Induction of Hairy Roots on Cultivated Soybean Genotypes and Their Use to Propagate the Soybean Cyst Nematode

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ABSTRACT

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Ten soybean (*Glycine max*) genotypes were evaluated for hairy root induction by four strains of *Agrobacterium rhizogenes*. Influence of inoculation site was assessed by infecting hypocotyls and cotyledons on germinated seedlings. The presence of opines in extracts of cultured roots was used to score transformed roots. A cucumopine strain, K599, induced hairy roots on 37% of the cotyledons infected on the 10 genotypes tested. Transformed root development after infection of cotyledons with the mannopine strain 8196 occurred at a frequency of 3% on four genotypes. Agropine strains 1855 and A4 induced hairy roots on 1% of cotyledons of different genotypes. No opine-positive transformed roots were induced from hypocotyl inoculations with any *A. rhizogenes* strain-soybean genotype combination tested. However, adventitious roots containing no detectable opines developed from hypocotyl inoculations both at the wound site and at a region directly below the cotyledons. Transformed roots differentiated from globular callus at the wound site on cotyledons infected with virulent *A. rhizogenes*. Opine-containing hairy roots were established permanently in tissue culture and exhibited typical hairy root morphologies and growth parameters. Infection of soybean cultivar Williams 82 hairy root cultures with second-stage juveniles or cysts of the soybean cyst nematode, *Heterodera glycines* race 3, led to the appearance of mature cysts about 3 wk later. The nematode was propagated by excising an infected root and transferring it to a fresh root culture.

Agrobacterium rhizogenes, the causal agent of hairy root disease, induces the proliferation of neoplastic, transformed roots (1,35,37). During infection, the T-region, a segment of the root-inducing (Ri) plasmid in *A. rhizogenes*, is transferred and stably integrated into the plant genome (5). Upon expression of this integrated T-DNA, transformed roots rapidly proliferate and synthesize certain low molecular weight carbon compounds called opines (25). Four opine-type Ri plasmids have been identified. Agropine-, mannopine-, cucumopine- and mikimopine-type Ri plasmids harbored in strains of *A. rhizogenes* induce transformed roots which synthesize the strain-specific opines (7,11,13,26).

Recently, hairy root cultures have been used to cultivate obligate root parasites. *Plasmodiophora brassicae* Woronin and *Polymyxa betae* Keskin, both obligate root-inhabiting fungi, can be propagated on transformed root cultures of sugar beet (19). Infections with vesicular-arbuscular mycorrhizal fungi, *Glomus mosseae* Gerdemann & Trappe and *Gigaspora margarita* Beker & Hall, have been obtained on hairy root cultures of *Convolvulus sepium* L. (20). In addition, the root-knot nematode, *Meloidogyne javanica*, has been propagated on transformed root cultures of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.) (32). Such cultures are being used for routine maintenance of the nematode and to study the parasitism of *M. javanica* by *Pasteuria penetrans* (Thorne) Sayre & Starr (33).

Soybean (*Glycine max* (L.) Merr.) is grown widely in the United States as a source of oil and high-protein meal. Annually, the soybean crop is valued at an estimated 11 billion dollars. *Heterodera glycines* Ichinohe, the soybean cyst nematode, occurs in Canada, the Peoples' Republic of China, Colombia, Indonesia, Japan, Korea, the Soviet Union, and throughout the soybean production areas of the United States (29). This obligate root parasite is a major yield-limiting pest of soybean in the United States (12).

The soybean cyst nematode can be propagated gnotobiotically on normal soybean root explants (14). However, this technique requires the continual establishment of root explants because these organs have a determinant period of growth in culture. Soybean hairy roots, which should exhibit indeterminate growth in tissue culture, could provide an alternative to normal root explants for monoxenic propagation and study of obligate soybean root parasites such as the soybean cyst nematode.

The few reports in the literature suggest that *A. rhizogenes*-induced hairy roots are difficult to establish on soybean. Responses of 26 genotypes of *G. max* to induction of hairy roots by *Agrobacterium* strain A136 harboring pRiA4b have been reported (23). Seven of the genotypes produced roots at the infection sites, another eight produced only small galls, and the remaining 11 did not respond to inoculations with this bacterial strain. However, attempts to culture these roots were unsuccessful. In addition, primary roots were not characterized with respect to opine content or other hairy root markers (23). Recently, Rech and co-workers (28) induced hairy roots on *G. canescens*, a wild *Glycine* spp. Permanent cultures could be established and the transformed roots were regenerable. However, hairy root cultures of the domesticated genotypes of *G. max* have not yet been reported.

