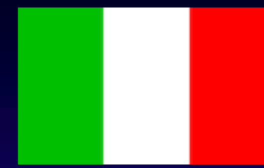


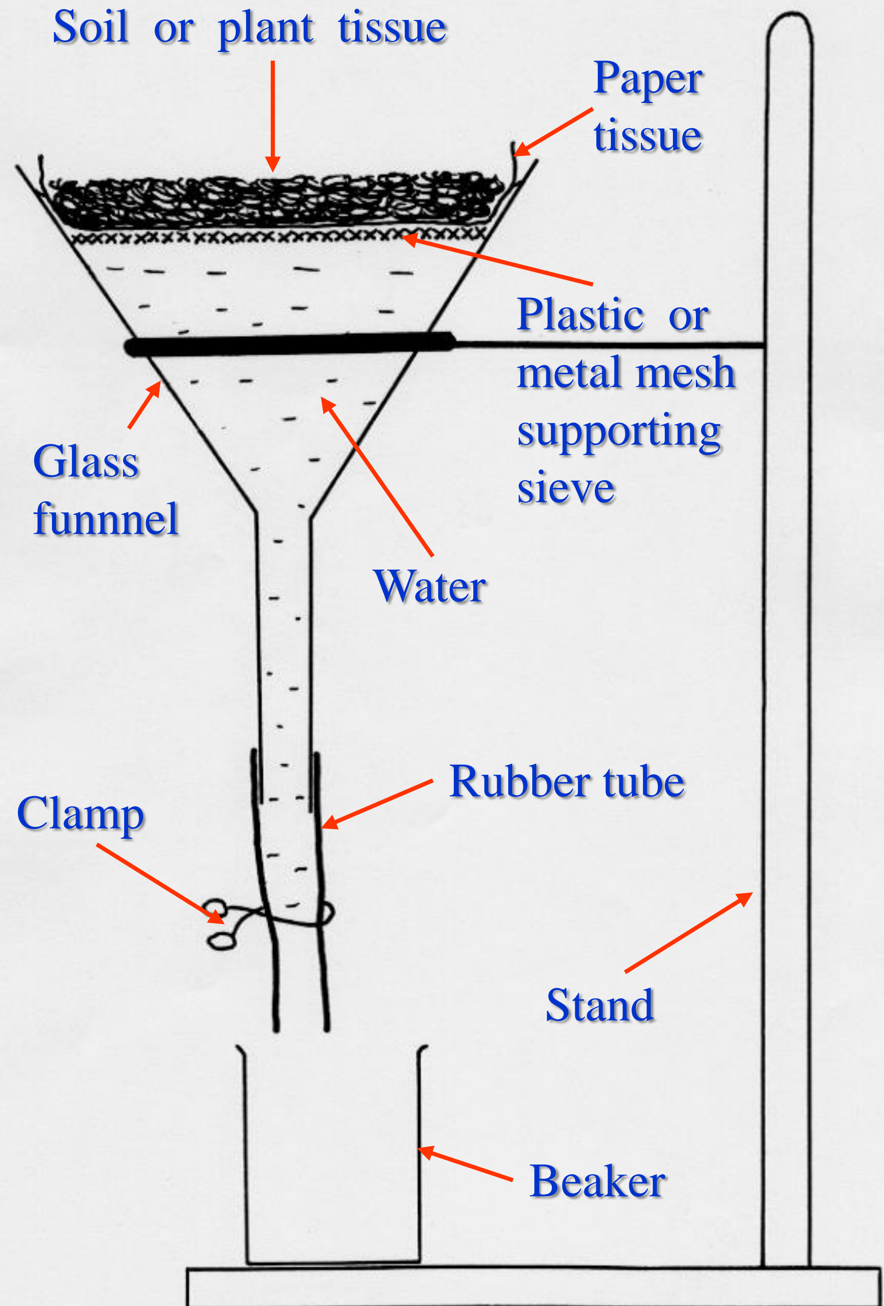
Twinning BA/12/IB/AG 01 “Further strengthening of capacities of phytosanitary sector in the fields of plant protection products, plant health and seeds and seedlings, including phytosanitary laboratories and phytosanitary inspections”

Training course on agricultural nematology

Mostar, March 7-11, 2016

SELECTED EXTRACTION TECHNIQUES FOR FREE EXTRACTION & DETECTION OF LIVING NEMATODES

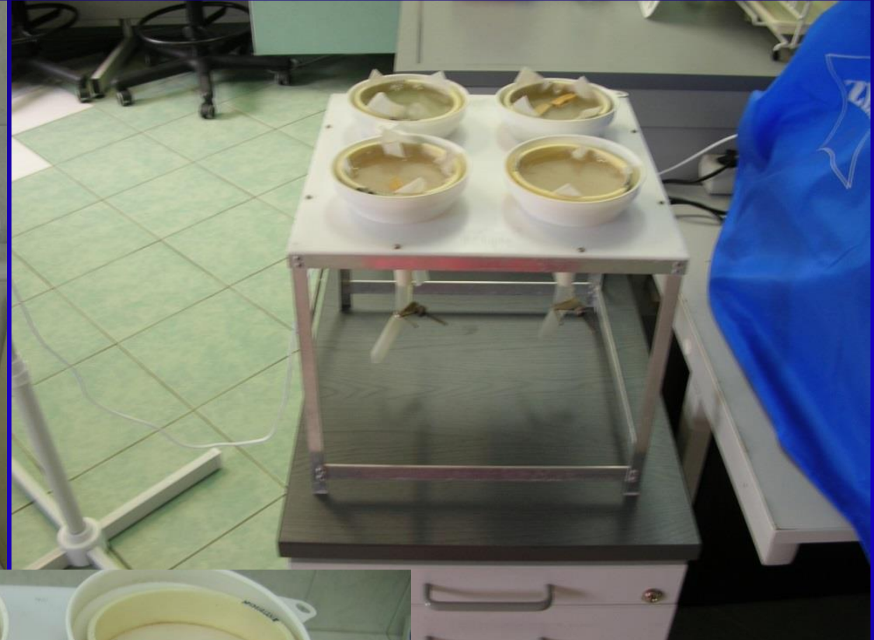
EXTRACTION FROM PLANT MATERIAL



Bearmann funnel apparatus for free living nematode extractionfrom plant material or soil (not proper for extraction of Longidoridae from soil)

