Infection by *Polymyxa betae* and *Plasmodiophora brassicae* of Roots Containing Root-Inducing Transferred DNA of *Agrobacterium rhizogenes*

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ABSTRACT

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Typical infections of *Polymyxa betae* and *Plasmodiophora brassicae* develop in root organ culture of their host, *Beta vulgaris* or brassicas, containing the root-inducing transferred DNA of *Agrobacterium rhizogenes*. In dual culture, the fungal life cycles consisted of two distinct phases. One occurred in the epidermis of the susceptible host, characterized by the production of primary encysted plasmodia, and the other occurred in the root cortical tissue characterized by the production of cystosori. The principal growth phases are the fragmentation and segmentation of the primary plasmodia and of the cystogenous plasmodia.

Additional key words: clubroot, hairy root, rhizomania.

Plasmodiophora brassicae Woron. and *Polymyxa betae* Keskin are obligate root-inhabiting organisms classified with the Plasmodiophorales (9,11). *Plasmodiophora brassicae* is the causal agent of clubroot in brassicas, *Polymyxa betae* is associated with the disease rhizomania, one of the most important diseases of sugar beet. Rhizomania is caused by beet necrotic yellow vein virus, which is transmitted by *Polymyxa betae* (12). The details of the life cycle of many members of the Plasmodiophorales are still uncertain; some stages resemble Myxomycetes. In most genera of the family, there are two major growth phases in the host root: the root hair phase, characterized by the production of zoosporangia and biflagellate zoospores, and the cortical phase (also termed the secondary or cystogenous phase), characterized by the production of resting spores. Both phases of the life cycle have plasmodia as growth forms (3,8,9,11,23).

The obligate nature of the Plasmodiophorales and the difficulty of obtaining inoculum have significantly retarded study of the host-parasite interactions and the breeding of resistant plants. The use of dual culture to study intransigent fungi was first suggested by Morel (14,15) who inoculated callus tissue cultures with zoospores of *Plasmopara viticola*, the causal organism of downy mildew. Since then, methods have been devised for growing several host-fungus combinations, and occasionally it has been possible to derive a pure culture of a fungus from such dual cultures (7).

The successful callus culture of *Plasmodiophora brassicae* starting from naturally infected tissue has been reported (6,8,18,20,22). However, until now, it has not been possible to infect healthy brassica callus *in vitro* with inoculum either of resting spores or plasmodia of *Plasmodiophora brassicae* (1). No similar reports exist of *Polymyxa betae* in tissues of their hosts. Presumably, failures have not been published.

As the endophytes require host roots for their development, we established them in dual cultures of host roots containing the root inducing (Ri) transferred DNA (T-DNA) of *Agrobacterium rhizogenes* strain A_4. This bacterium was originally isolated by Peter Ark (University of California, Berkeley) from naturally infected roses exhibiting hairy root symptoms (13). In axenic continuous culture, genetically transformed roots differ from normal roots; they grow faster and are phytohormonally independent. The remarkable growth potential in culture of roots containing the Ri T-DNA of *A. rhizogenes* was first observed by Tepfer and Tempé (19). The first infections of Ri T-DNA root cultures with *Polymyxa betae* and *Plasmodiophora brassicae* are described here.

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MATERIALS AND METHODS

Establishment of transformed roots. Moore et al (13) and Tepfer and Tempé (19) described the technique of transformed roots.

Bacterial culture media. A. rhizogenes, strain A_4 (ATCC 31798), was cultivated for 2 days at 28 C in liquid medium containing (in grams per liter): yeast extract (Difco), 1; K_2HPO_4, 0.5; NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.004; adjusted to pH 6.9.

Inoculation of plant material. For dual culture with *Polymyxa betae*, we transformed roots of sugar beet (*Beta vulgaris* L.) and red beet (*B. vulgaris*). Fresh roots of beets were soaked in a $HgCl_2$ solution (0.2%) for 5 min, then washed with sterile water, cut into disks, and placed on a 1% agar plate. Each disk was inoculated with 0.1 ml of cell culture (about 10^8 bacteria). For dual culture with *Plasmodiophora brassicae*, we transformed roots of rapeseed (*Brassica napus* L.) and of mustard (*Brassica hirta* Moench.). Plant shoots were grown under sterile conditions on Murashige and Skoog's (MS) medium (17) but containing 0.33 g/L^{-1} of NH_4NO_3 and 0.38 g/L^{-1} of KNO_3. The shoots were wounded with a scalpel and inoculated with a drop of cell culture of *A. rhizogenes*.

