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A method for the detection of the resting sporangia of potato wart disease *(Synchytrium endobioticum)* in the soil of old outbreak sites

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Zusammenfassung, Résumé p. 230

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Summary

A method is described for the sampling of soils of old potato wart outbreak sites and the extraction of the resting sporangia of the causative fungus *Synchytrium endobioticum* (Schilb.) Perc. by a process of wet-sieving and chloroform flotation. A procedure for isolating individual spores for germination test or further study is also described. The method has been successfully used to recover spores from soils from outbreaks which occurred from 4–70 years previously and has made possible the study of long-term survival and decline of resting spore populations in field soil.

Introduction

In Scotland almost 10 000 ha of arable land are subject to one of the several government orders relating to potato wart disease caused by *Synchytrium endobioticum* (Schilb.) Perc. These orders (Anon., 1912–1973) restrict potato growing on land where wart disease has occurred and designate such land as 'scheduled'. Cultivation of potato cultivars known to be immune from wart disease and approved by the Department of Agriculture was permitted on scheduled land, but on land scheduled since the 1973 Order no potatoes may be grown and on the remaining fields of the farm, which are designated as a 'safety zone', only approved immune cultivars are permitted.

These restrictions have successfully prevented spread of the disease in Scotland to the extent that there have been only 4 occurrences on farms in the last 20 years and only one in the last 10 years. Noble & Glynne (1970) report an isolated finding of resting sporangia remaining viable for 10 years but Olsen (1961) and Hartman (1955) showed that spores can remain viable for much longer periods and McDonnell & Kavanagh (1980) claim viability, as assessed by Glynne's acid fuchsin test (Glynne, 1926) for 31 years. From these observations and from the author's work (unpublished) it seems likely that the pathogen may survive in soil for up to 30 to 40 years.

Of 317 outbreaks of wart disease recorded on farms in Scotland, 215, involving nearly 6000 ha, occurred before 1924 and are therefore at least 60 years old. After that time it is unlikely that they contain viable sporangia. Before considering 'descheduling' such farms, effective techniques must be available to sample the soil and extract remaining

resting spores for examination and viability. The techniques published by Glynne (1926), Mygind (1954 & 1961), Nelson & Olsen (1964), Marcus (1969), Pratt (1976) and Potoček (1977) were developed for qualitative testing and those of Nelson & Olsen and Potoček destroy the viability of the spores. None of these methods was suitable for investigating old outbreak sites with a view to descheduling them.

The methods described here were used to recover resting sporangia from outbreak sites of a wide range of ages and they detail the isolation of single spores for viability testing or critical microscopical examination.

Materials and methods

The distribution of the wart pathogen in arable fields is known to be patchy and unpredictable, probably arising from the random planting of diseased seed tubers. Therefore it is necessary to sample most old outbreak sites methodically to maximize the chances of recovering resting sporangia in sufficient numbers to gain a clear impression of their state of preservation. Occasionally, detailed site records or the proximity of a garden implicated as a source allow intensive sampling of the original focus of infection.

The field is divided into regular units of not more than 0.41 ha (1 acre), less than this if the field is small, and 50 cores of soil per unit taken with a 2.5-cm diameter auger from the 7.5–15 cm soil layer from evenly spaced sites in the unit to give a bulk sample of ca 1 kg. When an area likely to contain resting spores occurs in a unit, such as an old potato field storage site, a gateway or land adjacent to a garden, this is sampled intensively and the remainder of the 50 cores taken evenly from the rest of the unit. These bulk samples are air-dried, crushed and sieved through a 3-mm mesh sieve to remove stones, roots etc., and after mixing a 100 g subsample is taken from each by the halving method of Thomson & Doyle (1955).

