A *HELIOTHIS* IDENTIFICATION KIT

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The aim of the *Heliothis* identification project is to develop a simple kit that can be used by field workers to distinguish quickly and reliably between individual eggs and small larvae of *Heliothis armigera* and *H. punctigera*.

BACKGROUND

These two species are the most commercially important insect pests of Australian cotton. While *H. punctigera* is present in large numbers early in the season and *H. armigera* is the major pest in late summer, a small proportion of both species can occur in December and January, during the economically critical flowering period. Choice of insecticide during this summer period is complicated by the presence of pyrethroid and endosulfan resistance in *H. armigera* and not *H. punctigera*.

Currently the industry adheres to a three-stage resistance management strategy (RMS). The aim of the strategy is to preserve the cheaper insecticides for use on cotton and other summer crops whilst minimising the selection of resistant *H. armigera*.

Applications of pyrethroids against *H. armigera* are permitted but they are effectively limited to only two or three per season. Since there are currently no "quick and easy" methods for species-typing,

eggs and young larvae, the RMS cannot take account of seasonal or geographical variations in species abundance. An identification kit would enable consultants and growers to ascertain the species composition of a particular egg-lay. A more flexible RMS based on such a kit would allow the choice of insecticide to be made to minimise resistance-selection and costs for each particular locality and time.

PROPOSED KIT FORMATS

Two different formats are under consideration. One is based on a 96-well plate format and is known in scientific jargon as an ELISA. This type of assay has been widely used in biomedical and veterinary science. It may be suitable for large or corporate cotton farms. The other format uses a special type of plastic sheet, onto which the eggs and larvae are squashed before testing. The scientific jargon for this type of test is an "immunoblot". The second format may better meet the requirements of all the cotton consultants, including those consulting for smaller family farms.

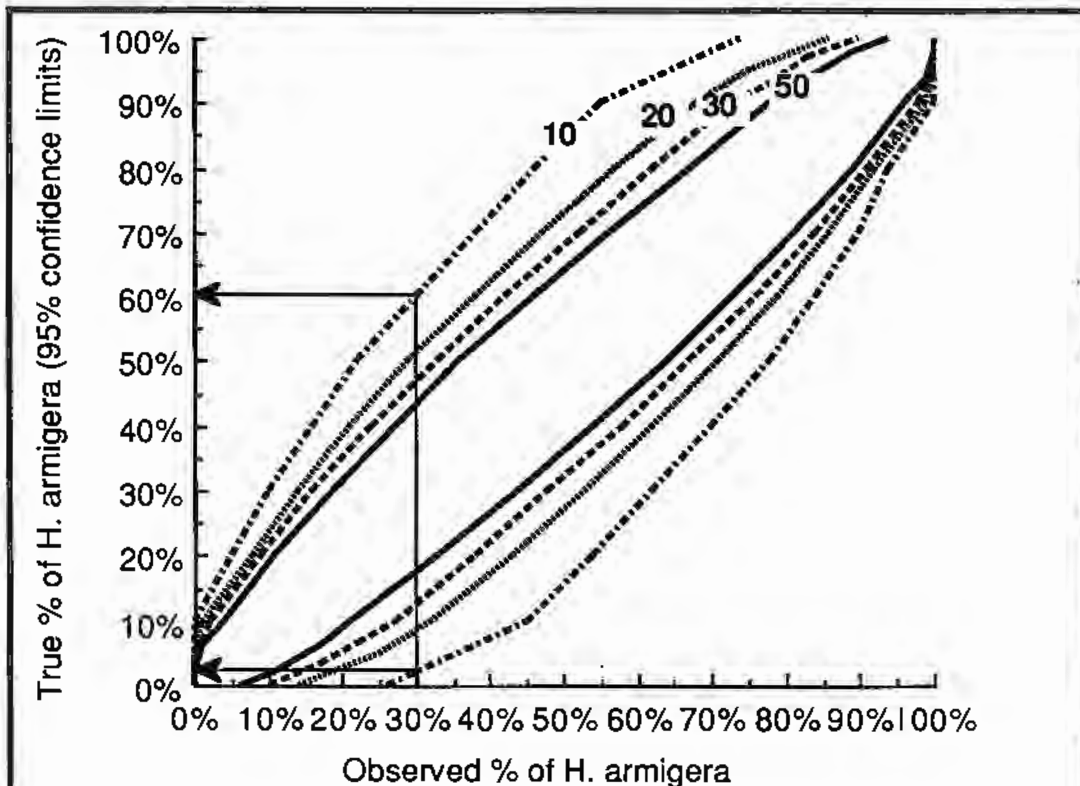
We have tested both formats successfully using antibodies we have generated against *Heliothis*. Whilst, at the time of writing, we do not yet have species-specific antibodies, the results tell us that identification of single eggs and larvae will be perfectly feasible given the right antibody. The actual complexity and speed of the assay will depend largely on the properties of the antibodies that can be obtained. The diagram below shows the sort of procedure that we are aiming for in the case of the immunoblot format.

