CHAPTER 3

MATERIALS AND METHODS

3.1 South African cultivation of *Pleurotus* species

3.1.1 Spawn

Spawn is prepared in a very sterile and carefully monitored environment. Growers mostly prepare their own spawn, but it is possible to obtain it through a commercial spawn laboratory. Strains used in commercial cultivation are kept viable through maintaining pure subcultures at 4°C as well as in LN_2. Pre-sterilised grain (wheat, rye, millet or sorghum are all used in South Africa, rye being the preferred grain) is used in the preparation of the spawn, which is done according to standard procedures (Eicker 1995).

3.1.2 Substrate and Pasteurisation

The study has been conducted on privately owned *Pleurotus*-farms in the central Gauteng and Western Cape provinces of South Africa. The substrate of choice on all the farms is wheat straw. The wheat straw substrate is obtained in bales of between 17 kg and 350 kg sizes. The quality of the straw is variable, with the grower depending on availability and transport costs rather than using preferred suppliers. It is then stored in an area providing protection from precipitation, wind and rodents. The actual preparation of the straw is done according to a method preferred by the grower, but the principles according to which it is done is the same for all methods.

Pasteurised wheat straw is used as the substrate. The straw is chopped into 5–10 cm lengths and wetted to obtain a 65-70 % moisture content (measured w/w). Batches of straw, predetermined and sufficient for each run, is pasteurised and used immediately. With a nitrogen content of *ca* 0.5%, slow release nitrogen supplementation is needed to bring it to 4-5%.

Other additives could include vitamins, insect growth regulators (IGR's), nematocides, pesticides, fungicides and a 1:1 lime + gypsum mixture. All supplementation, if used, is added before pasteurisation. Heat labile pesticides are added later during the cooling down period. During pasteurisation heated steam (kept at 75-80°C) is forced through for 8-10 hours. Throughout pasteurisation the moisture content is kept at 70%. After cooling down polypropylene growth containers are filled with prepared substrate whilst 2–4 % spawn is added simultaneously. Before it is divided among the growth containers, a typical pasteurisation batch weighs 1.5 tonnes (Eicker 1995, Van Greuning 1995).

3.1.3 The growth system

Once again, although the same cultivation principles are applied, the cultivation methods are tailored after each individual grower's preference and facilities. Space is not a problem on most mushroom farms, so that the growing system is dictated by available labour and environmental control costs. This results in the use of mainly two different growth systems. In the one system 50 kg polypropylene columns or tubes (so-called Zadrazil sausages) are each tied onto an upright stand or support made from square tubing and standing on the ground. Smaller polypropylene bags with only an 8kg capacity are used in the other system, where tiered rows are hung, in groups of five, from the shelf above.

In all systems, new bags are filled with the prepared substrate and incubated until homogenous colonisation (spawn run) took place. Upon completion of the spawn run, the bags are pierced in several places using a 25mm punch. Clusters of sporocarps will be borne from these holes.

Incubation, growth, harvesting and packaging are done indoors. Insulated growth areas are used in order to control the climate of the crop environment. These may be permanent buildings or double-layer plastic greenhouse tunnels, where fibreglass insulation gets packed between the plastic layers. It has been found that polystyrene sheets makes good insulatory material as well.

3.2 Isolation of Animal pests

3.2.1 Materials and Methods

3.2.1.1 Nematodes and Mites

Nematodes were collected by plating the substrate directly onto CMA plates. Small pieces of substrate were placed at one end of the CMA plate only and sealed with Parafilm. The opposite part of the plate not containing the substrate was covered with foil. The plates were incubated in a room at 22°C. Nematodes moved towards the darkened area within 24–48 hours. After making sure no mites or fungal contamination was present, a small block of eelworm-infested agar was transferred to a fresh CMA plate. Plates containing live specimens were sent to the Biosystematics Division of the Agricultural Research Council of South Africa for identification.