The subsample is soaked overnight in a 4-litre beaker containing 2 l 0.1 % Triton X100 (iso-octyl phenoxy polyethoxy ethanol ethylene oxide, BDH Chemicals Ltd, Poole, England) before fitting the beaker with a 2-mm mesh gauze screen and a laboratory stirrer (Fig. 1). The stirrer is started and one side of a double-headed peristaltic pump (Glen Creston Ltd, Stanmore, England), running at 100 rpm, gradually applies the soil slurry to the top of a 20-cm diameter sieve stack consisting of 300, 180, 100, 75, 35 and 23.5 μm mesh aperture sieves arranged in descending order of mesh size. The last three sieves each have a coarse (0.5 mm aperture) backing gauze fitted in close contact with the underside of the mesh.

Mechanical wet sieving is carried out with a Pascall 'Inclyno' Model 1 sieve shaker (Pascall Engineering, Crawley, Sussex) and is continued until the water draining from the bottom of the sieve stack is almost clear – this usually takes ca 40 min. During sieving, the volume of slurry pumped from the extraction beaker is replaced with water from a static supply, the first 5 litres of which contains 0.1 % Triton X100, by the second peristaltic pump head. On completion of sieving the remaining contents of the extraction beaker – water, coarse sand, small stones and the contents of the delivery tube – are added to the top sieve of the stack before it is fitted with a water spray head whose 7 hollow cone jets wash the sample. This is operated for 5–10 min with the shaking action to complete the fractionation of the sample and clean the last two fractions.

The sieve stack is dismantled and the material washed from the 35 and 23.5 μm mesh

Fig. 1. Apparatus used for resting spore extraction and wet sieving (the water level in the extraction beaker has been lowered to show gauze screen). Double pump (centre) transfers soil slurry from the beaker to the top of the sieve stack and also maintains a constant water level in the beaker.

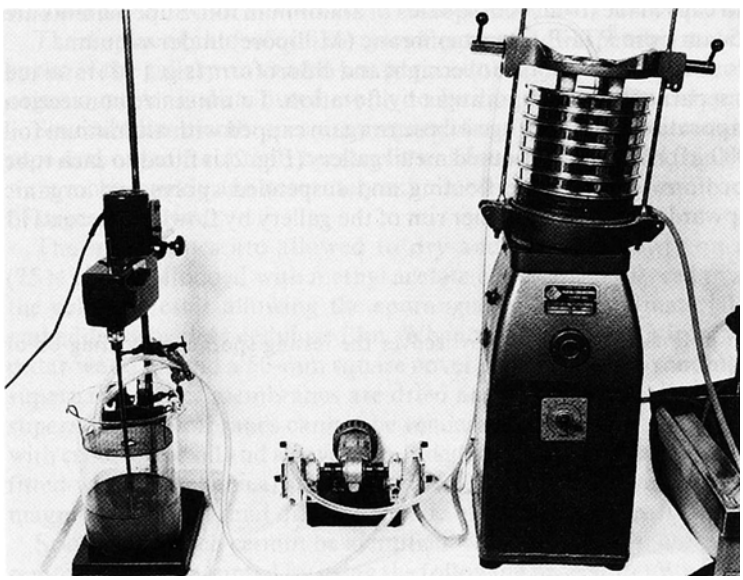

Abb. 1. Für die Dauersporen-Extraktion und Nassiebung verwendeter Apparat (wasserstand im Extraktionsbecher zur Sichtbarmachung des Gazesiebes gesenkt). Doppelpumpe (Mitte) führt Bodenschlämmung vom Becher zur Spitze des Siebsatzes und erhält auch einen konstanten Wasserstand im Becher.
Fig. 1. Appareil utilisé pour l'extraction des spores et le tamisage humide (le niveau d'eau dans le becher a été abaissé pour montrer le tamis métallique). La pompe double (au centre) transfert le dépôt de terre du becher au sommet de la colonne de tamisage et maintient un niveau d'eau constant dans le becher.

sieves into 50 ml polypropylene centrifuge tubes which have been prepared by trimming any roughness from the top inside edge, dipping in 2 % Arquad 18:50 antistatic solution (AKZO Chemie UK Ltd, Hersham, Walton-on-Thames, England) and draining dry. The soil fractions are washed from the sieves with a fine jet made from a G 20 (0.5-mm bore) hypodermic needle and approximately 4 ml of soil particles added to each tube. Remaining soil particles and sporangia trapped in the sieve meshes are released by briefly immersing the mesh horizontally in water in an ultrasonic cleaning bath (Kerry Ultrasonics Ltd, Hitchin, England), washing them from the sieve and adding to the remainder of the respective fractions.