Bearmann funnels (Poland)

(phot. SPHSIS, Voivodship Laboratory in Warsaw, Poland



Modified Bearmann funnels (Poland)

(phot. W. Karnkowski)

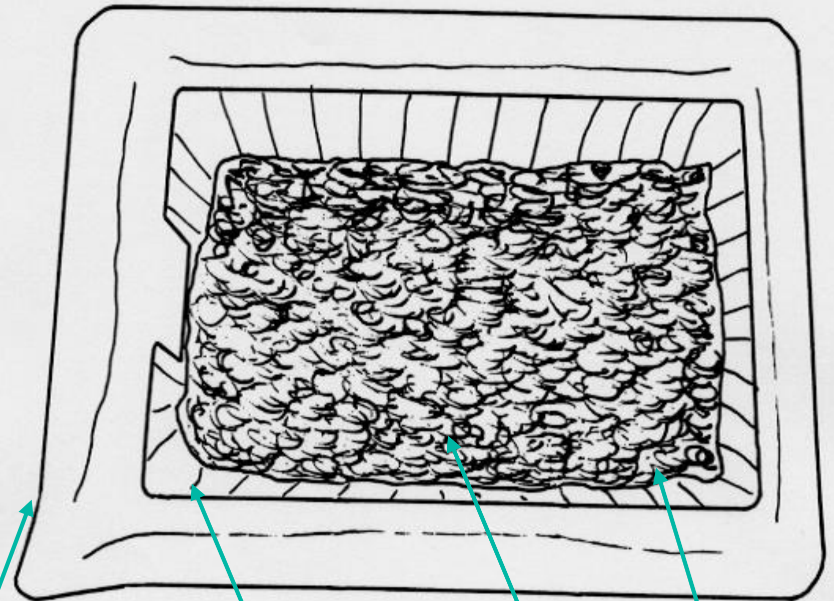
Alternative Barman (Oostenbrink dish)

(phot. W. Karnkowski)

Whitehead Tray

(EPPO Website)

Top view



Photographic developing dish

Soil or plant tissue

Plastic coated wire-letter dish

Paper tissue

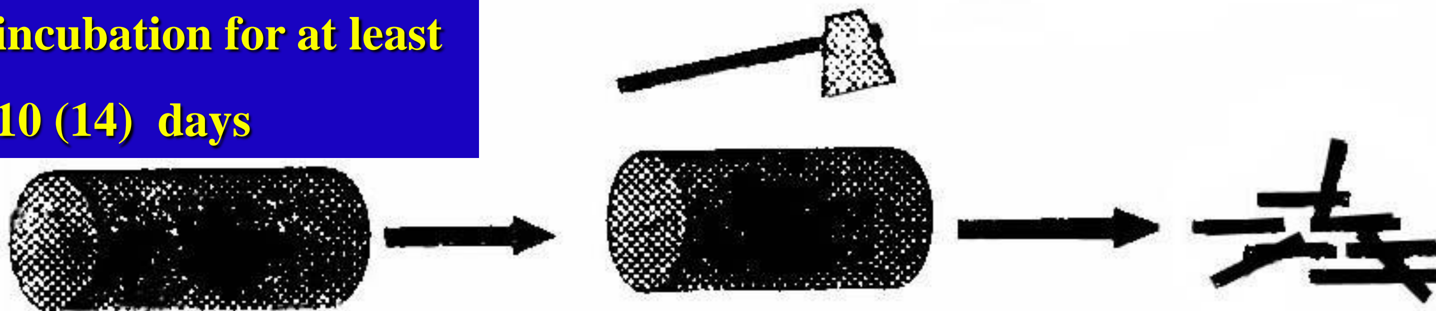
Side view

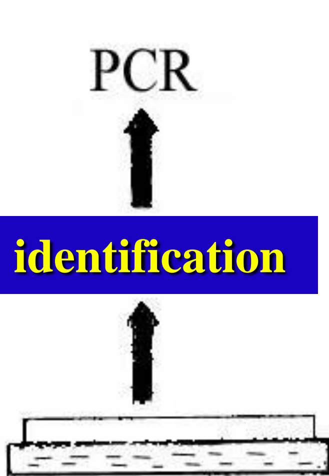
Water level

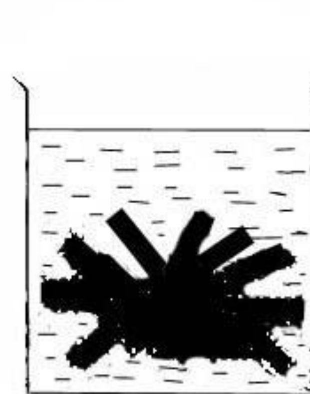
Wood/wood products analyses for the presence of *Bursaphelenchus xylophilus*

incubation for at least

10 (14) days







extraction for at least

3 days

Incubation of wood samples in incubator for at least 10 days at the temperature 25-30°C (at winter where the temperatures are below 0°C this time should be enlarged to minimum 3-4 weeks). It allows for multiplication of nematodes and so make them more detectable (phot. W. Karnkowski)

Nematode extraction from wood (first stage) (phot. W. Karnkowski)

Nematode extraction from wood (second stage) phot. W. Karnkowski)

Root incubation

This method for the extraction of **motile** nematode stages is recommended for extraction of endoparasitic nematodes, such as migrating endoparasites (*Radopholus* spp., *Pratylenchus* spp.) from roots.

