Procedure for the mass-rearing of the parasitic nematode Deladenus siricidicola for release against the Sirex noctilio woodwasp.

The basic procedure is that the Kamona strain of Deladenus siricidicola is kept frozen in liquid nitrogen and is thawed out when it is needed for the mass-rearing of the nematodes for field releases or to produce another generation for storing again. The thawed nematodes are plated onto fresh agar containing the Amylostereum areolatum fungus. The petri-dishes now containing both the nematodes and fungus have sections of agar removed to inoculate other petri-dishes containing fresh agar. From these cultures the fungus and nematode-containing pieces of agar are added to a brown rice/raw wheat mixture in Erlenmeir flasks to which water had been added and then autoclaved at 121 °C for 30 minutes on a hardgoods cycle. These inoculated flasks are then stored at 23 °C for about 6 weeks when the ingredients have turned black and the nematodes are ready for harvesting. Harvesting involves merely adding water to the flasks, shaking them and draining off the liquid. This is repeated three times for each flask. Excess water is then drained off (the nematodes settle on the bottom) and sampling takes place by drawing off an aliquot and counting the number of nematodes under a stereo-microscope. The amount of liquid is then adjusted until there are 1 million nematodes in 10 ml of water. The nematodes are despatched in sealed plastic bags each containing 5 million individuals. They are best kept at 5 °C.

Freezing: for storage of the Kamona strain
A 10 % glycerol solution is made up using sterilised water. A flask of nematodes is harvested by adding water, swirling it around and emptying out the ± 1 million nematodes suspended in water. Contamination is minimised at every step of the procedure. The nematode suspension is placed in a crystalising dish 110 mm in diameter and 60 mm high. The amount of water in the dish is reduced by draining off (pouring off) the excess water until the harvested nematodes are in the smallest possible amount of water. Sterilised water is added to the crystalising dish so that 75 ml are present including the nematodes in solution. 75 ml of the 10 % glycerol solution is then added i.e. it is now a 5 % glycerol solution. The whole 'brew' is completely mixed and then left in a laminar-flow bench for ± 6 days until all the water has evaporated.

Aliquots of ½ ml are then taken and placed in each of the liquid nitrogen cryo-vials which had previously been autoclaved. These cryo-vials are then placed in the metal 'ladders' which are placed in the storage bins filled with liquid nitrogen which must be topped up weekly. This equipment is used by the artificial insemination industry and should be available in South Africa.

About 3 - 4 days before thawing the nematodes out for mass-culturing it is necessary to prepare an agar plate with the fungus (dissected from a female Sirex wasp) so that the

nematodes can start off immediately feeding on the fresh fungus. About 1/10 ml of the nematode/glycerol mixture is added to each agar plate. When thawing out the nematodes it is necessary to do this as fast as possible but without killing them i.e. place them in tepid water at ± 28 °C and agitate them gently.

Preparation of agar:
The potato dextrose agar (Oxoid, Code CM139) is too rich in nutrients which causes the fungus to grow too fast and it is thus diluted with purified agar (Oxoid, Code L.28) in the following ratio for batches of 400 ml (i.e. per 500 ml Erlenmeir flask):

7.8 g PDA + 2 g purified agar + 400 ml distilled water i.e. half the recommended recipe of the Potato dextrose agar; half of 39 g in 1 l water.

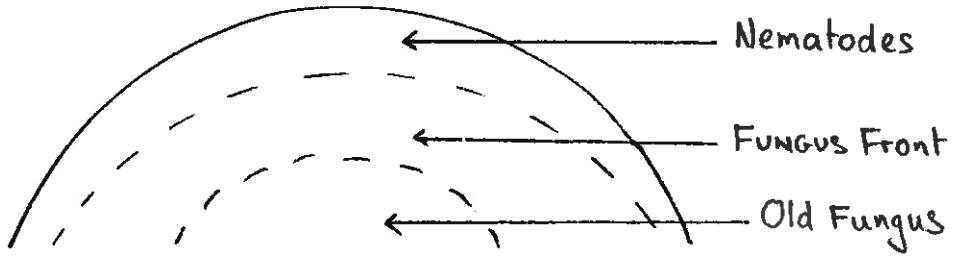
The mixture is agitated until evenly suspended and then a stopper is used to seal the flask. Tin foil is then placed over the stopper and neck of the flask. The flask is autoclaved at 121 °C for 20 minutes on a liquid cycle. The agar is allowed to cool until it is possible to comfortably hold the flask in the hand (± 55 °C). A flask with 400 ml of agar will give enough for 20 petri-dishes.