FIG. 1. EXAMPLE OF TARGET KIT PROCEDURE FOR END-USER

1. Collect samples.	Tear off leaf bearing egg or larva and squash the sample onto plastic membrane. Repeat until sufficient samples have been collected.
2. Block.	Place plastic membrane into a solution of dried skim milk powder. Approx. 30-60 min?
3. Wash 5 min.	Wash the membrane.
4. Incubate with enzyme-antibody conjugate. (10-30 min).	Incubate the membrane with enzyme-linked antibody
5. Wash 3 x 5 min (total of 15 min.)	Thorough washing is essential to remove all the antibody-enzyme complex that is not specifically bound.
6. Incubate membrane with substrate 10-30 min.	When controls change colour stop the reaction by washing the blot.
7. Score positives and negatives.	Make decision on insecticide use.

The number of eggs or larvae that must be tested is an important consideration in designing the kit. The number chosen will depend on the degree of error that growers and consultants are willing to accept in their estimate of the total percentage of *H. armigera* present on the crop. The levels of error associated with varying numbers of individuals to be tested are illustrated in Fig. 2.

FIG. 2. HOW MANY SAMPLES SHOULD A KIT ACCOMMODATE?



There are a number of issues that need to be considered to apply the ID kit effectively in the field. One of these is how many eggs or larvae should be sampled from each field or management unit. The figure illustrates some preliminary calculations. The horizontal axis gives the observed % of *H. armigera* as estimated by a consultant using the ID kit. Statistical theory dictates that this sample percentage can only be an approximation to the true percentage throughout the whole field. By reading off the vertical axis from the curves one obtains the upper and lower limits of the true percentage of *H. armigera* that could be present in the field for cases where 10, 20, 30 or 50 individuals have been tested.

Let us say the consultant observes 30% of the eggs in a field to be *H. armigera* and 70% are *H. punctigera*. If the consultant's estimate was based on only ten eggs (i.e. 2 *H. armigera* and 8 *H. punctigera*), then the true percentage of *H. punctigera* could be as low as 3% or as high as 60% (see lines). Not very useful information! If the sample had been based on 30 eggs (i.e. 6 *Heliothis armigera* and 24 *Heliothis punctigera*) then the true percentage would fall between 8% and 50% or for a sample of 50 eggs, between 14% and 47%. Even if the consultant's estimate was based on 100 eggs, the true value could lie between 18% and 43%. That is, the results become more precise the greater the number of eggs that are tested. Note however, that the precision improves by only a small amount if we increase our sample from 30 to 50 individuals.

It is evident from Fig. 2 that the sample size to be employed in the kit depends on how confident you want to be in your estimate of *H. armigera* numbers. This will depend on factors such as the size of larvae present and the absolute density of eggs/larvae as well as the percentage of *H. armigera* which are resistant. These issues need further discussion between researchers and end-users. It is also important to know whether or not both pest species distribute eggs in a cotton crop in the same way. If they do not, this will affect the way in which samples must be collected. Dr. Gary Fitt (CSIRO, Narrabri) is investigating this problem.

SCIENTIFIC STRATEGIES

The genetic differences between *H. armigera* and *H. punctigera* are well established (Daly and Gregg 1985) and the adults may be readily distinguished by inspection. Decisions regarding insecticide usage must be taken before larvae develop beyond the early instars but the young larvae and eggs can only be resolved using electrophoresis (Daly and Gregg 1985). Although electrophoresis is a standard laboratory technique it is not readily adapted for field use.