PROCEDURE

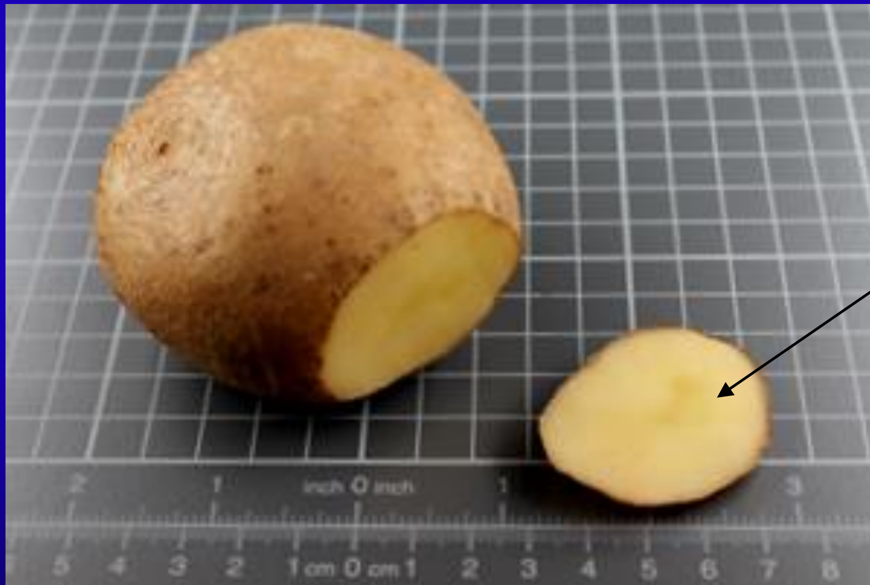
- Wash roots free from adhering soil and cut into short pieces (5-10 cm)
- Approximately 5 g of roots are moistened and placed in a polythene bag or glass jar that is closed and incubated for 3-4 days at room temperature (20°C); during this period, most of the nematodes will leave the root tissue
- Wash the roots and the inside of the bag or jar with a small amount of water and collect the water containing the nematodes in a beaker
- Pass the nematode suspension in the beaker over a 20 or 25 μm sieve to reduce the volume of water
- Examine nematodes at 25-40x magnification.

Enzymatic digestion of roots and potato peels

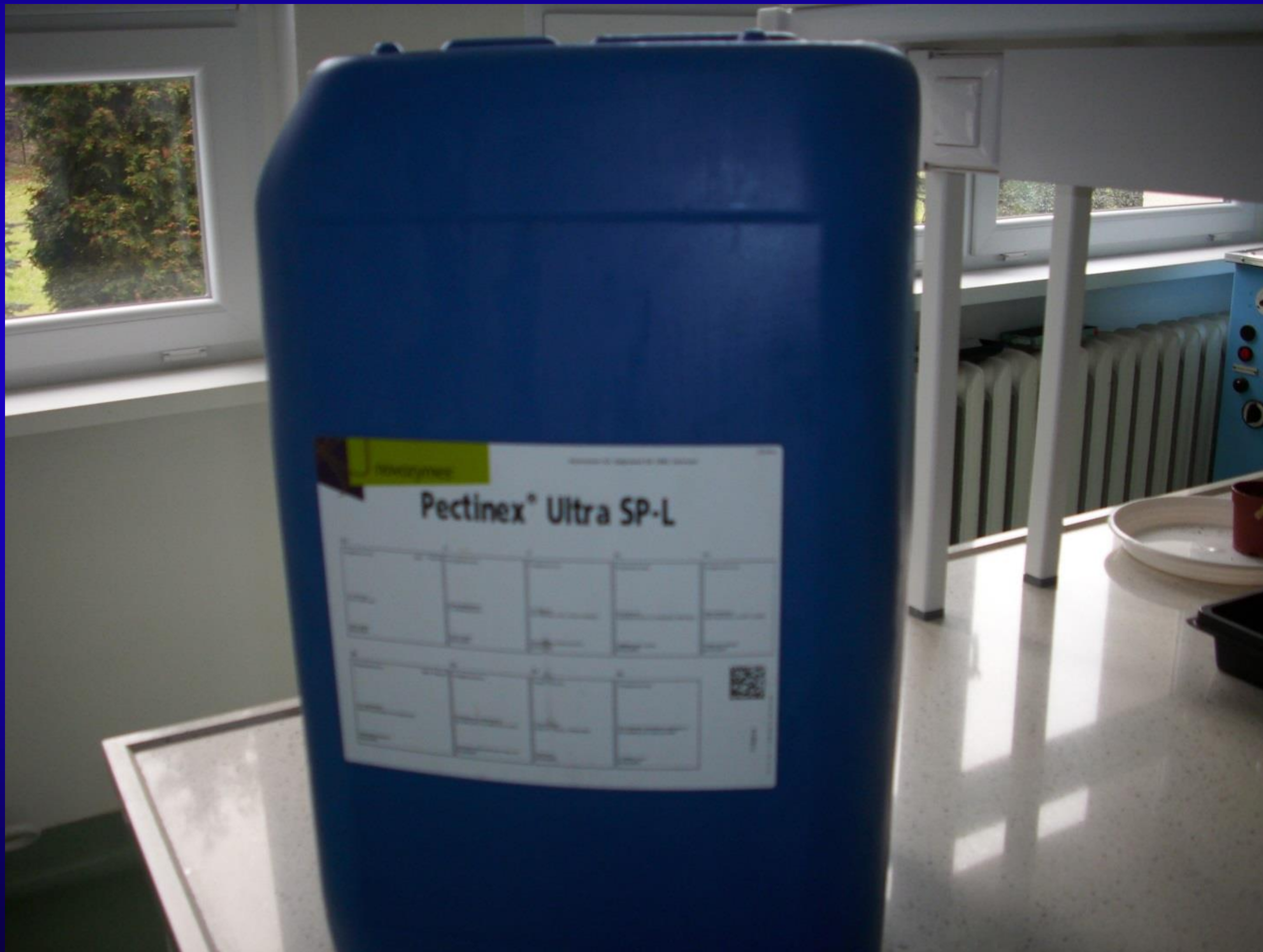
- This method recovers both **motile and immotile** stages of migratory and sedentary endoparasitic nematode species from plant tissues.
- In Poland we use French modification of extraction of *Meloidogyne* from potato peels.

