Early Development of Pythium polymorphon on Celery Roots Infected by Meloidogyne hapla

James L. Starr and James R. Aist

Former Research Assistant and Assistant Professor, respectively, Department of Plant Pathology, Cornell University, Ithaca, NY 14853. The senior author's present address is Department of Plant Pathology, North Carolina State University, Raleigh 27607.

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ABSTRACT

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Meloidogyne hapla-infected and *M. hapla*-free celery roots were inoculated with *Pythium polymorphon;* samples taken at 24-hr intervals were observed by interference-contrast light optics. Root-knot galls were colonized by 24 hr after inoculation, but invasion of *M. hapla*-free roots was not noted until 48 hr. A greater percentage of galled than of nongalled root segments was colonized at 72 hr. After ingress, *P. polymorphon* colonized both galled and nongalled root segments at apparently similar rates. The fungus seemed to invade nongalled portions of galled roots from infected galls. Aqueous extracts of galled and nongalled roots, either autoclaved or filter-sterilized and added to 1% glucose, were equally supportive of *P. polymorphon* growth in vitro. The results do not support the concept that galled roots provide nutrients for growth of *P. polymorphon* that are deficient in nongalled roots, but do suggest that factors attractive to the fungus may originate in the galls.

Additional key words: disease complexes, histopathology.

In an earlier paper (10) a root disease complex of celery that involved *Pythium polymorphon* Sideris and *Meloidogyne hapla* Chitwood was identified; *P. polymorphon* caused greater necrosis of celery roots in the presence than in the absence of *M. hapla*. Necrosis began in the galls and spread to adjacent nongalled tissue. In other reports of root disease complexes that involved soilborne fungi and root-knot nematodes (3, 7, 9), the galls were heavily colonized by the fungi and syncytia were extremely susceptible. It has been suggested (1, 5, 8, 11) that the root disease complexes of this type occur because the galls are rich in nutrients available for fungal growth. Such an hypothesis implies that nongalled roots are nutrient-deficient. The primary objective of this study was to compare the development of *P. polymorphon* on celery roots infected by *M. hapla* to that on *M. hapla*-free roots. In addition, the suitability of galled and nongalled root extracts for growth and sporulation of *P. polymorphon* was examined.

MATERIALS AND METHODS

Four-wk-old celery seedlings, *Apium graveolens* var. *dulce* 'Utah 52-70', were transplanted to 10-cm diameter pots that contained approximately 400 cc of autoclaved organic soil (Carlisle Muck). The soil was infested with 10^4 *M. hapla* eggs obtained by the method of Hussey and Barker (4). After incubation for 2 wk at 21 C with a 12-hr photoperiod (21, 520 lux) the roots were washed free of soil, excised, surface-sterilized for 3 min with 1% NaClO, rinsed in sterile water, and placed in sterile moisture chambers. In two tests the entire root system from *M. hapla*-infected and noninfected plants were used; in another test, only individual galls and root segments from *M. hapla*-free plants were used. The roots were inoculated with a 2-ml suspension of *P. polymorphon* sporangia and oospores (2:1) prepared by macerating 2-wk-old cornmeal agar (CMA) cultures in 50 ml of sterile distilled water for 5 sec in a food blender. The inoculum concentration was determined with a haemocytometer and adjusted to 6.5×10^4 propagules/ml.

Inoculated roots were incubated at 23 C. Samples were prepared for histology at 24-hr intervals, and consisted of individual galls with some adjacent nongalled tissue (1-3 mm total length) for *M. hapla*-infected tissue and 2-mm root segments from *M. hapla*-free roots. Samples were fixed in 5% glutaraldehyde, postfixed in 2% OsO_4, dehydrated in an acetone series, and embedded in Epon-Araldite. Serial sections, 20 μm thick and mounted on glass slides, were examined and photographed in immersion oil using Nomarski interference-contrast optics.

To examine effects of root extracts on growth of *P. polymorphon*, media were prepared from aqueous extracts of roots inoculated 3 wk earlier with 10^4 *M. hapla* eggs (galled roots) and from noninoculated (nongalled roots). Macerates of frozen root samples were mixed with distilled water (1:2, w/v), incubated for 10 min at 4 C, and

filtered through Whatman No. 1 filter paper.