This paper describes 1) an investigation into genotype, pathogen, and infection parameters necessary to induce hairy roots on *G. max*, 2) the establishment and characteristics of soybean hairy root cultures, and 3) the use of these cultures for the axenic propagation of the soybean cyst nematode.

MATERIALS AND METHODS

Soybean genotypes. The 10 genotypes of *Glycine max* used in this study were acquired from R. L. Bernard, curator, USDA Northern Soybean Germplasm Collection, University of Illinois at Urbana-Champaign, Urbana. Soybean seeds were surface sterilized by soaking in 2.1% sodium hypochlorite for 20 min followed by two 10-min washes in sterile distilled water. Seeds then were plated onto sucrose water agar (5.0% sucrose in 0.8% agar) medium (SWA) to allow germination and to select for sterile seeds. Germinating seeds were transferred to 25- × 150-mm test tubes containing 10 ml of SWA.

Bacteria. Four strains of *A. rhizogenes* were evaluated for their ability to induce transformed roots on 10 soybean genotypes. Two agropine-type strains, A4 and 1855, and one mannopine strain, 8196, were from our collection. The cucumopine strain, K599, was obtained from Allen Kerr, Waite Institute, Glen Osmond, 5064—South Australia. Nonpathogenic strain NT-1 is *A. tumefaciens* strain C58 cured of its Ti plasmid (34). Bacterial strains were grown in yeast extract-mannitol liquid medium (27) with aeration at 28 C.

Plant inoculations. Soybean seedlings were inoculated after the emergence vegetative stage (10). The onset of vegetative stage in the 10 selected soybean seedling genotypes varied between 6 and 15 days after plating seed on SWA. Inoculations were performed with a scalpel previously dipped into an overnight culture of the strain of *Agrobacterium* being tested. Cotyledons were inoculated by cutting the abaxial face several times to form a checked wound site. Hypocotyl segments were inoculated by making 2.0-cm-long longitudinal cuts. Twenty seedlings of each genotype were inoculated at each site for each bacterial strain tested. Inoculated seedlings were returned to 25- × 150-mm test tubes and incubated in growth chambers under cool-white fluorescent lighting for a 16-hr photoperiod at 25 C.

Establishment of root cultures. Cotyledons and hypocotyls with root primordia were transferred to 25 ml of liquid MonMor medium in 25- × 100-mm culture plates. MonMor medium consisted of Monnier's salts (17) containing Morel's vitamins (18), 86 mg L^{-1} of ferric-sodium salt EDTA according to Murashige and Skoog medium (21) and 20.0 g L^{-1} of sucrose. The pH was adjusted to 5.8 before autoclaving for 20 min at 118 C and 1.0 g cm^{-2}. After autoclaving, the medium was cooled to approximately 45 C and carbenicillin at 500 mg L^{-1} was added to inhibit