PROCEDURE

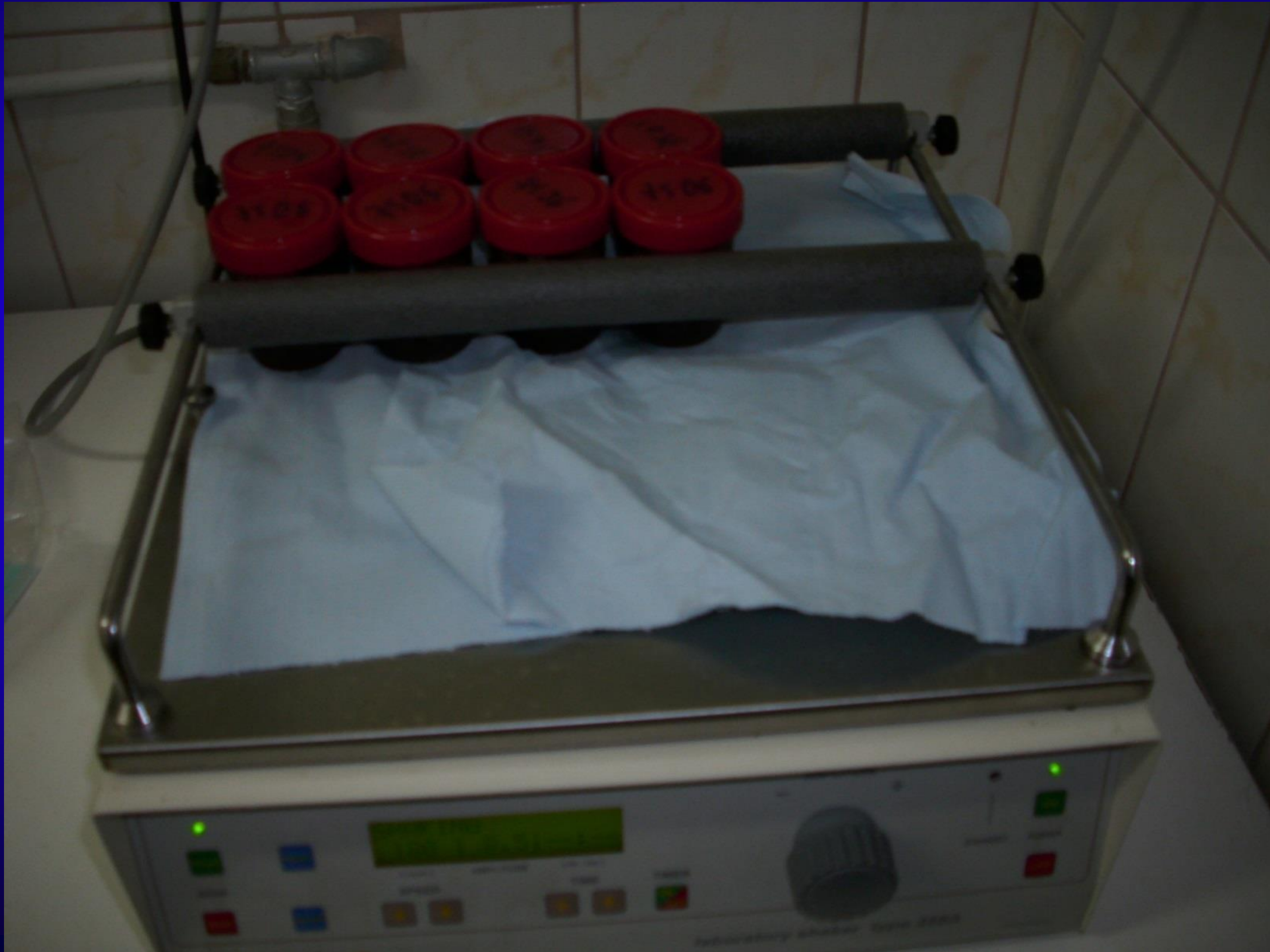
- Prepare potato peels and place them in in 100-500 ml plastic cups (phot. M. Saldat).



- Fill cups with enzymatic solution (300 ml Celluclast 1.5 L + 150 ml Pectinex Ulra SP-L + 550 ml water) mixed thoroughly.
- (phot. W. Karnkowski)



- Incubate plant tissue + enzymes in closed cups at room temperature for at least 12 hrs at 100 rpm on an orbital shaker.
- (phot. W. Karnkowski)



- Extract nematode stages from the potato suspension with sieve set (0.6 mm aperture sieve where the remains of plant material are collected; 0.16 mm aperture sieve for female colection and 0.02 mm aparture sieve for male and juvenile collection), or cetrifugal extraction.(phot. W.Karnkowski)

- Analyse extract under stereoscopic microscope
- (phot. W.Karnkowski).

Maceration & centrifugal flotation

a) This method is well suited to recover both **motile and immotile** nematode stages from plant tissues. In a first step, nematodes are liberated from plant tissue by maceration in a blender.

b) They are then separated from the macerated plant tissue by centrifugal flotation using a solution of specific weight higher than the nematodes.