The tubes containing soil and water are centrifuged at 2000 *g* for 10 min and to avoid loss of spores the supernatants are decanted through a 47-mm diameter 8-μm pore

cellulose ester filter membrane (Millipore (UK) Ltd, Park Royal, London) under vacuum. The soil pellets are dehydrated by adding acetone, with thorough mixing, and centrifuging at 2000 *g* for 5 min. Three repetitions of this treatment are usually required to complete dehydration. To minimize evaporation of acetone during centrifugation, the tubes are covered with caps made from 5-cm squares of aluminium foil. Supernatants are decanted through a 5-μm pore PTFE filter membrane (Millipore) under vacuum.

Residual acetone is allowed to evaporate overnight and chloroform (s.g. 1.48) is mixed with the dried soil to separate the wart sporangia by flotation. To minimize convection currents set up by evaporation the centrifuge tubes are again capped with aluminium foil and centrifuged at 2000 *g* for 20 min. A spouted metal gallery (Fig. 2) is fitted to each tube in turn and the chloroform carrying the floating and suspended spores and organic matter is displaced upwards and over the inner rim of the gallery by flowing Arcton 113

Fig. 2. Apparatus used in flotation procedure to recover the resting sporangia floating on or suspended in the chloroform.

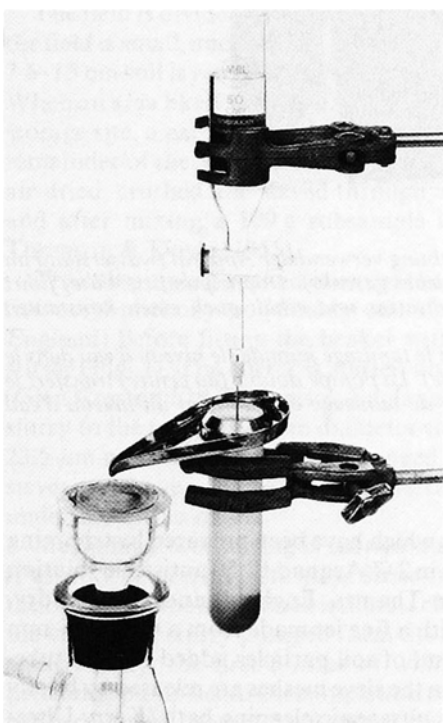

Abb. 2. Apparat, welcher für die Flotations-Prozedur zur Gewinnung der in Chloroform suspendierten oder treibenden Sporen verwendet wurde.
Fig. 2. Appareil utilisé dans le processus de flotaison pour recueillir les sporanges restants, en flotaison ou en suspension dans le chloroforme.

(s.g.1.63) (trichlorotrifluoroethane; Imperial Chemical Industries Ltd, Runcorn, England) slowly through a 14-cm long G 20 hypodermic needle to the lower region of the centrifuge tube (Fig. 2). The lower end of the hypodermic needle is sealed and small apertures cut through the wall immediately above the seal allow the Arcton 113 to flow laterally from the tip of the needle without disturbing the pelleted soil particles.

The displaced chloroform carrying floating and suspended material is directed via the spout of the gallery on to an 8-μm pore cellulose ester membrane under vacuum and the organic matter is distributed evenly to facilitate microscopical examination.

