place a one cm sized larva in a plastic vial.

Step 2. Pour 0.5 ml buffer (provided with the kit)

Step 3. Crush the larva in buffer with the pestle provided.

Step 4. Place the dip-stick into the homogenate as per the instructions provided.

Step 5. Wait for 10 minutes until the strip is saturated with the capillary flow of the solution

Step 6. Two clear purple bands (as shown in Fig.4.5) indicates a resistant larva. Only one purple band at the upper portion of the strip indicates susceptible larva.

Step 7. Calculate results from 20-40 strips to determine resistance frequency in the region

Patents have been applied for and these kits will be commercially available shortly. Similar strips are under development for other insecticide groups.

2. Improved filter paper test for detecting and quantifying increased esterase activity in organophosphate resistant mosquitoes (Diptera: Culicidae). Pasteur and Georghiou, (1989).

This was the first filter paper spot test developed, to detect esterase B mediated resistance in mosquitoes.

3. Diagnostic assays based on esterase mediated resistance mechanisms in western corn root worm, Diabrotica virgifera virgifera, (LeConte). Zhou et al. (2002).

Resistance to methyl parathion among Nebrasca western

corn root worm, *Diabrotica virgifera virgifera*, populations was found to be associated with increased hydrolytic metabolism of an organophosphate insecticide substrate. A non-denaturing PAGE method was proposed as a resistance diagnostic method to detect the frequency of methyl parathion resistant individuals in field populations.

4. Dot-blot test for identification of insecticide-resistant acetylcholinesterase in single insects. Dary et al. (1991).

A simple test was devised to detect insecticide resistance using insects or enzyme from individual insects blotted on nitrocellulose filter papers.

The test is based on staining residual insecticide insensitive acetylcholine esterase on nitrocellulose papers with the Karnovsky and Roots staining technique.

Fig 4.6. Diagram of an immunoblot test
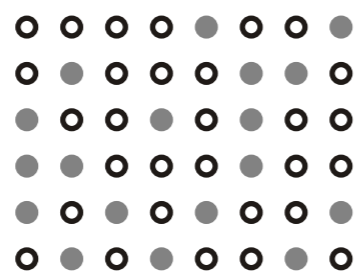
Insecticide concentrations which inhibit the sensitive AChE are used to allow the insensitive AChE remain on the blots as residual enzyme. Insecticide treated and control membranes are stained and compared to distinguish resistant and susceptible insect genotypes (Fig. 4.6).

5. A microfluorometric method for measuring ethoxycoumarin-O-deethylase activity on individual Drosophila melanogaster abdomens: Interest for screening resistance in insect populations. Sousa et al., (1995).

A method was developed to measure ethoxycoumarin-O-deethylase (ECOD) activity on individual *Drosophila melanogaster* abdomens in microtitre plates. Pyrethroid resistant insects were found to exhibit high ECOD activity and could be easily distinguished from the susceptible insects.

6. Immunological detection of p450 mediated pyrethroid resistance in German cockroach, Blatella germanica, (L). Scharf et al. (1998).

A single protein (p450) band of $M_r = 49,000$ was purified from a cypermethrin resistant strain of the German cockroach, *Blatella germanica*. The purified protein was found to have N-demethylation properties and was over-expressed in the resistant strains. Polyclonal antibody was raised in mice and was used to detect pyrethroid resistance in German cockroaches, using western blots.

7. Cytochrome p450-associated insecticide resistance and the development of biochemical diagnostic assays in Heliothis virescens (F). Rose et al., (1995).

A microtitre plate based assay was developed to detect *H. virescens* resistance to cypermethrin and thiodicarb (Fig 4.7).

Fig 4.7 Microtitre plate test to detect cytochrome p450

Insecticide resistant larvae showed higher rates of metabolism with methoxyresorufin, *p*-nitroanisole (PNA) and *p*-nitrophenyl acetate (PNPA) substrates, as compared to susceptible larvae. Microtitre plate assays were conducted using PNA and PNPA as monooxygenase and esterase substrates, respectively. Both assays measure the same end point, i.e formation of *p*-nitrophenol

8. A simple biochemical assay for glutathione S-transferase activity and its possible field application for screening glutathione S-transferase-based insecticide resistance. Vontas et al. (2000).

A simple iodometric titration test procedure was developed as a quantitative assay for visually determining GST activity in individual insects.

9. A molecular diagnostic for endosulfan insecticide resistance in the coffee berry borer Hypothenemus hampei (Coleoptera: Scolytidae). ffrench-Constant et al., (1994).

A molecular diagnostic method PASA (PCR-mediated amplification of specific alleles) was described. The method is based on the use of degenerate primers in PCR to amplify a section of the cyclodiene resistance gene *Rdl* from *H. hampei* (Fig. 4.8)
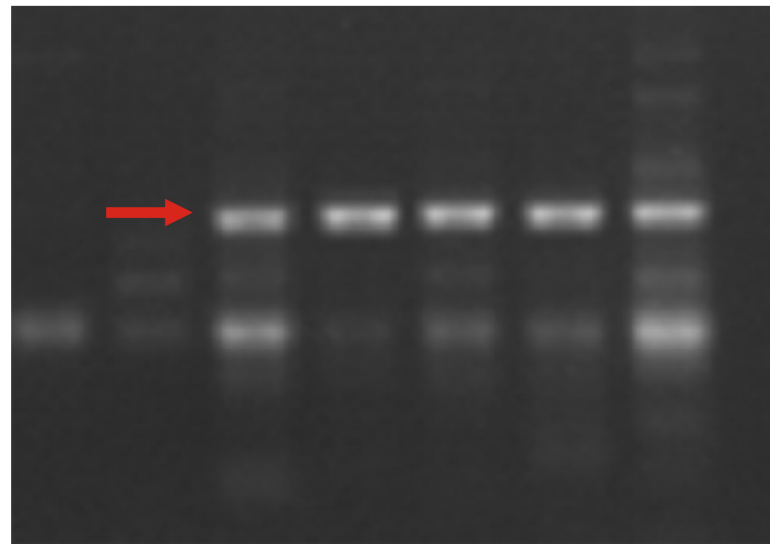

Fig 4.8 PCR test test to detect specific alleles