PROCEDURE

- Wash plant tissue and cut into pieces about 1 cm long. Mix the sample carefully if only part of it is used for nematode extraction
- Macerate plant tissue in a blender at about 12,600 rev min^{-1} for 30 s
- Pour the resulting suspension through a 1200 µm sieve and collect in a beaker
- Wash the plant tissue on the sieve carefully with water to collect all nematodes
- Centrifuge the collected washing water containing the nematodes with 1% kaolin powder (approx. 1 spoon, depending on size of tubes) at approx. 1800 g for 4 min; time and g-force is not that critical, as long as a stable pellet is achieved; time length's of 2-5 min and g-forces of 700 to 2900 g can be used
- Discard the supernatant and re-suspend the sediment in a $MgSO_4$ solution (or similar extraction fluid) with a density of 1.15-1.18
- Centrifuge again at 1800 g for 4 min
- Pour the supernatant through a sieve of maximum 20 or 25 µm aperture size and rinse very well with water to remove the excess salt or sugar solution
- Transfer the nematodes from the sieve into a glass beaker
- Examine nematodes at 25-40x magnification

Blender and centrifuge used in France (phot. W.Karnkowski)

STAINING OF NEMATODES IN PLANT TISSUE

PROCEDURE

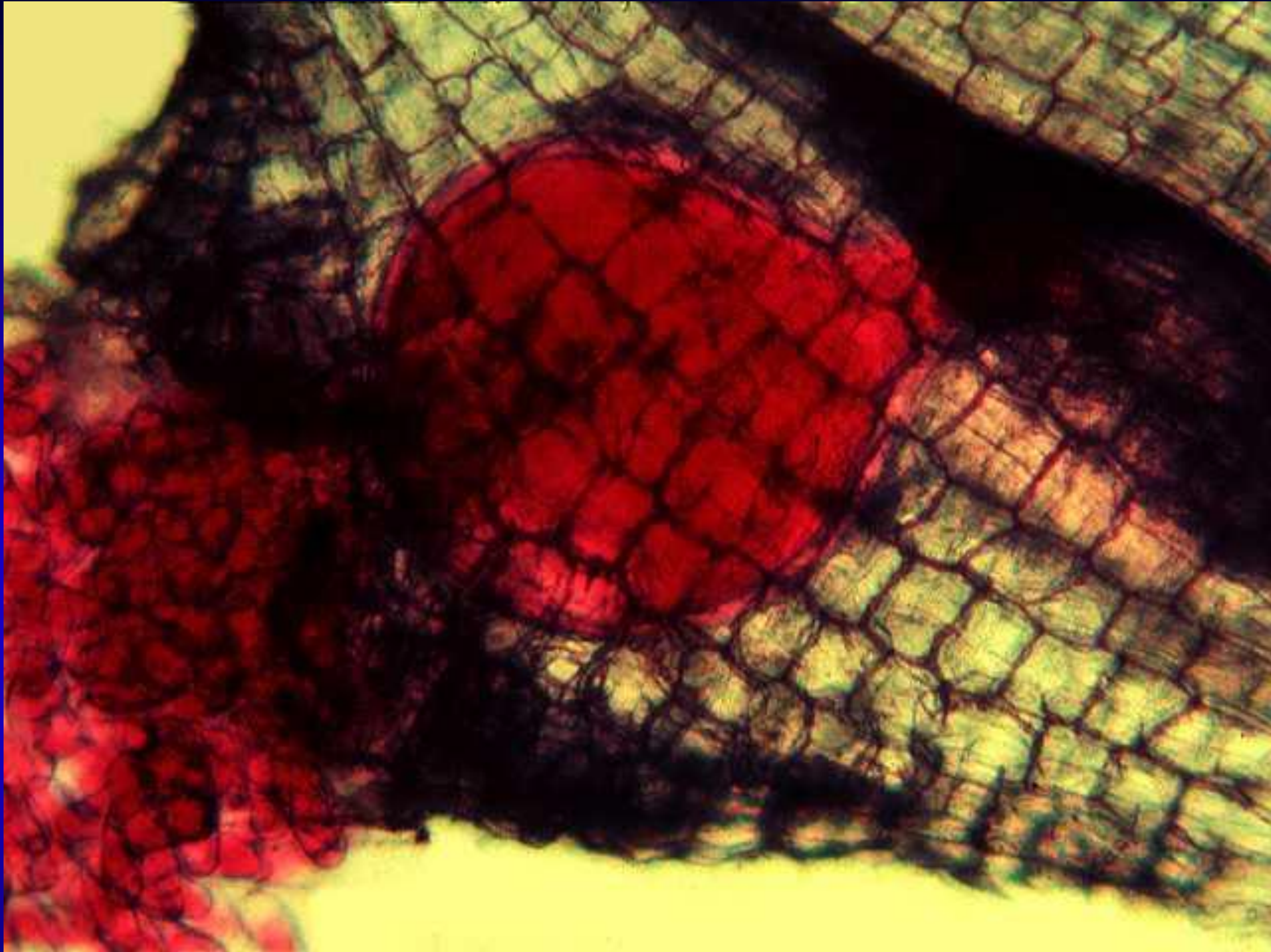
- Prepare two solutions:
  - lactoglycerol:
    - lactic acid 100 ml;
    - glycerol 200 ml
    - distilled water 100 ml
  - lactoglycerol + stain
    - lactoglycerol + 0.5% stain (acid fuchsin, cotton blue, or picric acid)
- Wash roots or other host tissue thoroughly under gently running tap water to remove debris and adhering soil particles. Small roots can be stained intact. Larger plant tissues should be cut into pieces 1-2 cm long, and thick material should be thinly sliced before staining.