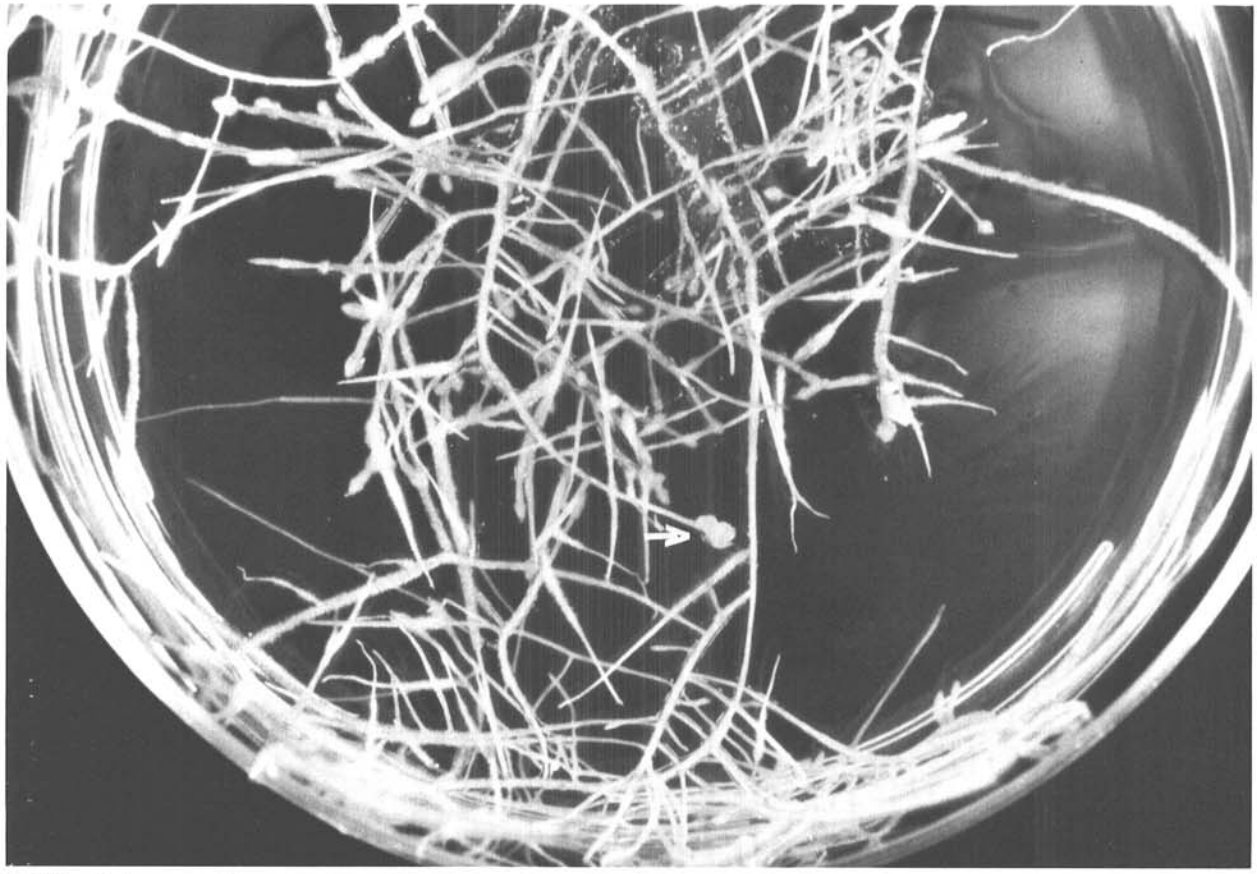

Fig. 1. Established hairy root cultures after 2 wk of growth on MonMor agar medium. Arrow indicates friable callus.

bacterial growth. For propagation of the soybean cyst nematode, approximately 2.0 g of verified transformed roots were subcultured from MonMor liquid medium to Lauritis medium (14,15) containing 16.0 g L^{-1} of Difco Bacto agar in 150- × 25-mm culture plates.

Opine analysis. For the detection of the mannityl opines, approximately 0.3 g of root tissue was macerated in 100 μl of 70% ethanol containing 10 μl of the electrophoresis running buffer (formic acid/acetic acid/water, 3:6:91, v/v/v, pH = 1.9). For detection of cucumopine, root tissue was macerated in distilled water. In each case supernatants were recovered following centrifugation. Twenty microliters of supernatant extract was spotted on Whatman 3 MM paper. The spots were allowed to dry, and the papers were wetted with the running buffer and subjected to high voltage paper electrophoresis (HVPE) at 4,000 V for 12–15 min. The electrophoretograms were dried in a stream of warm air until no odor of acetic acid could be detected.

TABLE 1. Frequency of hairy root induction on cotyledons of genotypes of *Glycine max* inoculated with one of four strains of *Agrobacterium rhizogenes*

	Opine positive roots / total roots[a] *A. rhizogenes* strain			
Genotype	K599	8196	1855	A4
Cartter	13/13[b]	0/0	1/2	0/3
Fayette	10/10	1/3	0/2	0/0
Franklin	1/1	0/0	0/0	0/0
Kent	10/10	1/3	0/0	0/0
Lee	3/3	0/0	0/0	0/0
Mandarin	17/17	1/2	0/0	1/2
Maple Arrow	15/15	2/4	1/3	1/6
Peking	1/1	0/0	0/0	0/0
Pickett	1/1	0/0	0/0	0/0
Williams 82	3/3	0/2	0/0	1/3
Total	74/74/200[c]	5/14/200	2/7/200	3/14/200

[a] In each case 20 cotyledons were inoculated with each strain of *A. rhizogenes*.
[b] Number of cotyledons yielding opine-positive roots/number of cotyledons producing roots at the wound site.
[c] Total number of cotyledons inoculated by each strain.