Several filter membranes are prepared from each tube, the number depending on soil type and organic content, because it was found that several thinly covered membranes were more quickly and accurately examined than a smaller number with a heavy deposit of organic matter.

The membranes are allowed to dry and lowered slowly on to a thin glass plate (75 × 63 mm) flooded with methyl acetate containing 10 % cedar wood oil. This softens the cellulose ester allowing the sporangia and organic material to become partially embedded in a clear cellulose film. When this film is dry, it is treated with 3–4 drops of cedar wood oil and a 50-mm square cover slip is applied to complete the mount. Water supernatant filter membranes are dried and mounted as above but the PTFE acetone supernatant membranes cannot be rendered transparent and are simply dried, treated with cedar wood oil and a cover slip added. Examination is carried out with a microscope fitted with a mechanical stage (modified if necessary to accept the glass plate) at ×100 magnification and final diagnosis made at ×400 magnification.

Sporangia which cannot be identified with certainty on the cellulose ester mounts are removed and remounted by using the following procedure. While the spore is retained in view at low power, the coverslip is prised away from the glass examination plate and removed. The spore, embedded in the cellulose film, is centred in the microscope field and the objective racked upwards. The area of cellulose membrane with the spore at its centre is defined by the microscope illumination and a small square (ca 2 × 2 mm) containing the spore is cut from it with a scalpel. This square is transferred to the centre of a 1.2-μm pore polyamide filter membrane (Sartorius, PO Box 142, Göttingen, W. Germany) on a vacuum filter apparatus and drops of methyl acetate applied to it until the cellulose is seen to dissolve. The vacuum is then applied to remove the solvent and dissolved membrane leaving the separated spore which is transferred, with a single-haired 'brush', to a drop of 50 % glycerine in water on a standard microscope slide. A cover slip is added to complete the mount. A spore so mounted can be examined by using an oil immersion objective if necessary. It can also be 'rolled over' by movement of the coverslip to check for plasmolysis of the zoospore primordia and it can be readily removed from the mount and washed for a later germination test if required.

Prevention of cross contamination

When soils from several old outbreak sites are examined, some perhaps with high concentrations of spores, the apparatus must be thoroughly cleaned between sites to avoid carry-over of sporangia. An ultrasonic cleaning bath has proved very efficient especially for cleaning fine mesh sieves, which are easily damaged by brushing and the mesh aperture size altered.

Sieves are therefore washed and then immersed in a solution of 4 % Decon 90 or Neutracon (Decon Laboratories Ltd, Brighton, England) in the ultrasonic cleaning bath for 5 min, turned over and treated for a further 5 min before rinsing. All other apparatus is treated in the cleaning bath for 10 min and to prevent spore adhesion the overflow gallery and stainless steel needle are polished between sites. If the apparatus is immersed in 10 % hypochlorite solution (Deosan; Diversity Ltd, Annan, Scotland) for 20 min after washing, any remaining sporangia contaminating the following sample can be recognized by their bleached appearance.

Effect of chemicals on viability of sporangia

The chemicals and solvents used in the extraction procedure were tested for toxicity by immersing sporangia of known percentage germination in them for at least three times the maximum contact time during processing. The chemicals were washed off or allowed to evaporate and the sporangia retested for percentage germination.

No reduction in germination was observed after treatment with chloroform, Arcton 113, acetone, methyl acetate, 0.1 % Triton X100, 50 % glycerine or cedar wood oil. Nevertheless, sporangia are not allowed to stand unnecessarily in solvents and sporangia required for subsequent testing are removed from the cedar wood oil mounts as soon as possible after identification.

Efficiency of detection of sporangia

It is difficult to assess the efficiency of recovery of sporangia from naturally infested soils but the following procedure was adopted as a suitable compromise. Fifty single resting spores were counted into each of 5 samples of dried field soil contained in 4-litre extraction beakers. The soil was mixed, wetted, mixed again and allowed to air-dry in an attempt to incorporate the sporangia artificially into soil aggregates. The samples were then processed as described. An average of 42 spores per sample were recovered, a detection rate of ca 84 %.