PROCEDURE

- Place analysed tissue in a beaker or other vessel (metal cup, etc.) filled with lactoglycerol containing stain.
- Boil solution containing stain for 0.5-2 min (delicate roots) up to 10 min (thick roots), on gas burner, thoroughly mixing the vessel content with a spatula.
- Pour out the vessel content over a 200 to 1000 µm aperture sieve.
- Rinse the host tissue for at least 45 sec in running tap water, and then place in a vessel (ie. beaker, conical flask with cottowool stoppers) filled with lactoglycerol (without stain).
- Perform „differentiation” for 24 h at room temperature or for 10-20 min under pressure about 1 atm (in autoclave or pressure cooker).

PROCEDURE

- Pour out the vessel content over a 200 or 1000 µm aperture sieve.
- Rinse the host tissue for at least 45 sec in running tap water, and then place in a Petri dish filled with water.
- After standing and „differentiation" nematodes are stained whilst plant tissue is at last partly discoloured.
- To find out endoparasites, mount a small amount of tissue on a microscope slide, cover it with a coverslip, and apply *gentle* pressure.
- To find out (detect) root-knot nematodes (*Meloidogyne spp*.), observe roots to find out stained par-shaped nematode females and egg-masses which may be dissected out with preparation needles for further analysis.



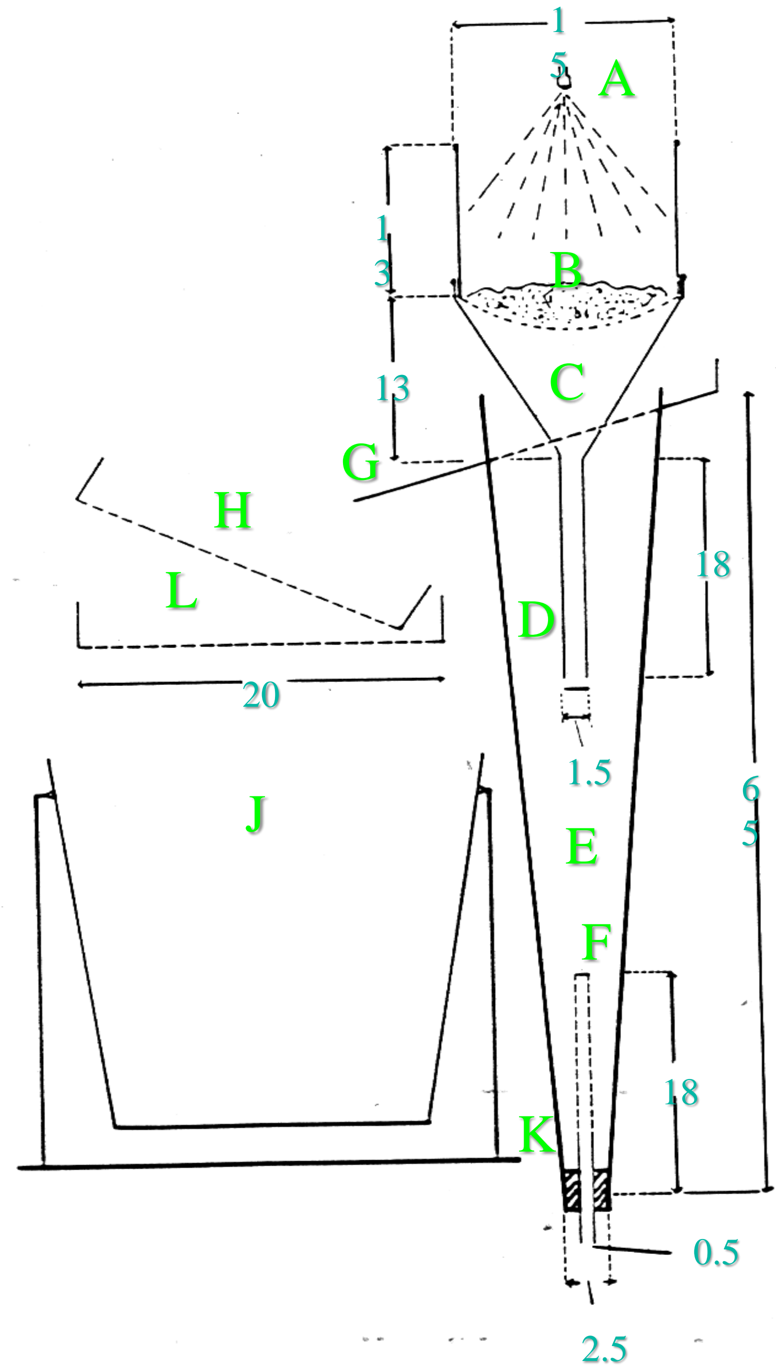
Stained *Meloidogyne* female with egg mass (source unknown)

EXTRACTION FROM SOIL

Standard Oostenbrink elutriator

Scheme

A. nozzle
B. soil sample
C. funnel
D. baffle plate
E. conical column
F. perforated tube
G. overflow spout
H & L. 45-53µm aperture sieves
J. pivoted bucket
K. plug