Mannityl opines were visualized with the alkaline silver nitrate reagents of Trevelyan and co-workers (31). Electrophoretograms were dipped in silver nitrate solution (4 g of silver nitrate in 20 ml of water diluted to 1 L with acetone) and dried thoroughly. The spots were developed by dipping in ethanolic NaOH (2% NaOH in 90% ethanol). The papers were subsequently dipped in Kodak fixer and rinsed with distilled water for 15 min (6).

Cucumopine and its acid-degradation product were visualized with the Pauly reagent by spraying the dry electrophoretograms lightly with a solution containing equal parts of sulfanylic acid (1.0% in 1 N HCl) and sodium nitrite (5.0% in water). Papers were allowed to dry and then sprayed with aqueous 15% sodium carbonate (8,24). Cucumopine and its acid-degradation product appear as reddish and bluish spots, respectively, as the paper is sprayed with sodium carbonate.

Spots were identified as opines by comparing their electrophoretic mobilities and staining properties with those of authentic standards. Mannopine, mannopinic acid, agropine, and agropinic acid were synthesized by Yves Dessaux in our laboratory. Cucumopine was syntheized from L-histidine and α-ketoglutaric acid (7) by Paul Hanselmann, also in our laboratory. Extracts prepared from normal leaf or root tissues or from authentic hairy roots of *Nicotiana tabacum* L. 'Xanthi NG' were included on electrophoretograms as negative and positive controls, respectively.

Propagation of *Heterodera glycines*. Soybean cultivar Williams 82 transformed root cultures, freshly transferred to plates containing Lauritis medium (14), were inoculated with six to eight gravid females of *H. glycines* race 3 from gnotobiotic culture (15). Alternatively, second-stage juveniles (J2) from pot cultures were collected and surface sterilized by soaking in a solution containing 100 mg L^{-1} of $HgCl_2$ and 1,000 mg L^{-1} of sterile streptomycin sulfate. Nematodes were washed twice with sterile distilled water by centifugation (16). Between 50 and 100 J2 were added to the subcultured transformed root cultures grown on Lauritis medium.

RESULTS

Differentiation of roots at inoculated sites. After approximately 10 days, globular callus tissue appeared at some of the wound sites of cotyledons inoculated with strains of *A. rhizogenes*. Extensive splitting of hypocotyls with no callus formation occurred