Culture of roots. Induced roots were explanted 4 wk after infection and cultured on 2% agar MS medium containing carbenicillin (0.5 mg/ml). Roots free of bacteria were maintained on agar medium without carbenicillin and were subcultured every 2 wk in the liquid MS medium (Fig. 1).

Opine assay. Root extracts were analyzed for opines as described (19). Mannopine and agropine were extracted by homogenizing roots with ethanol (1 ml/g of fresh weight) and centrifuged at 15,000 × g for 10 min; 10 and 20 μl of the supernatant and the standard samples (authentic mannopine and agropine extracted from transformed tobacco calli) were spotted on Whatman 3MM paper and electrophoresed (about 100 V/cm, 20 min). The buffers used were formic acid, acetic acid, and water (30:60:910, v/v/v). The dried chromatogram was stained with an alkaline silver nitrate reagent and shows that the transformed roots produced agropine and mannopine (Fig. 2). These compounds were absent in nontransformed roots. Because the opine synthesis of plants infected with *A. rhizogenes* is encoded by T-DNA of the plasmid Ri (2,21), the present result indicates stable maintenance of T-DNA in the transformed roots.

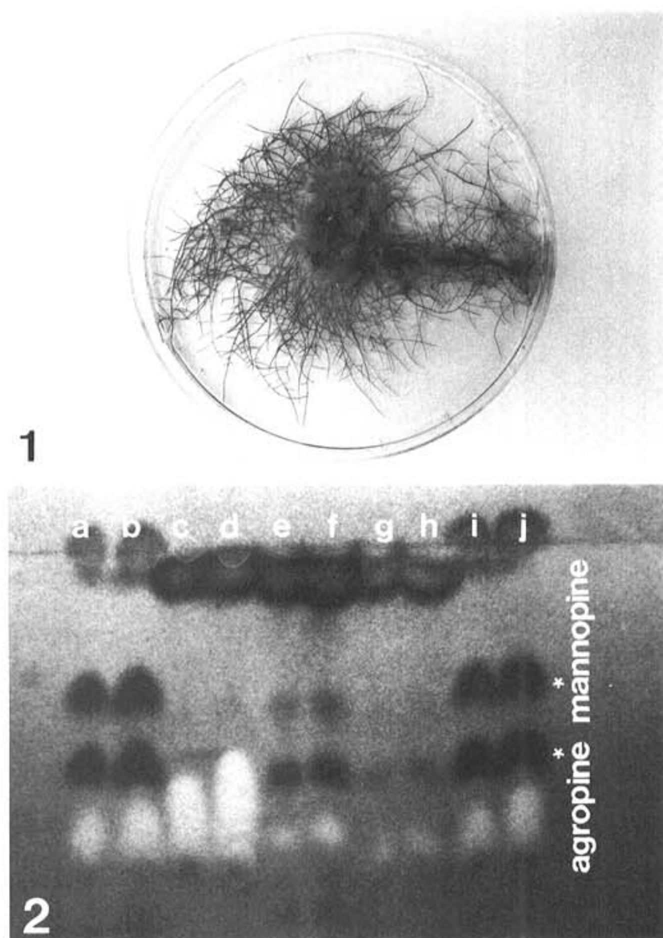
Establishment of fungal inocula. The major problem in dual culture of obligate parasites and their hosts is to avoid bacterial contamination: It is advisable to start with clean preparations of active parasites, freshly extracted from infected plants.

Inoculum of Plasmodiophora brassicae. Methods have been described for isolating plasmodia (10) or spores (4) of *Plasmodiophora brassicae*. Cabbages were grown in soil infested with resting spores of *Plasmodiophora brassicae*. Mature clubroots were removed from infected plants, washed with tap water, surface-sterilized in the $HgCl_2$-Tween solution for 20 min, and washed with sterile water. As the resting spores are free in host cells, not united in cystosori, they can be liberated from club tissues by grinding in a Waring blender. The crude extracts were centrifuged at $400 \times g$ for 5 min. The turbid supernatant containing bacteria and host constituents were discarded, and the residues were resuspended in sterile distilled water. Resting spores were extracted from this suspension by passing through a series of graded sieves. The suspension of spores was filtered through an 8 μm Millipore filter, which is small enough to retain spores, 10–15 μm in diameter. The spores were then resuspended in sterile water to give a final concentration of 10^9 spores per milliliter estimated with a hemocytometer. Numbers of bacteria remaining after the purification procedure were estimated by standard agar plate counting techniques. Plate cultures of brassica roots were inoculated with approximately 10^6 spores.