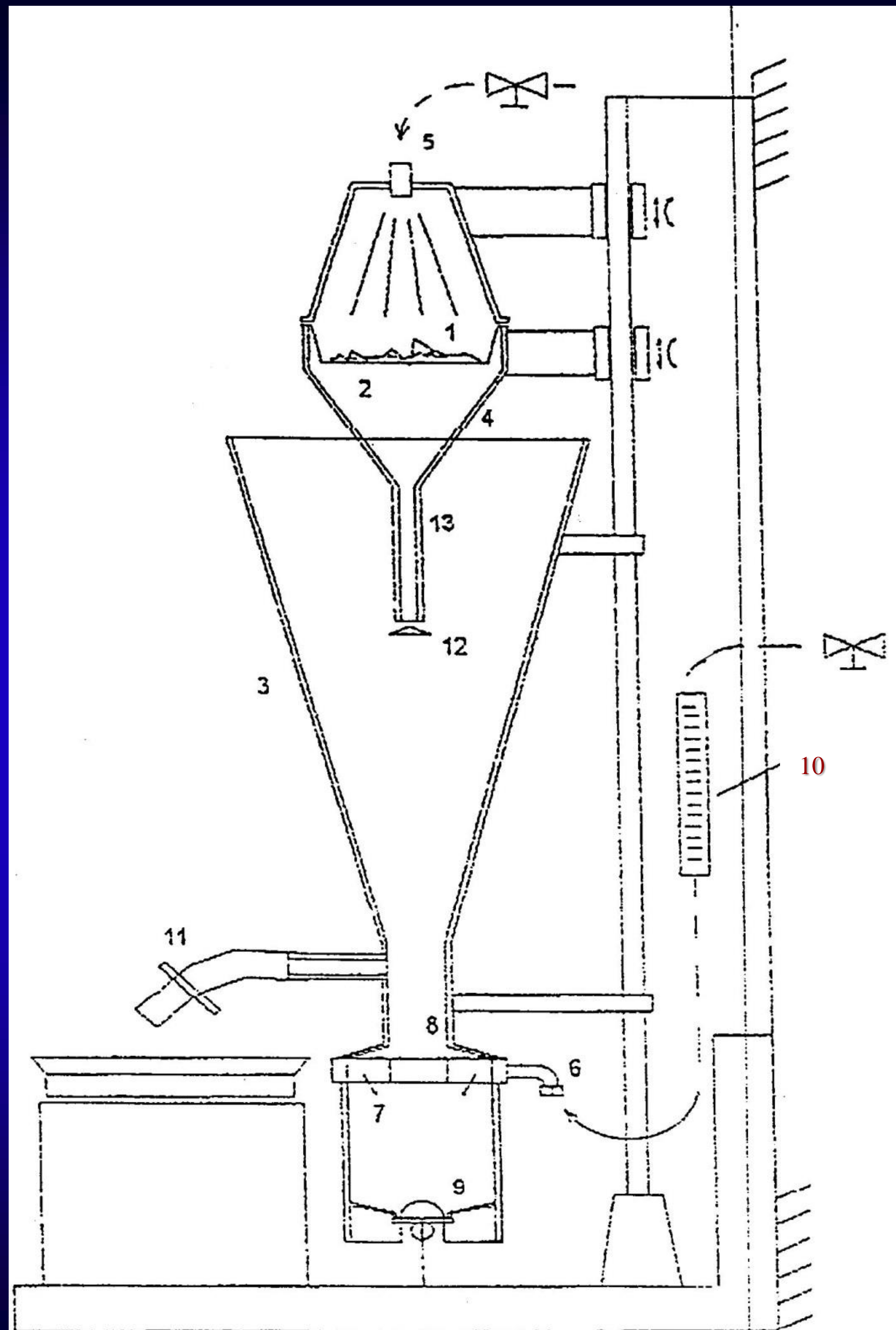


Standard Oostenbrink elutriator made of stainless steel (EPPO Website)

Modified Oostenbrink elutriator - scheme

1. soil sample
2. sieve with 1 mm mesh width;
3. extraction chamber
4. cone
5. rinsing nozzle
6. upward waterflow
7. ring nozzle
8. stricture
9. bottom valve
10. flowmeter
11. hose clamp
12. cone outlet
13. minimal final water level during extraction

45-50 µm aperture sieves are used for collecting all nematdes; 160 – 200 µm aperture sieves are used for collection of large nematodes (ie. *Longidoridae*)

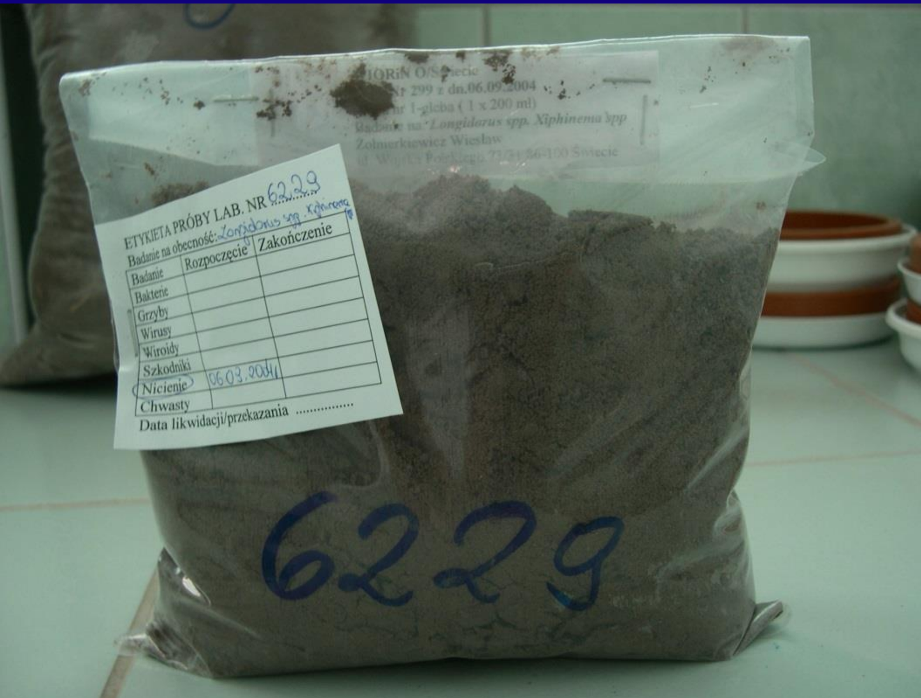


Modified Oostenbrink Elutriator

(phot. W. Karnkowski)

Taking a sub-sample

(phot. SPHSIS, Voivodship Laboratory in Katowice, Poland)



Extraction

(phot. W. Karnkowski)

Collection of extracted material on sieves
(phot. SPHSIS, Voivodship Laboratory in Katowice, Poland)

The material collected on sieves should be placed on Baermann funnel or Oostenbrink dish for further analyses

(phot. left W.Karnkowski; right SPHSIS, Voivodship Laboratory in Katowice, Poland).