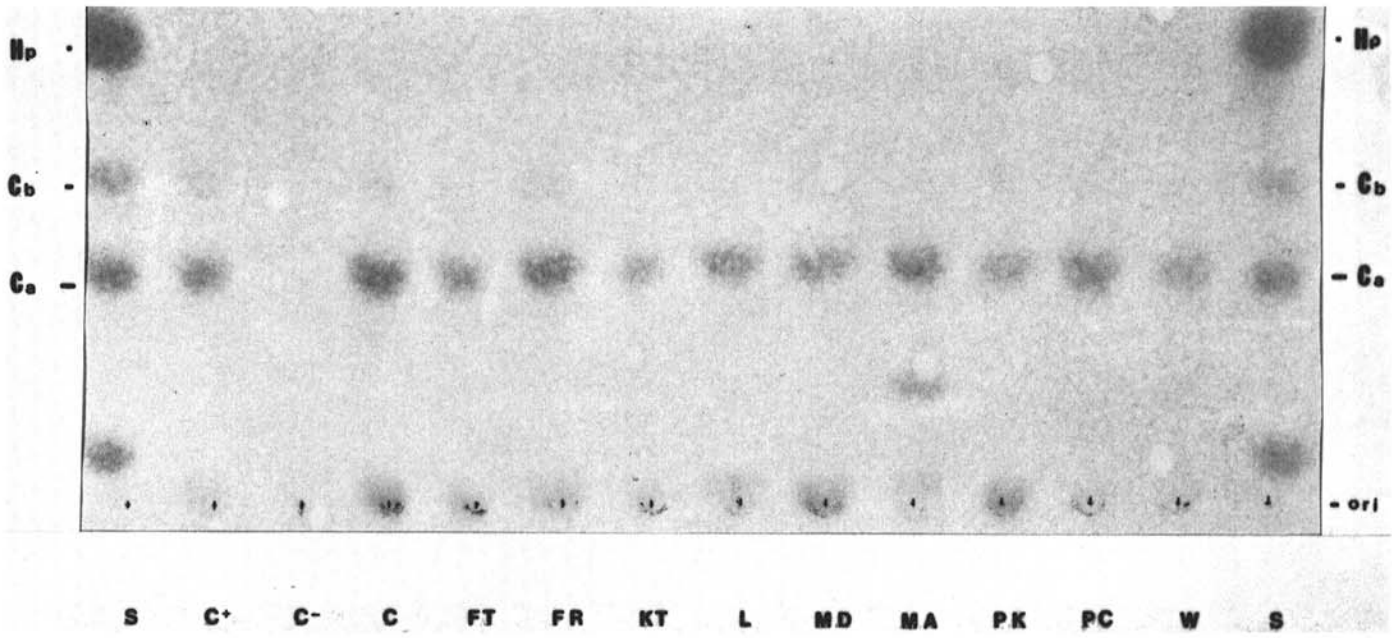

Fig. 2. Electrophoretic analysis of extracts from transformed roots of soybean incited by *Agrobacterium rhizogenes* strain K599. Standards (S) are: cucumopine (Ca), acid-degradative product of cucumopine (Cb), and histidine (Hp). (C+), Extract of tobacco hairy roots induced with cucumopine strain K599. (C-), Extract from normal tobacco roots. Other lanes contain root extracts from roots induced on genotypes: Cartter (C), Fayette (FT), Franklin (FR), Kent (KT), Lee (L), Mandarin (MD), Maple Arrow (MA), Peking (PK), Pickett (PC), and Williams 82 (W).

on inoculated hypocotyls with all bacterial strains tested. Fifteen to 25 days after inoculation of cotyledons with strains of *A. rhizogenes*, root primordia differentiated from globular callus tissue. Hypocotyls inoculated with virulent strains of *A. rhizogenes* or the nonpathogenic strain NT-1 gave rise to roots at the inoculation site and at a region about 0.5 cm below the cotyledons. Roots that developed from hypocotyls did not contain detectable opines in their cell extracts (data not shown).

When root primordia had elongated to approximately 2.0 cm, the entire hypocotyl or cotyledon was dissected from the seedling and transferred to liquid MonMor medium containing carbenicillin. Approximately 10% of the roots failed to grow in liquid MonMor medium after excision from the seedling. After 1 wk, clonal lines were established by subculturing single roots. While some subcultured roots failed to elongate, most of the roots showed growth rates of approximately 0.5 cm per 24 hr. When transferred to solid medium, many of the roots formed a small amount of friable callus at root tips (Fig. 1).

Roots containing opines were scored as being transformed (see below). Hairy root cultures were established by subculturing 4-cm segments of root meristem to 25 ml of liquid or solid MonMor medium. Hairy root cultures could be routinely maintained on solid MonMor medium by subculturing at 3-wk intervals. Hairy root cultures agitated at 60 rpm in liquid MonMor medium grew rapidly and subculturing was necessary every 10 days.

Efficiency of different strains of *A. rhizogenes*. Strain K599 was the most efficient at inciting hairy roots on cotyledons of the 10 soybean genotypes tested. This strain induced transformed roots on 5–85% of the infected cotyledons, depending on genotype (Table 1). Cucumopine, the indicator opine associated with tissues transformed by strain K599, was present in extracts from all roots tested (Fig. 2).

Root formation following inoculation with agropine strains 1855 and A4 occurred at frequencies of 3 and 7%, respectively (Table 1). However, the absence of opines in extracts indicated that most of these roots were not truly transformed (Table 1 and Fig. 3). Mannopine strain 8196 induced roots at a frequency of 7%, and only 35% of these were found to contain mannopine and mannopinic acid (Table 1 and Fig. 3).