Growth of *P. polymorphon* on media prepared from root extracts was compared by three methods, each test being conducted at least twice. With the first method, extracts from 60 g (fresh weight) of tissue were added to 1.0 liter of distilled water and autoclaved for 15 min at 121 C, 1.4 kg/cm^2. The medium was dispensed into 125-ml flasks (40 ml/flask) and each flask was seeded with three 4-mm diameter plugs taken from the margin of a colony of *P. polymorphon* growing on CMA. After incubation on a wrist-action shaker (100 cycles/minute) at 23 C, the mycelial mats were collected on tared filter paper at 24 hr intervals, dried overnight at 90 C, and then the dry weight was determined. The second method was designed to detect noncarbon-source nutritional differences. Extracts were centrifuged at 93,000 *g* for 2 hr at 4 C, and the supernatant liquid was collected and sterilized with a Millipore filter (0.22 μm pore size). Extract from 40 g (fresh weight) of tissue was made up to 1.0 liter. One percent glucose was added because insufficient growth occurred without glucose. This medium was used as previously described for determination of dry weight increase over a 72-hr incubation period. With the third method, in order to know the approximate concentration, extracts from 60 g (fresh weight) of tissue were made up to 1.0 liter in 1.5% agar, autoclaved, and dispensed into 15-cm diameter petri plates (10 ml each). Each plate was seeded with a 4-mm CMA plug of *P. polymorphon,* and linear growth was measured during a 72-hr incubation period at 23 C. After 96 hr, the same cultures were flooded with cotton-blue in lactophenol for 24 hr, the excess stain was removed, and the entire contents of the petri plate were macerated with 50 ml of water in a food blender for 10 sec. The numbers of sporangia and oospores per milliliter of the resulting suspensions were determined with a haemocytometer.

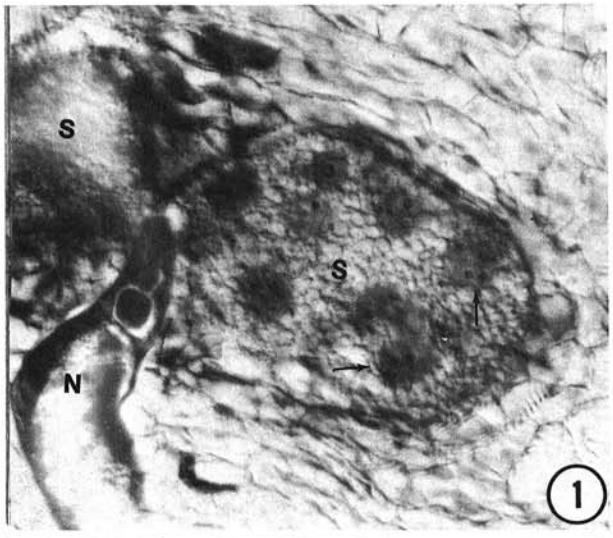
RESULTS

Development of P. polymorphon on M. hapla-infected celery roots.—Since similar results were obtained when either entire root systems or root segments were inoculated, the results were combined (Table 1). Galls not inoculated with *P. polymorphon* contained swollen, second-stage larvae of *M. hapla* associated with well-developed syncytia (Fig. 1) that contained dense cytoplasm and numerous multinucleolate nuclei. By 18 hr after inoculation of root segments with *P. polymorphon*, sporangia had formed germ tubes, zoospores were not seen, and oospores failed to germinate. Penetration of galls occurred 24 hr after inoculation with the fungus (Fig. 2), and appressoria were absent in 12 examples observed. Colonization of galls was rapid and hyphae grew intracellularly; frequently the hyphae reached the syncytial complex by 48 hr after inoculation (Fig. 3). At this time cytoplasm in colonized syncytia appeared less dense than that in noncolonized syncytia, nuclei were no longer detectable, and the fungus had invaded (apparently from the galls) the nongalled portions of *M. hapla*-infected roots (Fig. 4). By 72 hr, *P. polymorphon* had colonized the entire galls, and syncytia were usually devoid of cytoplasm, although vestigial amounts (Fig. 5) occasionally were seen. Sporulation occurred in galls by 72 hr after inoculation (Fig. 6).

TABLE 1. Summary of the histological study of the development of *Pythium polymorphon* on *Meloidogyne hapla*-infected and *M. hapla*-free celery roots

Treatment	Time after inoculation with *Pythium* (hr)	Samples examined (no.)	Samples colonized by *P. polymorphon*	Pertinent observations
M. hapla	. . .	10	0	2nd-stage larvae associated with well-developed syncytia
P. polymorphon	24	23	0	–
	48	24	6	Hyphae restricted to outer cortical cells
	72	23	6	All tissues colonized, fruiting bodies not present
M. hapla + *P. polymorphon*	24	16	10	Ingress by direct penetration
	48	17	12	5/12 samples had colonized syncytia
	72	12	9	Complete cytoplasmic breakdown observed in syncytia *P. polymorphon* fruiting bodies were present.