Pouring of agar into petri-dishes:
The warm (± 55 °C) flask containing the liquid agar is surface-sterilised by squirting 70 % alcohol over it and then it is placed on the laminar flow bench. The petri-dishes in their sealed plastic bags are similarly sterilised and placed on the bench.

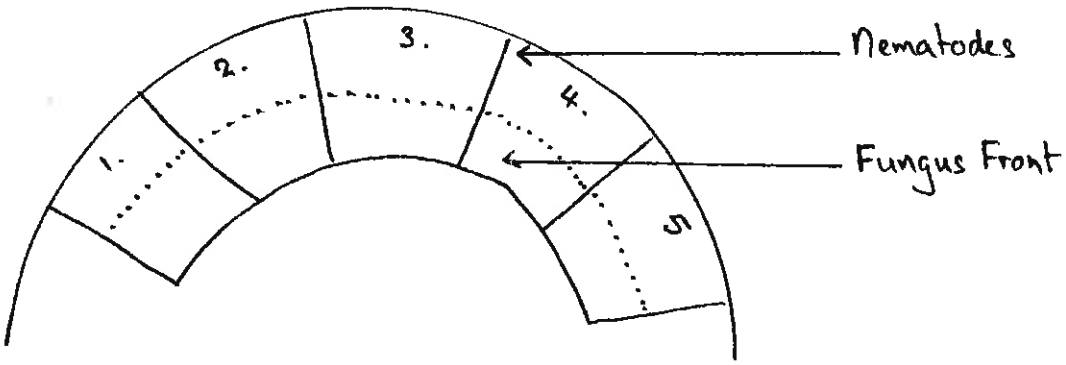
The tin foil and bung are removed from the flask and the mouth of the flask is drawn over a bunsen-burner flame to sterilise it before the agar is poured.

Agar is poured into the petri-dishes until it fills the bottom completely as it spreads over the dish. Twenty petri-dishes are prepared at a time (amount of agar per flask), they are stacked in batches of 10 and left on the laminar flow bench to solidify.

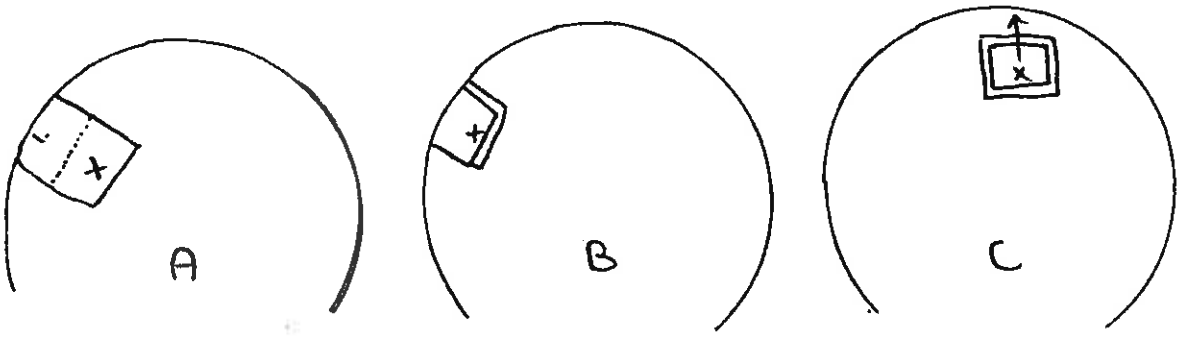
Inoculating the agar with nematodes:
From the petri-dishes inoculated with the thawed out Kamona strain, about 5 sections are cut out with a flamed-sterilised spatula from the outer margin where both the fungus and nematodes occur. The nematodes are found in an arc just beyond the fungus front as it spreads over the agar.



Each of these five sections are again divided into half and the one half placed on top of the other before being placed into the petri-dish containing the fresh agar. This doubling up is to minimise the contact area on the surface of the fresh agar so that the fungus growth is slowed down as it initially grows faster than the nematodes can reproduce.



The growing front of the fungus must be placed facing the edge of the petri-dish containing the fresh agar so that the fungus is forced to double back on itself and so retarding further the speed of growth of the fungus to allow an equivalent growth in the numbers of nematodes.



A = section of infested agar to be transformed with X representing the growing front of the fungus and the dots where the segment is cut in half, the lower half containing mostly fungus and the upper half mostly nematodes.

B = the lower half is excised and placed on the upper half to minimise the surface area of the fresh agar to be inoculated.

C = the segment is placed on the fresh agar with the older fungus facing the direction of the eventual growth and the most actively growing fungus facing the rim of the petri-dish. The active fungus will grow towards the rim and then will double back on itself and spread onto the

wider agar plate.

