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Methods for isolating the alder *Phytophthora*

By J.-C. Streito, G. Jarnouen de Villartay and F. Tabary

Laboratoire National de la Protection des Végétaux, Unité de Mycologie Agricole et Forestière: 38 rue Sainte Catherine 54043 Nancy cedex, France; E-mail: jean-claude.streito@agriculture.gouv.fr

Summary

Phytophthora disease of alder (*Alnus* spp.) is now widespread in Europe. In France, difficulties were initially encountered in attempts to isolate the pathogen from necrotic bark tissue but, with experience, a high degree of success was achieved by direct plating onto agar media. Both corn meal agar and a selective medium derived from PARBHy were satisfactory. Success depended largely on the activity of the fungus in the bark tissue and some evidence was obtained that this varied from year to year. However, autumn was found to be a generally good period for isolation. It is possible to store collected samples at 7 ± 4°C for some days. The alder *Phytophthora* was isolated from the water of two rivers with a baiting method using alder twigs but the efficiency of the method was low.

1 Introduction

A severe dieback of alder (*Alnus* spp.) associated with an undescribed *Phytophthora* species was first reported in 1993, in England (Gibbs et al. 1994; Gibbs 1995). The causal organism was described as an unusual form of *Phytophthora cambivora* and, pending a full description, named the 'alder *Phytophthora*' (Brasier et al. 1995). In France, studies on the alder *Phytophthora* began in 1996 (Streito et al. 2002). The main problem was the frequent failure of isolation attempts. In 1997, 151 samples from necrotic bark lesions were processed by F. Tabary and J-C. Streito (LNPV), but only 15 isolates of the alder *Phytophthora* were obtained. As a result of these difficulties several aspects of the method were studied during 1998, in order to improve the success rate. This note presents the results of this work.

2 Materials and methods

2.1 Isolation from bark necrosis

Diseased trees were selected according to the external symptoms: small, yellow leaves, dieback and tarry exudations on the lower stem (Gibbs 1995). Samples were collected from necrotic lesions at the base of symptomatic trees. For each tree, a piece of bark with attached wood, 10 cm × 10 cm × 3 cm, was taken from the outer edge of the necrotic lesion, wrapped in a plastic bag and brought to the laboratory at ambient temperature.

There they were usually wrapped in damp paper and left in the laboratory at room temperature (25 ± 5°C) overnight before processing. To test the effect of storage on isolation success, samples from 17 trees were divided in two. Half of each sample was left at room temperature overnight and then analysed and the other half was stored at 7 ± 4°C and analysed after 4–29 days.

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At the time of isolation the outer bark was removed with a knife and discarded. The sample was then washed for a few minutes under running tap water and dried with filter paper. The whole surface was disinfected quickly with 70% ethanol and dried again under sterile conditions.

Small pieces (2 mm^2) of inner bark tissue taken from various points around the outer edge of the lesions were then plated directly on corn meal agar (CMA; Difco Laboratories Ltd, Sparks, MD, USA; 17 g CMA in 1 l twice distilled water) or onto a selective medium derived from PARBHy (ROBIN et al. 1998), the composition of which is shown in Table 1. With many samples both media were employed. For each sample, four plates of CMA and four plates of selective medium were used. Each plate was inoculated with eight chips of bark.

Plates were incubated under the following conditions: 12 h daylight at 20 ± 3°C, 12 h dark at 18°C ± 3°C. Colonies were observed after 3 days, by direct observation through the bottom of the plate (magnification 100×), and then once a week for 3 weeks. The alder *Phytophthora* is easily recognized by its ornamented oogonia (BRASIER et al. 1995).

2.2 Baiting in river

Sections cut from 1-year-old shoots of healthy alder (diameter 1 cm, length 10 cm) were joined together to form a small raft, moored to the bank and left to float on the surface of various rivers in north-east France, including the Sarre and the Moselle. After about 1 week (in summer) and about 4 weeks (in winter), necrosis appeared in the bark at the ends of the sections and around injuries. Isolations were attempted using the method described above.

3 Results and discussion

3.1 Isolation from bark

The same basic isolation method was used in both 1997 and 1998, and the results are shown in Table 2. In 1997 the alder *Phytophthora* was obtained from just 10% of the 151 samples. The success rates improved markedly in 1998 when 60% of the 173 bark samples yielded

Table 1. **Composition of the specific culture medium**

17 g corn meal agar (DIFCO) in 1 l twice distilled water
Sterilization 122°C for 20 min, when temperature reaches 45°C add:
0.1 mg pimaricin
10 mg rifampicin
250 mg ampicilin
15 mg benomyl (benlate 50%)
50 mg hymexazole (tachigaren 75%)

Table 2. **Efficiency of *Phytophthora* detection from trunk necrosis in 1997 and 1998**

Year	1997	1998
Number of samples	151	173
Isolation yielding alder *Phytophthora*	15	108
Percentage positive detection	9.9	62.5

the alder *Phytophthora*. In the two years, the samples often came from the same sites and sometimes from the same tree. *Phytophthora* was frequently isolated in 1998 from locations where it was not detected in 1997.