The kit under development utilises an approach now commonly applied to the identification of many different human or animal diseases as well as many other instances where very sensitive and specific detection of molecules is required. There are two elements to such a kit. The first is a specific detecting agent, an antibody, which will bind strongly to only one of the two species. The second element is a set of reactions which allows a user to observe

that binding has occurred. In this case a set of reactions which causes a change in colour is the most appropriate choice.

Antibodies as detecting agents

The detecting agents are antibodies which are generated by the immune cells of mammals. Their principal role in the body is to bind to foreign material: viruses, bacteria and other parasites, as a preliminary to their elimination. However any large molecule that is "foreign" can cause the production of antibodies when it is introduced into a mammal and we are exploiting this property of the mammalian immune system in developing the kit. In this case we immunise mice with proteins and other large molecules prepared from the homogenised eggs and larvae of only one *Heliothis* species. The immunisation process is exactly analogous to the tetanus, 'flu and other jabs used to protect humans from disease. Since *Heliothis* is genetically very different from the mouse, the mouse makes many different antibodies to the foreign *Heliothis* proteins.

Species-specific antibodies

Two closely related species such as *H. armigera* and *H. punctigera* will have a similar complement of proteins i.e. there are very few proteins that are not present in both species. However at least some small portions of some of the proteins are known to vary between the two species (Daly and Gregg 1985; pers. comm. Dr. Matthew Greenstone). Antibodies themselves bind to quite small portions (antigenic determinants) of proteins and other large molecules. Most of the antibodies made by the mice bind to antigenic determinants that are the same in both species of *Heliothis* but there

are a small number of antibodies that are species-specific. Our task is to isolate this small subset of antibodies, grow them up in large quantities and incorporate them into a useable kit.

Monoclonal antibody technology

We take mouse-cells that secrete antibodies, make them immortal and grow them in hundreds of cup-shaped depressions in a plastic sheet. Each cup is 5 mm in diameter and contains the descendants (a clone) of a single original cell. This means that only a single type of antibody is made in any individual cup. Antibodies that are produced by this system are called monoclonal antibodies (MAbs). Most of the cups contain cells that have lost the ability to make antibodies or make antibodies which are not of the correct specificity. We test for antibodies in every cup, first to determine whether they can recognise either species of *Heliothis* and then to see if they are specific for just one of the species. Because the cells that produce the antibodies are immortal, once we obtain an antibody with the desired characteristics we will be able to grow it up in unlimited quantities and it will have consistent properties.

Both the immunisation and cell-culture steps are laborious and time-consuming processes and may have to be performed many times to obtain the antibodies we want. This is because we are seeking a relatively rare population of specific antibodies. To speed up the process and eliminate non-specific antibodies, we are employing the techniques of *in vitro* immunisation and differential immunisation but the details of these are beyond the scope of this paper.

Making the results of the test visible

There are many minor variations in the way this can be done. The choice between them often depends on whether simplicity or maximum sensitivity is the target. Again the final choice of system will depend on the characteristics of the MAbs that we obtain. Our preferred option would be to provide the MAb in a form in which it was linked to an enzyme. The function of the enzyme is to convert a colourless chemical into a coloured one. The result, for a kit designed to score *H. armigera* as positive, is a coloured spot at every point where an *H. armigera* egg or larva has been squashed. *H. punctigera* squashes would be blank in such a system. The schematic below shows the principle of one possible assay system of this type.

PROGRESS TO DATE

In the first phase of the project we have concentrated on generating immune mice and have achieved this successfully. We are now engaged in making the antibody-secreting cells from those immune mice into immortal cell lines that can be grown outside the the animal. So far we have only observed antibodies which recognise *H. armigera* and *H. punctigera* approximately equally. However it is very early days in this regard. Within the next few months, as more data becomes available, it should become clear whether specific antibodies that recognise the early life-stages of *H. armigera* will be obtained.

REFERENCES

Daly, J., and P. Gregg. 1985. *Bull. ent. Res.* **75** : 169-184.

FIG. 3. PRINCIPLE OF SQUASH IMMUNOBLOT