The procedures here described will detect single spores only in the size range 74–23.5 μm. In laboratory studies, thin-walled sporangia of less than 23 μm have occasionally been seen but they are thought to be short-lived in field conditions and have never been observed in soils from old outbreaks. No sporangia larger than 70 μm have been observed.

When soils from recent outbreaks are processed, warted potato host tissue may not be completely decomposed and adjacent infected cells remain attached to one another. An accurate assessment of the resting sporangia population will then be obtained only when the fractions held by the 75-μm and 100-μm sieves are examined for clumps of sporangia.

Discussion

Efficient fractionation of soil samples necessitates the breakdown of soil aggregates into individual particles. Comparisons between wet and dry sieving with samples of a medium loam soil showed that aggregates break down more readily by using wet sieving which yields 30 % more particles in the resting spore size range.

Breakdown is further improved in the early stages of soil extraction by using the non-ionic wetting agent Triton X100 which also reduces spore losses on the apparatus due to static charges. No wetter is used during the later stages of sieving because the reduction of surface tension diminishes the rate at which water passes through the smaller sieve meshes. The addition of a coarse backing gauze on the 75, 35 and 23.5 μm sieves greatly increases their rate of sieving.

The rate and efficiency of sieving is dependent on the type of shaker employed. The intense jolting action of the Pascall Machine shakes water droplets forcibly from the lower surface of the sieve creating a suction effect which gives almost 30 % more particles in the spore size range than a vibrating shaker.

It is important to abide by the manufacturer's recommendations regarding the maximum charge for each sieve mesh and select a total sample size such that no fraction of it will overload its respective sieve. Preliminary tests indicated that 105 g of medium loam soil was the maximum that could be handled without exceeding the recommended load of 9–10 g for the finest mesh sieve and a 100-g sample was standardized since it gives rapid and reproducible results with both clay and organic soils.

Sieving efficiency is improved when the sample is applied gradually to the sieve stack and this is achieved by pumping the soil slurry progressively from the extraction beaker where the finer particles, which are more easily suspended, are sucked up and applied first to the unobstructed sieves where they can pass without hindrance to the mesh which retains them.

A medium loam soil gives an average of 15–20 mounts for examination including the water and acetone supernatant mounts but numbers vary from 8–60 from each 100-g soil sample depending on soil type. Clay soils process rapidly and give few filter membrane mounts while peaty organic soils require more time and yield more mounts.

Glynne (1926) used chloroform flotation to separate resting sporangia from silica particles but tests of her method revealed that up to 70 % of sporangia could be lost during this stage of the process. After development of this stage however, using Arcton 113 and the overflow gallery as described, over 90 % of sporangia are routinely recovered. It is clear from the foregoing that sporangia can be lost on any item of apparatus which they contact. It follows that apparatus should be kept to a minimum and in this method, after sieving, spores contact only the polypropylene tubes, overflow gallery and hypodermic needle. Losses on the tubes are minimized by antistatic treatment and on the last two items by maintaining them in a finely polished condition.

The described methods have been in use in this laboratory for several years and have proved to be effective for the examination of soils from over 20 old outbreak sites ranging in age from 4–71 years. In each case, sporangia or their identifiable remains were recovered enabling studies to be made on the long-term survival and decline of populations of sporangia in field soils. The method does not employ chemicals which are hazardous to the operator or toxic to the sporangia.

Acknowledgement

I would like to thank Mrs J. Quinn for excellent technical assistance and patience during the development of this technique.