Fig. 1-8. Development of *Pythium polymorphon* on *Meloidogyne hapla*-infected and *M. hapla*-free celery roots. **1)** Second-stage *M. hapla* larva (N) associated with syncytia (s) which are characterized by dense cytoplasm with numerous nuclei (arrows) each with several nucleoli (×320). **2)** Direct penetration of a gall by hyphae of *P. polymorphon* (×590). **3)** Hyphae of *P. polymorphon* (arrows) invading syncytia 48 hr after inoculation with the fungus. Note disintegration of syncytial cytoplasm (×320). **4)** Colonization of nongalled portion of *M. hapla*-infected root by *P. polymorphon* 48 hr after fungus inoculation (×230). **5)** Colonization of syncytia by *P. polymorphon* 72 hr after fungus inoculation. **6)** *Pythium polymorphon* oogonium, with antheridia, in gall tissue 72 hr after inoculation with the fungus (×270). **7)** *Pythium polymorphon* hyphae (arrows) within cortical cells of *M. hapla*-free root 48 hr after inoculation with the fungus (×290). **8)** Colonization of *M. hapla*-free root 72 hr after fungus inoculation (×320).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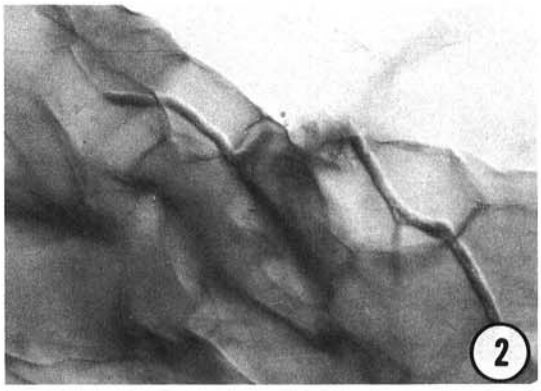
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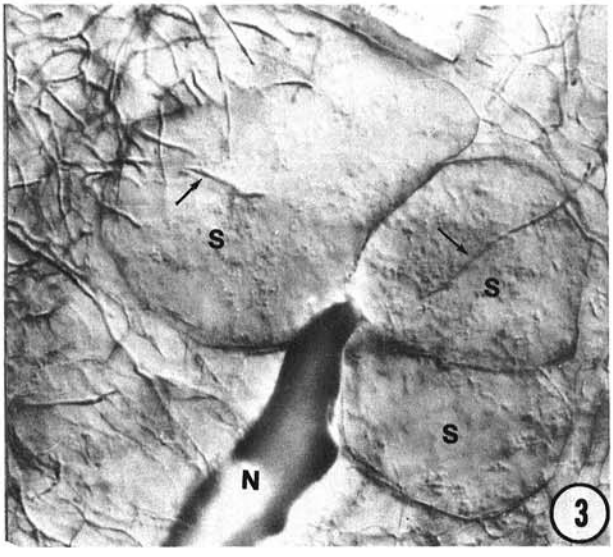
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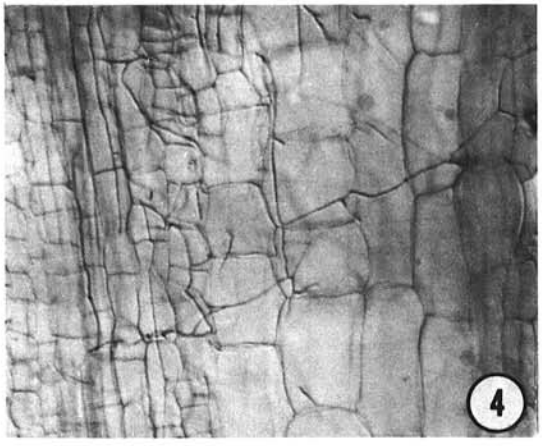
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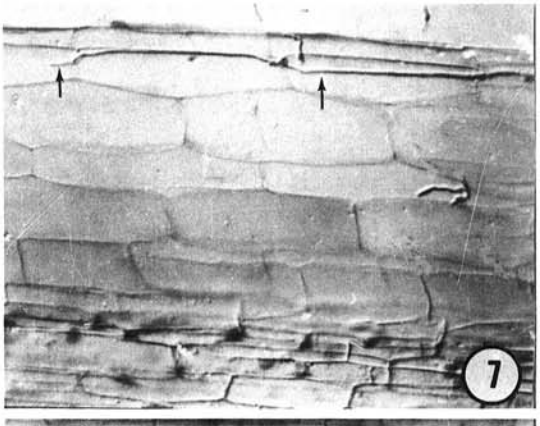
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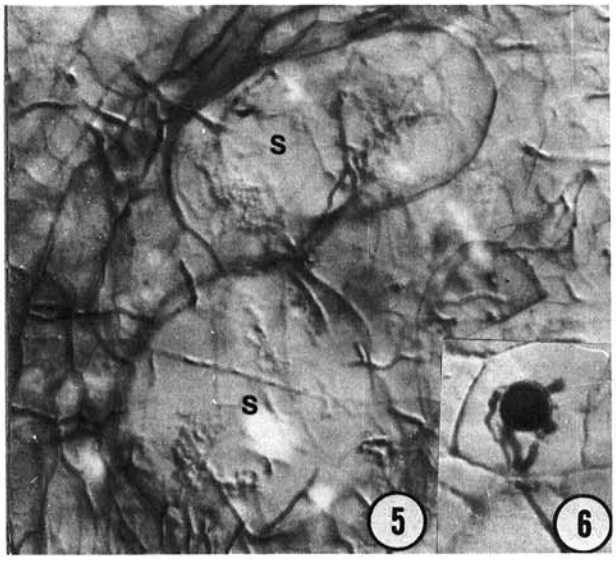
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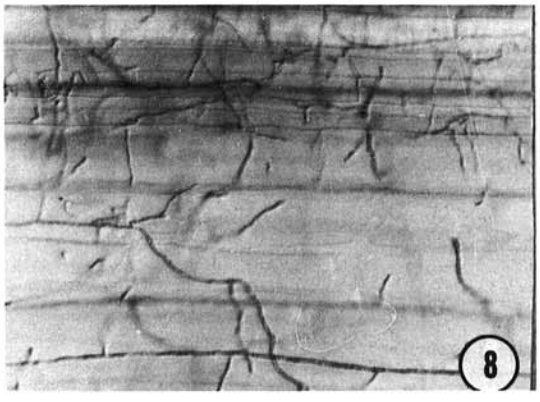
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Development of P. polymorphon on M. hapla-free roots.—As observed for galled roots, sporangia germinated directly by 18 hr after inoculation of *M. hapla*-free roots with *P. polymorphon*. However, colonization of roots was not detected until 48 hr after inoculation in contrast to galled roots which were colonized by 24 hr. Although actual penetration sites were not detected, early hyphal growth was apparently intracellular and parallel to the root axis, one to two cell layers beneath the epidermis (Fig. 7). By 72 hr, hyphae colonized the cortex extensively and had reached the central vascular cylinder (Fig. 8), but sporangia and/or oogonia were not observed. Estimated mycelial development in nongalled roots at 48 and 72 hr after inoculation was similar to that in galled roots at 24 and 48 hr, respectively; the apparent rates of colonization after ingress were comparable. However, the percentage of galled samples colonized was greater than the percentage of *M. hapla*-free samples colonized (Table 1).