Inoculum of Polymyxa betae. The cysts of *Polymyxa betae* are aggregated in host cells to form sori (Fig. 3A and B); the intact infected roots of sugar beet containing cystosori were the most easily handled starting material from which to initiate dual cultures. Sugar beets were grown in soil infested with *Polymyxa betae*. Infected roots were surface-sterilized as described above. Pieces of the roots in a drop of water were then placed near the transformed roots for 24 hr after which they were removed and subcultured in the liquid medium. The short period of contact with the surface-sterilized infected roots guards against contamination by other fungi in these roots. But many preparations of the transformed roots were contaminated by bacteria and also by *Olpidium*-like fungi and Protozoa. Only the few free from such contamination were used in subsequent experiments.

RESULTS AND DISCUSSION

Many parasitic plasmodia occurred in the epidermal host cells 4–5 days after inoculation with *Plasmodiophora brassicae* or *Polymyxa betae*. The exact method of entry and presence or absence of primary zoospores could not be observed. The plasmodia were surrounded by a host membrane and contained many lipid droplets and organelles. Between 7 and 10 days after inoculation, the cytoplasm of plasmodia became segmented. Each segment was rounded off, developed a fairly thick wall, and became a cyst (Figs. 3–5). *Polymyxa betae* is strikingly similar to *Plasmodiophora brassicae* in its early stages of development. The cysts, 10 or more in a cell, globular, spherical, 10–30 μm in diameter, were confined to the epidermal cells, and at maturity (10–15 days) the tissue was totally infected, as shown in Figure 3A. The epidermis sloughed during root growth and the cysts were liberated into the medium (Fig. 3B). The primary phase in dual culture was similar to those occurring in naturally infected cabbage (9) or beet (11).



Figs. 1 and 2. Transformed root organ cultures and opine analysis. **1,** Axenic transformed roots of red beet obtained from primary hairy roots incited by *Agrobacterium rhizogenes*, strain A_4 (ATCC 31798), 10-day-old culture in Murashige and Skoog's medium (17). (Petri dish, 144-mm diameter.) **2,** Opines in axenic transformed roots. Lanes a, b, i, and j, authentic mannopine and agropine; lanes c and d, nontransformed roots; lanes e and f, red beet transformed roots; lanes g and h, *Phaseolus vulgaris* used to establish dual culture of vesicular-arbuscular mycorrhizal fungi (16).