The precise choice of sample material appeared to be the most important factor in explaining these differences. Tarry spots can remain on the trunk for a long time, with the lesions beneath often being old and dry: isolation success was very low from such material. By contrast, isolates of *Phytophthora* were obtained easily from fresh active lesions. In these the necrotic tissues are moist and are continuous with healthy tissues. Commonly, old and fresh necrosis occur together on the same lesion.

In 1997, fresh necrosis was not observed in the north-east of France before July and the best period for sampling was September and October In 1998 fresh bark killing was observed as early as June and successful isolations were obtained at that time. The success rate continued high until the end of October. This suggests that the activity of the pathogen can vary from year to year. In January 1999 a successful isolation was made from Charentes (western France).

3.1.1 Cultural media

Isolation of the alder *Phytophthora* onto both CMA and the selective medium was equally efficient. Thus in 102 samples from which the alder *Phytophthora* was obtained, it was isolated on both media in 95% of cases. Although observations were easier to make on the selective medium because of the sparse contamination, the *Phytophthora* grew slowly on it. In contrast, on CMA there were many contaminants but *Phytophthora* grew faster and could therefore be identified sooner.

3.1.2 Storage of the samples

The comparison between samples analysed immediately and those stored for varying periods in the refrigerator showed that stored samples could maintain good viability. Although the data showed some variability, isolation success after 12 days storage was, at 60%, as high as in the non-stored control sample. The two samples stored for 29 days showed 40% isolation success, as compared with 65% in the controls.

3.2 Baiting in river water

In 1998 the alder *Phytophthora* was isolated once from the river Sarre and several times from the Moselle, between May and September. This was a very low success rate: in total less than 1% of fragments of necrotic bark yielded the alder *Phytophthora*. Other fragments yielded a variety of *Phytophthora* species, principally a *Phytophthora gonapodyides*-like species.

4 Conclusion

When the necrosis is fresh and the pathogen active, isolation of alder *Phytophthora* is easy and methods involving direct isolation onto agar can be readily used. CMA or a selective medium are equally suitable. It is possible to preserve samples in the fridge at 7 ± 4°C for some time without a serious loss of viability. There is no particular time of year for sampling, as the activity of the fungus varies from year to year. However, autumn is usually a good period. Although the successful isolation of the pathogen from river water is of interest, it seems that the alder twig tissues may not be selective enough for much use to be made of the 'raft technique' in ecological and pathological studies of the disease.

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Résumé

Méthodes pour isoler le Phytophthora de l'aulne

Une nouvelle maladie de l'aulne due à un *Phytophthora* a été découverte au Royaume Uni en 1993. Dans un premier temps, l'isolement de l'agent pathogène a été difficile. Par la suite, avec l'expérience, une méthode simple et efficace a été mise au point. Elle consiste à placer des morceaux de tissus prélevés en limite de nécrose au niveau du tronc dans un milieu gélosé. Un milieu sélectif (dérivé du PARHy) et le Corn Meal Agar ont été utilisés avec la même efficacité. Le choix des tissus et le niveau d'activité du champignon sont les deux facteurs de réussite les plus importants. Les échantillons peuvent être prélevés toute l'année à condition que le champignon soit actif. L'automne semble cependant être l'époque la plus favorable. Les échantillons peuvent être conservés en bas du réfrigérateur (7 ± 4°C), sans incidence sur l'analyse, pendant quelques jours. Une technique de piégeage à partir de baguettes d'aulne a permis d'isoler le *Phytophthora* de l'aulne directement dans l'eau de deux rivières. L'efficacité de cette méthode est cependant assez faible.

Zusammenfassung

Isolierungsmethoden für Phytophthora an Erle

Die *Phytophthora*-Erkrankung der Erlen ist heute in Europa weit verbreitet. In Frankreich traten zunächst Schwierigkeiten beim Nachweis des Erregers in Rindennekrosen auf, aber mit zunehmender Erfahrung konnte durch direktes Auflegen von Gewebestücken auf Agar-Medien gute Erfolge erzielt werden. Sowohl CMA als auch ein Selektivmedium (modifiziertes PARBHy) waren ähnlich gut geeignet. Der Isolierungserfolg hing stark von der Pilzaktivität im Rindengewebe ab, die von Jahr zu Jahr unterschiedlich sein kann. Der Herbst scheint jedoch die geeignetste Jahreszeit für die Isolierung zu sein. Die Proben können nach der Entnahme einige Tage im Kühlschrank (7 ± 4°C) gelagert werden. Die Erlen-*Phytophthora* konnte auch direkt aus Wasser von zwei Flüssen mit Erlenzweigen als Köder isoliert werden; diese Methode hatte jedoch eine sehr geringe Erfolgsrate.

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