In vitro growth of P. polymorphon on root extracts.—There was no difference in mycelial dry weight of autoclaved, galled, and nongalled root-extract media. By 48 hr, dry weight was about 50% of that on corn meal broth and did not increase further on any of the media. Growth of *P. polymorphon* on the two filter-sterilized extracts in 1% glucose also was similar (Fig. 9). The added glucose probably caused growth on the extracts to be greater than that on corn meal broth and to continue for the 72-hr incubation period. When extracts were added to agar and autoclaved, there was no difference in the linear growth rate between the two media (Fig. 10). The rate was greater, however, than that on CMA. The extract from galled roots stimulated significantly greater oospore production than did non-galled root extracts of CMA (Table 2). Effects on sporangium production were inconsistent.

DISCUSSION

That *P. polymorphon* colonized a greater percentage of root galls than nongalled root segments agrees with the earlier observation (10) that the fungus can be isolated from root galls with greater frequency than from nongalled roots. This could be due to the larger surface area of root galls as compared to that of nongalled root segments, because *P. polymorphon* would have a greater chance of coming into contact with the galls. However, the histological studies indicated that root galls were colonized by the fungus up to 24 hr earlier than were nongalled roots; thereafter, the rate of fungal development appeared to be the same. Collectively, these observations suggest that *M. hapla* may increase infection by *P. polymorphon* because root galls are more attractive to the fungus than are nongalled roots. Golden and Van Gundy (3), studying the interaction of *M. incognita* and *Rhizoctonia solani* on tomato and okra, found that hyphae were attracted to galls chemotropically, and that they formed sclerotia only on the galls. Wang and Bergeson (11) found significant increases in the amounts of sugars in the root exudate of *M. incognita*-infected tomato roots compared to those in healthy roots; this might be involved in the attraction of fungi to root-knot galls.