Suspension for microscopic analyses

(phot. left W.Karnkowski; right SPHSIS, Voivodship Laboratory in Katowice, Poland).

Extraction of Free Living Nematodes

Sieving Technique

(a UK presentation)

Extraction of Free Living Nematodes

- Equipment
- Baermann funnels
- netting 200µ x 50 µ
- 90µ & 250µ sieves
- 2 basins (bowls)
- 2 beakers 200ml
- 1 pan (rectangular bowl)

Extraction of Free Living Nematodes

Extraction of Free Living Nematodes

- Soil sample 1- 200 g.
- Break up soil with jet of water and fingers.

Extraction of Free Living Nematodes

- Mix sample thoroughly by pouring from basin to basin.

Extraction of Free Living Nematodes

- After mixing, immediately decant through the 250μ sieve.
- Collect decanted mixture which has passed through sieve in second basin.

Extraction of Free Living Nematodes

- Repeat mixing the soil solution a few times until everything is again in suspension.

Extraction of Free Living Nematodes

- Decant through 250µ sieve.
- Discard soil pellet.

Extraction of Free Living Nematodes

- Wash contents from 250μ sieve into beaker.

Extraction of Free Living Nematodes

- Mix soil solution again.
- Leave for 20 - 30 seconds.

Extraction of Free Living Nematodes

- Decant suspension through 90µ sieve.
- Sieve should be wet before use and suspended in water to level of mesh before suspension is poured through.

Extraction of Free Living Nematodes

- Mix suspension again.
- Leave for 30 seconds.
- Decant through 90µ sieve.
- Repeat third time if necessary.

Extraction of Free Living Nematodes

- Wash contents of 90µ sieve into second beaker each time solution is decanted.

Extraction of Free Living Nematodes

- Set Baermann sieve in pan.
- Pour from beaker containing solution from 250µ sieve.
- Remove sieve from pan and place in funnel.

Extraction of Free Living Nematodes

- Pour contents of pan into second beaker containing debris from 90µ sieve.
- Place Baermann sieve with filter inlay into pan.
- Pour contents of second beaker through sieve.

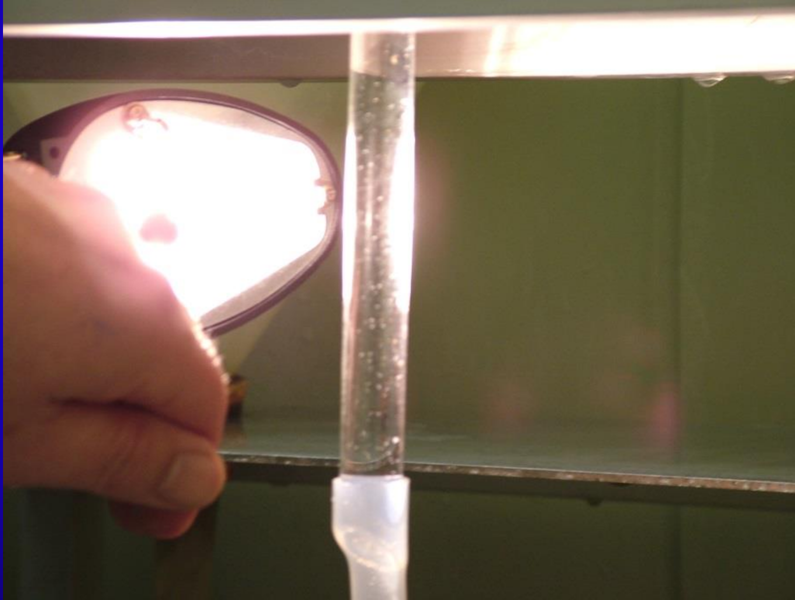
Extraction of Free Living Nematodes

- Transfer sieve to funnel before solution has drained through.

Extraction of Free Living Nematodes

- Top up funnels with room temperature water until it just touches the base of the sieves.
- Ensure there are no air spaces between sieve and filter paper.

Extraction of Free Living Nematodes



- Leave for 24 - 48 hours.
- Examine contents of bottom vial.

Analysis of an extract under stereoscopic microscope

(phot. SPHSIS, Voivodship Laboratory in Bydgoszcz, Poland).

Centrifugal flotation

a) It is the only method that allows extraction of **motile and immotile** nematodes from soil.
b) Nematode specimen are brought into a suspension with a specific gravity greater than their own, which is about 1.08, so they will float and heavier soil particles will sink.
c) Centrifugation is used to speed up the separation of the sinking fraction and floating fraction. It is also used to clean extracts obtained by sieving or elutriation.

PROCEDURE

- Fill 1000 ml centrifuge tube with up to 250 ml soil
- Add about 400 ml water plus a spoon of kaolin; kaolin forms a visible white layer that separates the sediment and nematodes from the supernatant (water and light organic material)
- Stir suspension thoroughly with stirrer or Vibro mixer to form a homogenous suspension
- Centrifuge tubes for approx. 4 minutes at 1800 g; time and g-force is not that critical, as long as a stable pellet is achieved; time length's of 2-5 min and g-forces of 700 to 2900 g can be used
- Gently pour off the supernatant and discard
- Re-suspend the pellet in about 400 ml of a $MgSO_4$ solution (or similar extraction fluid) with a density of 1.15-1.18
- Centrifuge tubes again at 1800 g for 4 min
- Gently pour the supernatant containing the nematodes over a 20 or 25 µm sieve
- Rinse the sieve immediately with water to remove the $MgSO_4$ solution
- Nematodes are transferred from the sieve in a glass beaker for examination at 25-40x magnification.