Batches of 10 inoculated petri-dishes are strapped together with an elastic band, turned upside down and placed at the bottom of a Sterilope paper packet whose entrance is then doubled over and sealed with tape. When placed on the shelf the petri-dishes must be upside down (i.e. agar facing downwards) so that any condensation falls away from the agar. The plates are then left for two weeks at 23 °C before they are ready to be transferred again - either to the wheat/rice mixture for mass culture or to perpetuate the culture and hence to petri-dishes with fresh agar.

Sterile procedure:
During the above preparations and in fact all preparations the following procedures are followed to ensure that there is no contamination of the cultures:
1. Disposable rubber gloves are put on and sprayed with 70% alcohol until dripping.
2. Working at the laminar flow bench the spatula is sterilised by heating over a bunsen burner and this is repeated after each transfer of petri-dish nematodes to other plates or to the mass rearing flasks.
3. The neck of the Erlenmeir flasks are heated briefly over a bunsen burner flame after taking off the tin foil and removing the stopper, and again before replacing the stopper.
4. It is important to work as deeply inside the flow chamber as possible and not to obstruct the flow with hands or other objects placed in the way of the cultures.
5. Wherever the inoculated agar touches the medium the fungus will grow, for this reason it must be placed directly in the middle of the culture flask without touching other spots (otherwise the fungus will proliferate too fast and swamp the nematodes).
6. The agar containing the fungus/nematodes is cut out, divided in two, and the one half is placed on the top of the other to minimise the surface area and slow the spread of the fungus (so that the nematodes can control it better).

Mass culturing on wheat/rice mixture:
It takes about six weeks for the nematodes to develop on the rice/wheat mixture which is ready for "harvesting" when most of the mixture has turned black and the nematodes start migrating onto the sides of the Erlenmeir flasks. The art is to get the correct fungus to nematode ratio - too much fungus and the whole flask is taken over, too many nematodes and they die from starvation. The fungus is seen as a frothy white growth on top of the rice/wheat mixture and has a very sweet smell.

A 50:50 by weight mixture of brown rice and raw wheat are mixed together and 100 g of this mixture is added to a 500 ml Erlenmeir flask. To this is added 150 ml tap water and a bung (steri-stopper) is placed on the flask and covered with aluminium foil to prevent any contaminants entering through

the bung. Oxygen must be able to enter hence the use of the steri-stoppers (or such equivalent) as bungs.

The flasks are then autoclaved for 30 minutes at 121 °C on a hardgoods cycle. When the flasks are removed from the autoclave it is necessary to loosen the mixture by shaking it up (by banging it against the palm of ones leather glove covered hand) to allow a greater surface area to be exposed for the fungus to grow on.

Inoculation of wheat/rice flasks with nematodes:
1. The laminar flow bench is cleaned with 70 % alcohol.
2. Each Erlenmeir flask containing the autoclaved wheat/rice mixture is sprayed with 70 % alcohol before placing them within the laminar flow bench. Similarly the packet in which the agar plates are held is sterilised before being placed on the flow bench. Rubber disposable gloves are worn when working at the flow bench and they are first sterilised with a 70 % alcohol spray before any flasks or plates are handled.
3. A packet is opened and the petri-dishes are placed with the agar facing upward.
4. The spatula is sterilised over a flame after each transfer of infected agar to the wheat/rice flasks.
5. The tin foil is removed from the flask and placed to one side; the stopper is removed and placed deep inside the flow bench; the lip of the flask is sterilised over the flame (and again just before replacing the stopper).
6. A section of the agar is taken from the petri-dish at the end where the nematodes are actively feeding; both the nematodes (clear area) and some fungus (yellow/brown area) must be included in the agar section; the agar is cut in half with the spatula and placed one half on top of the other to minimise the surface area in contact with the wheat/rice nutrient or else the fungus will grow too prolifically.
7. Using the spatula, the two halves of agar are lifted or speared and placed in the centre of the flask on top of the wheat/rice mixture. Other parts of the mixture must not be touched with the agar because the fungus will grow wherever it comes into contact with the agar.
8. Only two flasks can be inoculated from one petri-dish because there is not usually enough of the nematode/fungus area for more flasks.
9. The lip of the wheat/rice flask is then sterilised over a flame, the stopper and tin foil replaced, and the flask is put on a tray outside the flow bench.
10. The flasks are then placed in a room at 23 °C for about six weeks.

Harvesting:
Harvesting is the extraction of the nematodes from the mass culturing on the wheat/rice mixture for despatch to areas where they are to be inoculated into the trees. Harvesting takes place when most of the wheat/rice mixture has been consumed by the fungus and has turned black (i.e. about 3/4 of the flask's contents are black). Many of the nematodes have

by this stage migrated onto the sides of the flasks. The average yield of nematodes per flask is 1.6 million.