Mites were collected in a similar way, except that water agar was used. The Petri dishes were sealed very carefully so as to prevent any unwanted mite infestation. A small block of PDA with *Cladosporium* mycelium was placed upside down opposite the substrate in the Petri dish. The plates were not covered with foil, but the mites were attracted to the mould within a few hours. They were scooped out into a clean Petri dish that was then sealed with Parafilm and sent to the Biosystematics division of the Agricultural Research Council of South Africa for identification.

3.2.1.2 Insects

The insects were captured directly from the substrate the in growth tubes as well as directly from tubes bearing fruit bodies. This was done by means of a small suction-type trap (aspirator) or "pooter", based on the method explained by Eardley & Dippenaar (1996). The trap was constructed from a McCartney bottle, short sections of glass tubing fitted through the bottle lid, fine nylon mesh and silicone tubing (Fig 2). Upon capture of a specimen, the McCartney bottle with the insect(s) was unscrewed from the pooter and fitted with a lid. A replacement bottle was put onto the trap.

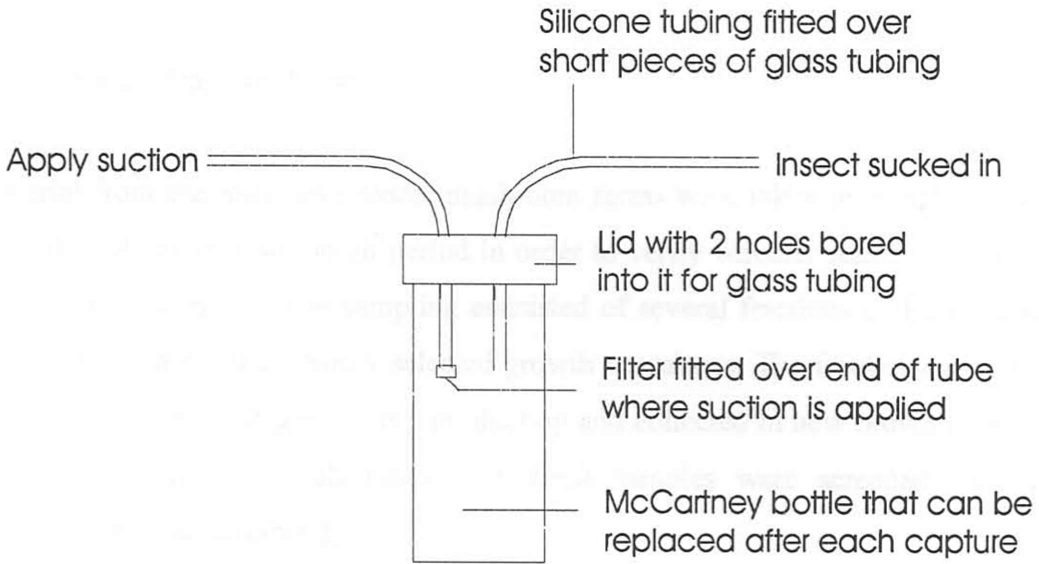

Figure 2 Outline of an aspirator-type insect trap known as a pooter

Larvae and pupae were collected in a clean Petri dish together with some moist substrate. The Petri dish was sealed with Parafilm as a safety precaution against accidental opening of the dish. The larvae and pupae were transferred to and incubated in a plastic food container with a ventilated lid in a room at 22 °C.

All insects were identified at the Department of Botany, University of Pretoria before being sent to the National Insect Collection in Pretoria for confirmation or to be identified to genus or species level, whichever was possible.

3.2.1.3 Gastropods

Slugs were collected by hand into a clean Petri dish. They were identified by the Department of Botany at the University of Pretoria. However, due to their infrequent occurrence and obvious relationship with conditions in the substrate that are too wet, the Biosystematics Division of the Agricultural Research Council of South Africa did not confirm their identification.

3.3 Competitive and Weed fungi

3.3.1 Isolation Methods

Material from the respective oyster mushroom farms were taken in samplings collected over all seasons in a 30 month period in order to verify whether seasonal fluctuation of the moulds occur. A single sampling consisted of several fractions collected at random from the substrate in randomly selected growth containers. The fractions were obtained from all the various stages of crop production and collected in new brown paper bags or sterile Petri dishes. At all times only fresh samples were screened for weed or competitive moulds (table 2).