Rapid cytoplasmic breakdown upon syncytial colonization, as noted here, appears to be a common characteristic of these complexes (3, 7, 9).

The in vitro growth assays did not yield evidence to support earlier implications (1, 5, 8, 11) that galled roots provide nutrients which are deficient in nongalled roots; growth of *P. polymorphon* was not enhanced by nutrients from galled roots. However, sporulation was greater on media supplemented with extracts from galled roots; similar results were reported by Brodie and Cooper (2) for

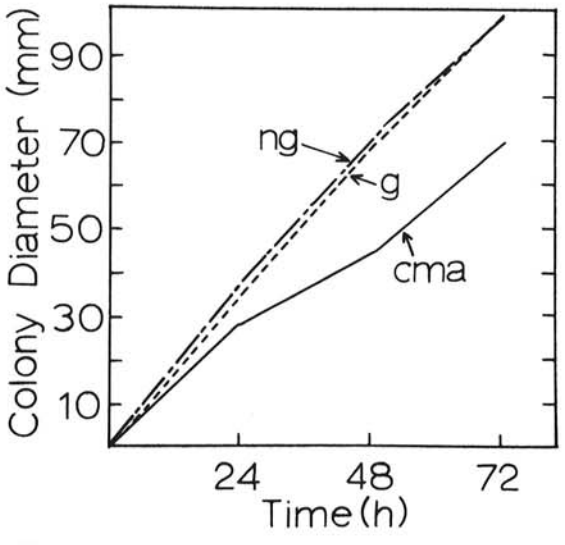

Fig. 9. Effect of filter-sterilized, galled (g) and nongalled (ng) root extracts in 1% glucose on growth (as dry weight) of *Pythium polymorphon* in broth. Corn meal broth (cmb) was used for comparison.

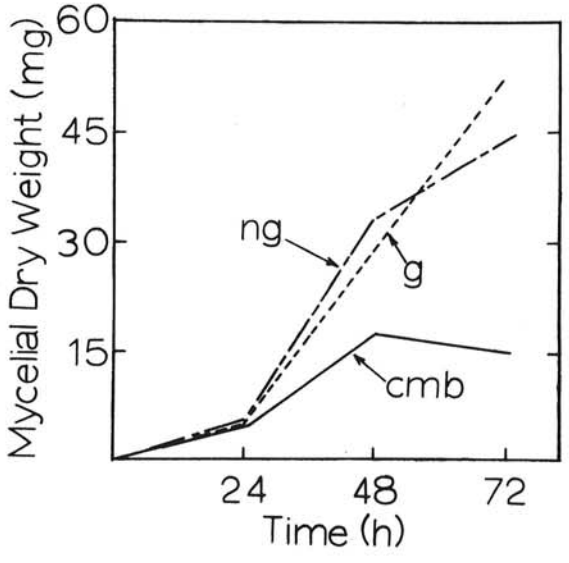

Fig. 10. Effect of autoclaved, galled (g) and nongalled (ng) root extracts on linear growth of *Pythium polymorphon* on agar. Corn meal agar (cma) was used for comparison.

TABLE 2. Effect of *Meloidogyne hapla*-infected and noninfected celery root extracts on the production of sporangia and oospores by *Pythium polymorphon*

Medium	First test		Second test	
	Sporangia (no.)	Oospores (no.)	Sporangia (no.)	Oospores (no.)
Corn meal agar	3,854[z] a	1,979 a	9,375 a	3,675 a
Nongalled root extract	5,125 a	33,875 b	9,750 a	60,750 b
Galled root extract	12,750 b	46,375 c	12,875 a	74,000 c

[z]Values are means of five replications, means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

a *P. debaryanum*/root-knot complex of cotton. On the other hand, Melendez and Powell (6) did detect increased mycelial growth of *Fusarium oxysporum* f. sp. *nicotianae* on media supplemented with extracts from *M. incognita*-infected tobacco roots. Since our histological data suggest that the galls themselves are preferentially attacked by *P. polymorphon*, future work should compare nutrients in galls to those in adjacent, nongalled segments of *M. hapla*-infected roots.

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