Zusammenfassung

Eine Methode zur Ermittlung von Dauersporangien des Kartoffelkrebses (Synchytrium endobioticum) aus Boden von alten Herden

Schottland hat fast 10 000 ha Ackerland, auf welchem Kartoffelbau durch Regierungsverordnung wegen des Vorkommens von Kartoffelkrebs (*Synchytrium endobioticum* (Schilb.) Perc.) eingeschränkt ist. In vielen Fällen sind die Beschränkungen seit über 60 Jahren in Kraft. Obwohl bekannt ist, dass der Erreger zwischen 30 und 40 Jahre lang lebensfähig bleibt, ergibt sich die Notwendigkeit einer Studie über die Langlebigkeit und den Rückgang von Populationen von Dauersporangien in alten Herden vor der Rücknahme legislativer Restriktionen für den in betracht kommenden Anbau von Kartoffeln. Die beschriebene Methode wurde für diesen Zweck für die Gewinnung von Dauersporen entwickelt.

Das Feld mit dem Krebsherd wird in Einheiten von 0,41 ha oder weniger unterteilt, ein 25 cm-Bohrer wird, für die Entnahme von 50 Bodenproben, gleichmässig über jede Einheit verteilt, für eine Sammelprobe von 1 kg verwendet. Diese wird luftgetrocknet, zerrieben, gesiebt und gemischt, dann wird eine 100 g-Einzelprobe für die Sporenextrahierung genommen. Unter Verwendung des in Abb. 1 gezeigten Apparates werden Einzelproben über Nacht in 0,1 % Triton X100-Lösung eingeweicht, um Bodenaggregate auseinanderzubrechen. Der daraus entstehende aufgerührte Schlamm wird stufenweise auf den oberen Teil eines Siebsatzes mit 20 cm Durchmesser gepumpt. Die 6 Siebe sind bei der Maschenweite entsprechend in abnehmender Grösse (300, 180, 100, 75, 35 und 23,5 μm) angeordnet. Die Nassiebung erfolgt mittels Schüttelapparat, bis das Wasser an der Basis des Siebsatzes klar ist. Die Bodenfraktionen werden gewaschen und das Material in Sporengrösse von den letzten beiden Sieben in 50 ml Zentrifugenröhrchen aus Propylen überführt, die mit antistatischer Lösung behandelt worden waren. Das sporenhaltige Material wird durch dreimalige Behandlung mit Aceton dehydriert; anschliessend wird zentrifugiert und die Sporangien durch Chloroform-Flotation separiert. Danach wird zentrifugiert und mit Hilfe des in Abb. 2 dargestellten Apparates mit Trichlorfluoräthan aufgeschwemmt. Sporangien und organische Masse werden gesammelt und gleichmässig auf eine 8 μm Celluloseester-Porenfiltermembran im Vakuum verteilt. Mikroskopische Präparate werden angefertigt, indem die Zellulosemembran auf eine 75 × 63 mm grosse Glasplatte aufgebracht wird und mit Methylacetat/Zedernölgemisch zum Klären und Erweichen der Membran und der teilweise eingebetteten Sporangien plus organischer Masse behandelt wird. Nach Abtrocknen wird die Membran mit 3–4 Tropfen Zedernöl behandelt und mit einem quadratischen 50 mm-Deckglas abgedeckt. Die Präparate werden mit Hilfe eines Mikroskopes mit mechanischer Führung (Kreuztisch) methodisch bei 100 × Vergrösserung durchkontrolliert. Die entgültige Diagnose erfolgt dann bei 400-facher Vergrösserung. Sporangien für kritischere Untersuchungen oder Keimtests können vom Präparat durch Ausschneiden kleiner Quadrate der Zellulose mit der Spore in der Mitte gewonnen werden. Dieses Quadrat wird auf eine Polyamidmembran plaziert und die Zellulose mit Methylacetat aufgelöst, wobei die separierte Spore für Präparierung oder Test auf Lebensfähigkeit übrig bleibt.

Der Apparat wird mit Ultraschall gereinigt, Kreuz-Kontaminationen werden durch Tauchen der Siebe in Hypochloridlösung vermieden.