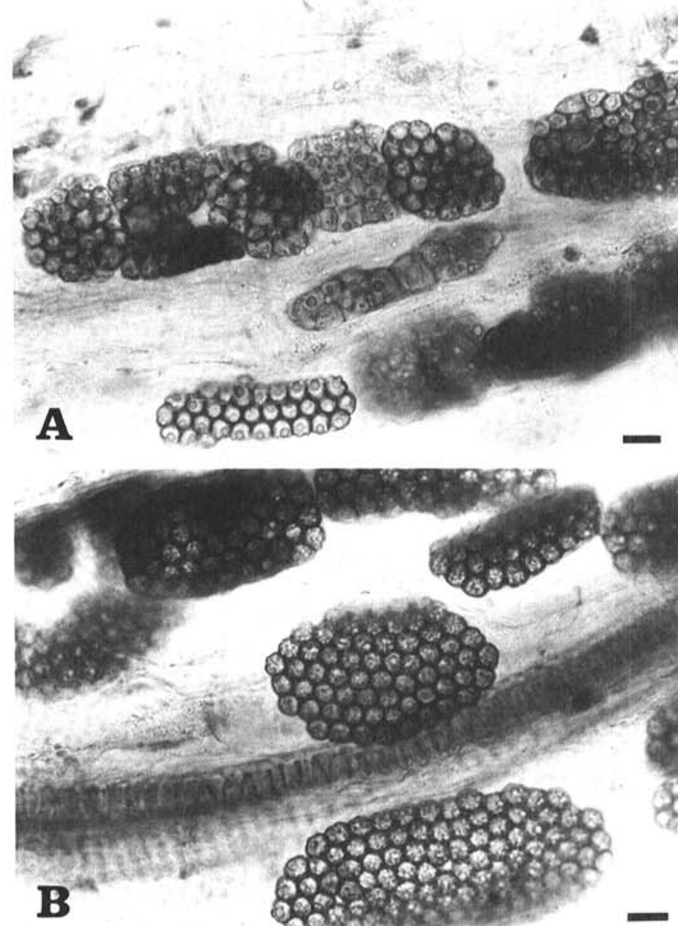


Fig. 3. Sugar beet root naturally infected by *Polymyxa betae* cystosori (bar = 20 μm). **A,** Cystosori during the maturation process. **B,** Mature cystosori near the central cylinder (roots stained by trypan blue).

Roots with primary cysts were maintained in liquid media for a further 10–15 days, but no secondary flagellate zoospores were ever seen. Typical secondary zoospores of *Plasmodiophora brassicae* have been described as easily distinguishable on the basis of swimming pattern and morphology (9); four to eight zoospores are produced in sporangiosori, each zoospore measuring 6 μm in diameter with a shorter, blunt flagellum 3–4 μm long and a longer flagellum with a whiplash tip 12–25 μm long. According to Keskin (11), secondary zoospores of *Polymyxa betae* were similar in shape, size, and structure to the zoospores of *Plasmodiophora brassicae*. In this study, one might suppose that these parts of the life cycle were absent because the parasites were arrested at their primary plasmodial stage. However, stages of the secondary phase were confirmed for *Polymyxa betae* by the presence of cystosori within the cortical tissue (Fig. 5A and B). Cystosori, one to several per cell, were highly variable in size and shape, being globular, irregular, rosette-like, elongated, and sometimes composed of linear series of cysts (Fig. 5B). These cystosori were not morphologically different from those found in natural infection (Fig. 3A and B); they can be regarded as young encysted plasmodia. In dual culture, these plasmodia did not complete their development and did not undergo meiosis to form resting spores.

The origin of cystogenous plasmodia within a transformed root was not clearly observed. They have been reported as developing from fused zoospores or amoebas or by coalescence of several amoebas or young plasmodia (9). Heim (5) reported a life cycle of *Plasmodiophora brassicae* that differed from the so-called orthodox type; the primary cysts germinated in the invaded tissue, transforming themselves into elongate cells, and fused to form the young plasmodia that could infect other healthy regions. She did not observe characteristic sporangiosori and zoospores and stated that *Plasmodiophora brassicae* can complete its life cycle within a parasitized root.

In dual culture, the cysts that resulted from the fragmentation of primary and secondary plasmodia were the most frequently observed stage. However, the possible effect of the MS medium on sporogenesis is not sufficiently documented to discount the occurrence of this stage in the life cycle.

Although the life cycle of the Plasmodiophorales is fairly well known, there are numerous differences in observations and interpretations. Coalescence of several amoebae or young plasmodia, plasmogamy of flagellate zoospores, caryogamy, and meiosis have been the subject of considerable discussion and controversy since the time of the Woronin's discovery (23). The problem is difficult to solve because of the parasitic nature of the species and the difficulty of culturing them outside their hosts.

Although at least one member of each of the families Peronosporaceae, Erysiphaceae, Pucciniaceae, Tilletiaceae, and Ustilaginaceae has been maintained in callus derived from its hosts (7), none of the attempts to grow either *Polymyxa* or obligate vesicular-arbuscular mycorrhizal fungi (16) on plant calli has been successful. These biotrophic endophytes remained ultimately dependent on some substance or condition in the root.

The transformed root dual culture technique is potentially valuable for studying the puzzling aspects of the Plasmodiophorales; all parts of the system can be observed microscopically so that the root hair and cortical phases can be studied in situ in monoxenic root organ culture.