Soybean genotypes. Efficiency of transformed root induction on cotyledons by strain K599 varied among the 10 soybean genotypes evaluated. Two genotypes, Mandarin and Maple Arrow, were quite responsive, yielding hairy roots in 75–85% of the infected cotyledons. Other genotypes, such as Franklin, Peking and Pickett, were relatively insensitive, showing infection rates of less than 10%.

Propagation of *H. glycines* race 3. Twenty to 25 days after inoculation (DAI) with gravid females and 16–20 DAI with J2, imbedded and emerging females were observed on Williams 82 hairy roots induced by strain K599 (Fig. 4A). Approximately 4–6 days after cyst emergence, first molting was observed followed by egg hatch and emergence of J2 (Fig 4B). Second-stage juveniles were observed migrating throughout the culture (Fig. 4C) and mature second-generation females were observed approximately 6 wk after inoculation (Fig. 4D). The nematode could be serially propagated by transferring infected hairy root segments to a fresh hairy root culture (data not shown).

DISCUSSION

The three variables tested, host genotype, strain of *A. rhizogenes*, and site of inoculation all proved important in the successful induction of hairy roots on soybeans. In general, cotyledon inoculations were more effective than stem or hypocotyl infections. This contrasts with results reported by Owens and Cress (23) who showed that stem inoculations were more effective than cotyledon infections. However, they did not characterize roots appearing at inoculation sites. Our observations that hypocotyl wound sites give rise to normal adventitious roots raises the question as to whether the roots appearing at their infection sites were truly transformed. In fact, our observations suggest that the genotypes of *G. max* tested have a propensity to form adventitious roots when inoculated with strains of *Agrobacterium*. This response depends on inoculation of *Agrobacterium* but does not require an Ri plasmid. Hypocotyl infections with strain NT-1 regularly gave rise to root proliferation at the wound sites and at a nonwounded collar region just below the cotyledons. Such roots from plants infected by *A. rhizogenes* and from plants infected with strain NT-1 contained no detectable opines. A few adventitious roots also developed from inoculated cotyledons. However, in such infections the nontransformed roots generally arose at the junction between the cotyledon and its petiole, distant from the actual wound sites. The roots forming at the wound site usually were transformed as judged by the presence of the marker opines.

Hairy root induction depended on the strain of *A. rhizogenes*. Strain K599 was by far the most effective in inducing hairy roots, with all soybean genotypes tested being sensitive to infection by this strain. The one mannopine-type and the two agropine-type strains of *A. rhizogenes* tested were much less effective at inducing hairy roots on soybeans (Table 1). These results are consistent with those of Byrne and co-workers (2) who failed to observe