Tap water is added to the flask so that it just covers the wheat/rice mixture. It is left for 10 minutes or so before the water is tipped out through a sieve into a large dish. This is repeated three times with each flask. This nematode/water mixture is allowed to settle for ± ½ hour before the excess water is tipped off (the nematodes sink to the bottom). The rest (the sediment) is poured into a measuring flask.

Counting nematodes:
The harvested nematodes are exported in a concentration of 1 million nematodes in 10 ml water. It is therefore necessary to know how many nematodes are present in the sediment that remains after harvesting and then bringing the ratio of nematodes to water to the correct concentration.
1. After washing the nematodes out of the Erlenmeir flasks, the sediment is allowed to settle out for half an hour. The excess water is then poured off and the sediment is poured into a measuring container and the reading taken e.g. 214 ml.
2. After measuring this initial volume it is then thoroughly mixed so that the nematodes are evenly distributed.
3. Using a Gilson "pipetman" P1000 with disposable tips, a 1 ml sample is taken and placed in 99 ml water.
4. From this, a 0.5 ml sample is taken and placed in a petri-dish which has lines scratched into its base the width of the field of vision through the stereo-microscope. This allows the number of nematodes in the patch of water to be easily counted by following the area demarcated between two lines.
5. The nematodes are counted, leaving out the dead ones which are invariable straight (the healthy ones are curved or twisted to varying degrees) and the adults which are very large in comparison to the juveniles.

e.g. 220 nematodes were counted
220 nematodes x 2 (for 1 ml)
440 nematodes x 100 (for 100 ml)
44 000 nematodes x 214 = 9 416 000 in 214 ml of water (i.e. 9.4 million)

The original sediment must now be brought up to 1 million nematodes in 10 ml water i.e. dilute. The nematodes are allowed to settle in a measuring cylinder and the excess water is removed until only 9 ml remains. This is then agitated (e.g. by blowing air (breath) through a pipette with the tip at the bottom of the cylinder) to ensure that the nematodes are evenly distributed in the suspension before it is decantered into the plastic bags in which they are transported.

Another example where the nematode numbers are known to be large: Initial volume = 265 ml. A 0.5 ml sample is taken after stirring to make sure the nematodes are evenly distributed. 99.5 ml water is added to this. A 1 ml sample of this dilution is taken to count. Count = 393 nematodes.

393 x 2 = 786 (for 1 ml)
786 x 200 = 157 200 (1 ml in 99 = 1:100 and 0.5 ml in 99 = 1:200)
157 200 x 265 = 41 658 000 or 41.6 million nematodes

Water must be added to the 265 ml to bring it up to 416 ml so that the ratio of 1 million nematodes in 10 ml can be attained.

Consignments

There are 25 million nematodes per consignment packed in five plastic bags (which permit oxygen exchange) each containing 5 million nematodes. They are packed with freezer blocks which are separated from the sachets by means of a layer of bubble plastic. They are stored at 5 °C to ensure their longevity. After 3 days there is a sharp increase in mortality.

Preparation of gel

Polyacrylamide gel or "Alcosorb" is used as the medium for inoculating the nematodes into the holes made by the punches. It is sold in powdered form (crystals the size of sugar) in South Africa as "Synpol" by Kynoch Soil Services in Randburg.

The ratio required is 2000 nematodes per ml of gel. This represents enough to fill a hole i.e. 50 holes per tree each containing 2000 nematodes i.e. 100 000 nematodes per tree.

2500 ml gel are needed for 5 million nematodes
1 g gel in 100 ml water
i.e. 25 g gel for 5 million nematodes
or 125 g gel for the entire consignment of 25 million nematodes

The following procedure is followed during inoculation:

1. One gram of gel added to 100 ml tap water is the required ratio.
2. Water of the required amount (measurement in a measuring cylinder) is added to the container.
3. The end of the plastic bag containing the nematodes suspended in water is snipped off and added to the water.
4. The required amount of gel is added and allowed to swell. The nematodes will be found in the water layer surrounding each gel particle. Once the gel is fully saturated it must be stirred to make sure that the nematodes are found throughout the gel (and that it has not formed clumps from which nematodes have been excluded). The gel provides the liquid film and the aeration necessary for the nematodes to survive.
5. Place in plastic squiggy bottles for squirting into the holes made by the punches (until the hole is full).
6. The nematode/gel mixture must be kept cool because heat will kill the nematodes.
7. The gel tends to be unattractive to the nematodes which tend to move out of it and so enter the tracheids. It takes about 24 hours for the nematodes to all enter the tracheids. It is best not to inoculate during rain which

tends to wash the gel out. This can be minimized by making the holes more to the sides of the log.