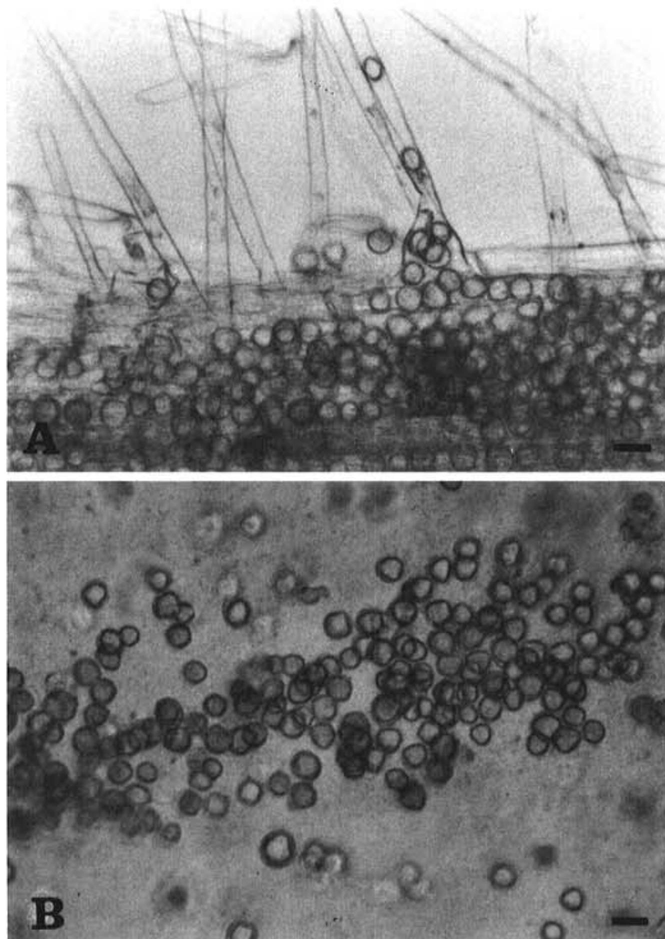


Fig. 4. Transformed root of *Brassica napus* showing the primary growth phase (bar = 20 μm). **A,** Primary cysts of *Plasmodiophora brassicae* within epidermic cells (photo of a living root). **B,** Cysts liberated into the medium.

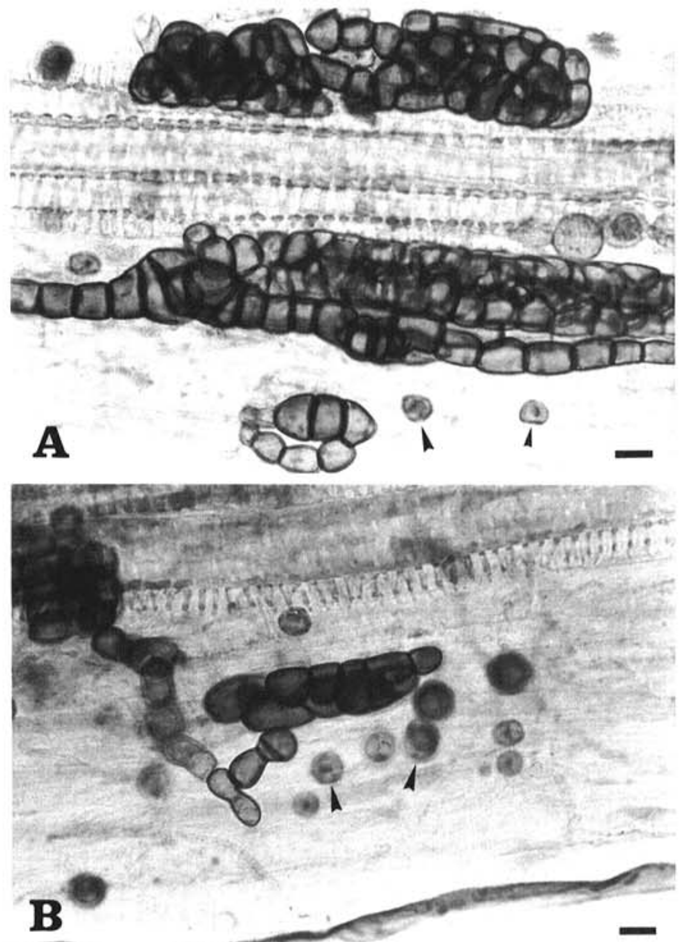


Fig. 5. Transformed root of *Beta vulgaris* showing the primary and the cystogenous growth phases (bar = 20 μm). **A,** Cystosori of *Polymyxa betae* in the cortical tissue near the central cylinder. **B,** Variation in size and shape of cystosori. Note the spherical primary cysts (arrows) remaining after staining with trypan blue.