STAGE OF PRODUCTION	PROCEDURE FOLLOWED
Sand or soil from storage facility for dry wheat	Aseptic collection of loose grain by hand, into sterile Petri dishes
Unsterile grain	
Sterile grain	
Unpasteurised wheat straw	
Pasteurised wheat straw	
Spawned substrate (in filled tubes)	Insertion of a very large pincer into randomly selected areas as well as areas with obvious contamination and chunks of substrate collected into either sterile Petri dishes or sterile brown paper bags.
Colonised substrate (in filled tubes)	
Fully productive tubes	

Table 2: Summary of the sampling procedures used to collect fractions from each monitored production stage.

During study three methods were used to isolate weed moulds from the collected material. The samples were treated as follows:

1. Dilution series of each fraction of the sample onto agar plates. The dilution series was done according to standard microbiological procedures, using a dilution range of 1:10, 1:100 and 1:1000.

2. Direct plating of each fraction of the sample onto agar plates. This was done by placing small amounts of material spaced evenly onto the agar surface.
3. Imbedding weighed quantities of fractions into cooled, but still molten agar.

The plates were incubated at 20°C, 25°C, 30°C, 35°C and 40°C. No temperature incubation lower than 20°C was done, since the environment within which the moulds are looked for, has a higher temperature range. Initial incubation was done in total darkness and once mycelial growth was observed, in black light at 25°C.

3.3.2 Media used for the isolation of moulds

For direct plating and imbedded material potato dextrose agar (PDA), Rose Bengal/PDA, oatmeal agar and water agar plates were used. O.5% Rose Bengal was added to the PDA plates only. Grain fractions were plated onto PDA with 0.25 % chloramphenicol (rather that 0.5 % Rose Bengal) added for increased bacterial inhibition.

The dilution series was done with sterile physiological Ringer's solution. 1 g material was weighed into sterile 250 ml Erlenmeyer flasks and 100ml Ringer's solution added with 1 drop of surfactant. It was shaken vigorously for 1min and the dilution series prepared from the resultant liquid. PDA plates with 0.5% Rose Bengal and 0.5 % surfactant was used for the dilution series. The surfactant in the PDA plates results in better delimitation of the fungal colonies.

3.3.3 Identification of isolates

After initial isolation and purification, the moulds were identified. Where an isolate was encountered more than once, only one isolate was kept, but the frequency with which it occurred was noted.

The slide culture method of Coetzee & Eicker (1990) was used. A 10x10mm PDA blocks were cut from a PDA plate. These blocks were stuck on the sterile inside surface of the lid of the Petri dish with the agar from which they were cut. A small quantity of spores of a mould was transferred to the four lateral sides of each block. A microscope

cover slip was placed on each agar block and the lid replaced on the dish. The agar plate + blocks was incubated in black light at 25 °C until sporulation was barely visible with a dissection microscope. At this point the cover slips was very carefully prised from the block and mounted on a drop of lactophenol blue on a microscope slide. The cover slip was sealed with a transparent nail cutex. With this method there is very little disruption of the spore-bearing structures. The agar plate ensures that the agar blocks in the lid do not become desiccated.

The semi-permanent mounts of the pure culture isolates were examined under a Nikon Optiphot light microscope, using Nomarski interference-contrast illumination. Identification was done according to several sources as applicable to each isolate.

3.4 Farming practice and hygiene

This was investigated through visitations to *Pleurotus* farms, assessment of the available facilities, interviews and monitoring of contamination by means of samples from the farms.

3.5 Meteorological information

The study has been conducted in a summer rainfall region (Gauteng), and a winter rainfall region (Western Cape). Information on the two study areas was drawn from weather stations in close proximity to each farm (courtesy of the South African Weather Bureau). In Gauteng meteorological information was therefore collected at the Jan Smuts weather station (Johannesburg International Airport) and in the Western Cape at the D.F. Malan weather station (Cape Town International Airport). The two weather stations where the data was collected are situated in the same floristic environments as the different farms.

Seasonal changes were investigated in order to find a possible correlation to infective episodes encountered in the various regions.