Die Methode konnte erfolgreich zur Untersuchung der Lebensfähigkeit und dem Rückgang von Populationen von Sporangien verwendet werden, wobei die untersuchten Bodenproben von Kartoffelkrebsherden 4 bis 71 Jahre alt waren.

Résumé

Methode pour la détection de sporanges de galle verruqueuse de la pomme de terre (Synchytrium endobioticum) dans les sols de zones anciennement contaminées

L'Ecosse a près de 10 000 ha de terre arable où la culture de la pomme de terre est limitée par

décision gouvernementale en raison de la présence de galle verruqueuse due à *Synchytrium endobioticum* (Schilb.) Perc. En de nombreux endroits, les restrictions sont en vigueur depuis plus de 60 ans alors que le pathogène semble se maintenir pendant 30 à 40 ans; il est donc nécesaire d'étudier la longétivité et la baisse des populations de sporanges dans les zones anciennement contaminées avant de considérer l'abrogation des restrictions législatives concernant cette culture. C'est pourquoi, la méthode décrite a été mise au point pour obtenir des sporanges.

Le champ contaminé est divisé en unités de 0,41 ha au moins et 50 prélèvements de terre sont effectués à intervalles réguliers à l'aide d'une tarrière de 25 cm sur chaque unité, afin d'obtenir des échantillons d'1 kg. Ceux-ci sont séchés à l'air, broyés, tamisés et mélangés et des sous-échantillons de 100 g sont utilisés pour l'extraction de spores. A l'aide de l'appareil présenté dans la fig. 1, les sous-échantillons sont immergés toute une nuit dans une solution de 0,1 % Triton X100 afin de séparer les agrégats et le dépôt résultant est maintenu en agitation et pompé vers le haut d'une colonne de tamisage de 20 cm de diamètre. Les mailles des 6 tamis vont en décroissant (300, 180, 100, 75, 35 et 23,5 μm). Le tamisage humide par secouage en à-coups est prolongé jusqu'à ce que l'eau à la base de la colonne soit claire. Les fractions de sol sont lavées et les particules correspondant à la dimension des spores et, provenant des deux derniers tamis sont transférés dans des tubes de propylène de 50 ml à centrifugation, préalablement traités avec une solution anti-statique. Ces particules sont déshydratées par 3 traitements à l'acétone, suivis d'une centrifugation et les sporanges sont obtenus après flotaison dans le chloroforme, centrifugation et séparation dans le trichlorotrifluoro-éthane, à l'aide de l'appareil illustré dans la fig. 2. Les sporanges et la matière organique sont recueillies et déposées uniformément sur une membrane sous-vide d'éther de cellulose avec des millipores de 8 μm. Les préparations microscopiques sont obtenues en appliquant la membrane de cellulose sur une plaque de verre de 75 × 63 mm montée dans un mélange méthyl-acétate/ huile de bois de cèdre qui clarifie et assouplit la membrane et fixe partiellement les sporanges et la matière organique. La membrane séchée est traitée avec 3–4 gouttes d'huile de bois de cèdre et recouverte d'une lamelle de 50 mm de côté pour compléter les préparations. Celles-ci sont observées méthodiquement à un grossissement de 100 avec un microscope équipé d'une platine à charriot et le diagnostic final des sporanges est fait au grossissement 400. Les sporanges destinés à un examen plus fin ou à des tests de germination sont retriés de la préparation en découpant un petit carré de cellulose avec la spore en son centre. Cette surface est placée sur une membrane de polyamide et la cellulose est dissoute avec le méthyl-acétate, laissant la spore disponible pour un nouveau montage ou un test de viabilité.

L'appareil est nettoyé par ultra-sons et les problèmes de contamination croisée sont évités en trempant les tamis dans une solution d'hypochlorite.

Cette méthode a été utilisée avec succès pour étudier la longévite et la baisse des populations de sporanges extraites de sols contaminés par la galle verruqueuse depuis 4 à 71 ans.

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