LITERATURE CITED

1. Buczacki, S. T. 1980. Culture of *Plasmodiophora brassicae* in host callus tissue. Pages 145-149 in: Tissue Culture Methods for Plant Pathologists. D. S. Ingram and J. P. Helgeson, eds. Blackwell Scientific Publications, Oxford. 232 pp.
2. Chilton, M., Tepfer, D., Petit, A., David, C., Casse-Delbart, F., and Tempé, J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. Nature 295:432-434.
3. Cook, W. R. I., and Swartz, E. J. 1930. The life-cytology and method of infection of *Plasmodiophora brassicae* Woron., the cause of finger and toe disease of cabbage and other crucifers. Philos. Trans. R. Soc. B 218:283.
4. Haji-Tinggal, S., and Webster, J. 1981. Technique for single spore infection by *Plasmodiophora brassicae*. Trans. Br. Mycol. Soc. 76:187-190.
5. Heim, P. 1955. Le noyau dans le cycle évolutif de *Plasmodiophora brassicae* Woron. Revue Mycol. 20:131.
6. Ingram, D. S. 1969. Growth of *Plasmodiophora brassicae* in host callus. J. Gen. Microbiol. 55:9.
7. Ingram, D. S., and Helgeson, J. P. 1980. Tissue Culture Methods for Plant Pathologists. Blackwell Scientific Publications, Oxford. 232 pp.
8. Ingram, D. S., and Tommerup, I. C. 1972. The life history of *Plasmodiophora brassicae* Woron. Proc. R. Soc. London, Ser. B 180:103-112.
9. Karling, J. S. 1968. The Plasmodiophorales, 2nd ed. Hafner, New York. 256 pp.
10. Keen, N. T., Reddy, M. N., and Williams, P. H. 1969. Isolation and properties of *Plasmodiophora brassicae* plasmodia from infected crucifer tissues and from culture callus. Phytopathology 59:637-644.
11. Keskin, B. 1964. *Polymyxa betae* n. sp., ein parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugendentwicklung der Zuckerrube. Arch. Mikrobiol. 49:348-374.
12. Langenberg, W. G., and Giunchedi, L. 1982. Ultrastructure of fungal plant virus vectors *Polymyxa graminis* in soilborne wheat mosaic virus-infected wheat and *P. betae* in beet necrotic yellow vein virus-infected sugar beet. Phytopathology 72:1152-1158.
13. Moore, L., Warren, G., and Strobel, G. 1979. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid 2:617-626.
14. Morel, G. 1944. Le développement du mildiou sur des tissus de vigne cultivés *in vitro*. C. R. Acad. Sci. Paris 208:50-52.
15. Morel, G. 1948. Recherches sur la culture associée de parasites obligatoires et de tissus végétaux. Ann. Epiphy. 14:123-234.
16. Mugnier, J., and Mosse, B. 1987. Vesicular-arbuscular infection in transformed root-inducing transferred DNA roots grown axenically. Phytopathology (In press)
17. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.
18. Strandberg, J. O., Williams, P. H., and Yukawa, Y. 1966. Monoxenic culture of *Plasmodiophora brassicae* with cabbage tissue. (Abstr.) Phytopathology 56:903.
19. Tepfer, D., and Tempé, J. 1981. Production d'agropine par des racines formées sous l'action d'*Agrobacterium rhizogenes*, souche A_4. C. R. Acad. Sci. Paris 292:153-156.
20. Tommerup, I. C., and Ingram, D. S. 1971. The life-cycle of *Plasmodiophora brassicae* Woron. in *Brassica* tissue cultures and in intact roots. New Phytol. 70:327-332.
21. White, F., Ghidosi, G., Gordon, M., and Nester, E. 1982. Tumor induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the genome. Proc. Natl. Acad. Sci. 79:3193-3197.
22. Williams, P. H., Reddy, M. N., and Strandberg, J. O. 1969. Growth of noninfected and *Plasmodiophora brassicae* infected cabbage callus in culture. Can. J. Bot. 47:1217-1221.
23. Woronin, M. 1878. *Plasmodiophora brassicae*. Urheber de Kohlpflanzen-Hernie. Jb. Wiss. Bot. 2:548.