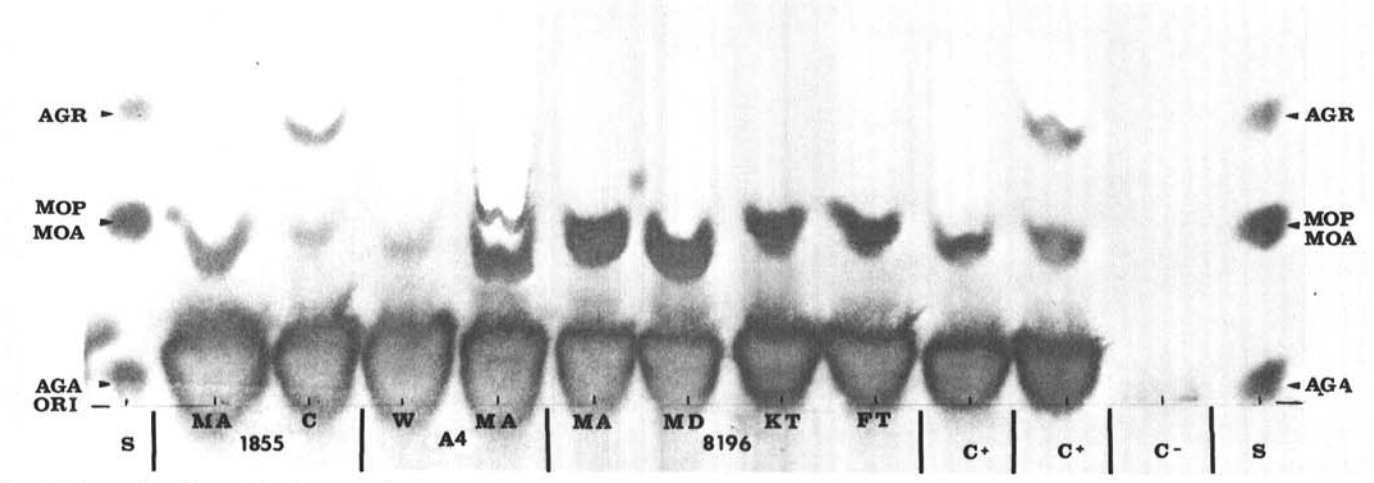

Fig. 3. Electrophoretic analysis of extracts from transformed roots incited by mannopine and agropine-type *Agrobacterium rhizogenes* strains. Standards (S) are: agropine (AGR), mannopine (MOP), mannopinic acid (MOA), and agropinic acid (AGA). Mannopine and mannopinic acid comigrate under these electrophoretic conditions. Other lanes contain root extracts from: Maple Arrow (MA) and Cartter (C) induced by strain 1855; Williams 82 (W) and Maple Arrow (MA) induced by strain A4; Maple Arrow (MA), Mandarin (MD), Kent (KT) and Fayette (FT) induced by strain 8196. (C+), Extracts from tobacco hairy roots incited by strains 8196 and 1855. (C−), Extracts from normal tobacco roots.

any hairy root induction on 17 genotypes of *G. max* by a strain of *Agrobacterium* containing pRi8196. Nor did strain 8196 induce hairy roots on *G. soja* or *G. canescens*. This is consistent with our observation that strain 8196 shows poor hairy root induction on the genotypes of *G. max* we tested (Table 1). However, our results contrast with experiments reported by Rech et al (28) on transformation of other *Glycine* spp. They observed that, although frequencies varied, a strain harboring the agropine-type Ri plasmid, pRi1855, was highly effective in transforming several accessions of *G. canescens*, *G. clandestina*, and *G. argyrea*. They also found hypocotyls to be more responsive than cotyledons. These differences may be due to dissimilarities in host plant species, chromosomal backgrounds of the bacteria, cultural conditions, or a combination of the three factors.

Hairy root formation also depended on the host plant genotype. Based on frequencies at which opine-positive roots arose, the 10 soybean genotypes tested could be divided into two groups. Genotypes Cartter, Fayette, Kent, Mandarin, and Maple Arrow were judged to be sensitive, showing frequencies of hairy root formation by strain K599 ranging from 50 to 85%. The remaining genotypes were relatively insensitive with transformation frequencies by this strain below 20%. Although the numbers are low, the few productive infections with the agropine- and mannopine-type strains of *A. rhizogenes* occurred most frequently on those genotypes susceptible to infection by strain K599 (Table 1).

Roots at wound sites were judged as transformed if opines were detected in cell-free extracts. Such opine-positive roots generally exhibited other phenotypes associated with true hairy roots including fast growth in culture, loss of geotropism, and lateral root branching (Fig. 1; 22,30). No morphological differences were noted among opine-positive roots of various *G. max* genotypes. When established in tissue culture, opine-positive hairy roots retained their transformed phenotypes. Furthermore, axenic root cultures could be maintained for at least 1 yr by transferring root tip cuttings from older cultures to fresh medium.

While identification based on opine content is sound for analysis of roots induced by the cucumopine and mannopine strains, it may underestimate the frequency of transformation by agropine strains. This is because, unlike cucumopine and mannopine strains, the opine biosynthetic genes in the agropine-type Ri plasmids are encoded on a T-DNA segment separate from that which encodes the *onc* genes (9,36). Thus, it is possible that some of the roots resulting from infection by the agropine strains were transformed but contained only the oncogenic T-DNA segment (3,4). However, the two agropine strains tested were inefficient at inducing either adventitious or transformed roots at wound sites (Table 1).

Hairy root cultures of Williams 82 inoculated with *H. glycines* race 3 produced mature cysts approximately 21 days after nematode inoculation (Fig. 4B). Root cultures could be infected with gravid females or with J2, although inoculation with the former was simpler and appeared to be more efficient. The time required for development of mature cysts was similar to that reported for *H. glycines* on axenic explant cultures of normal soybean

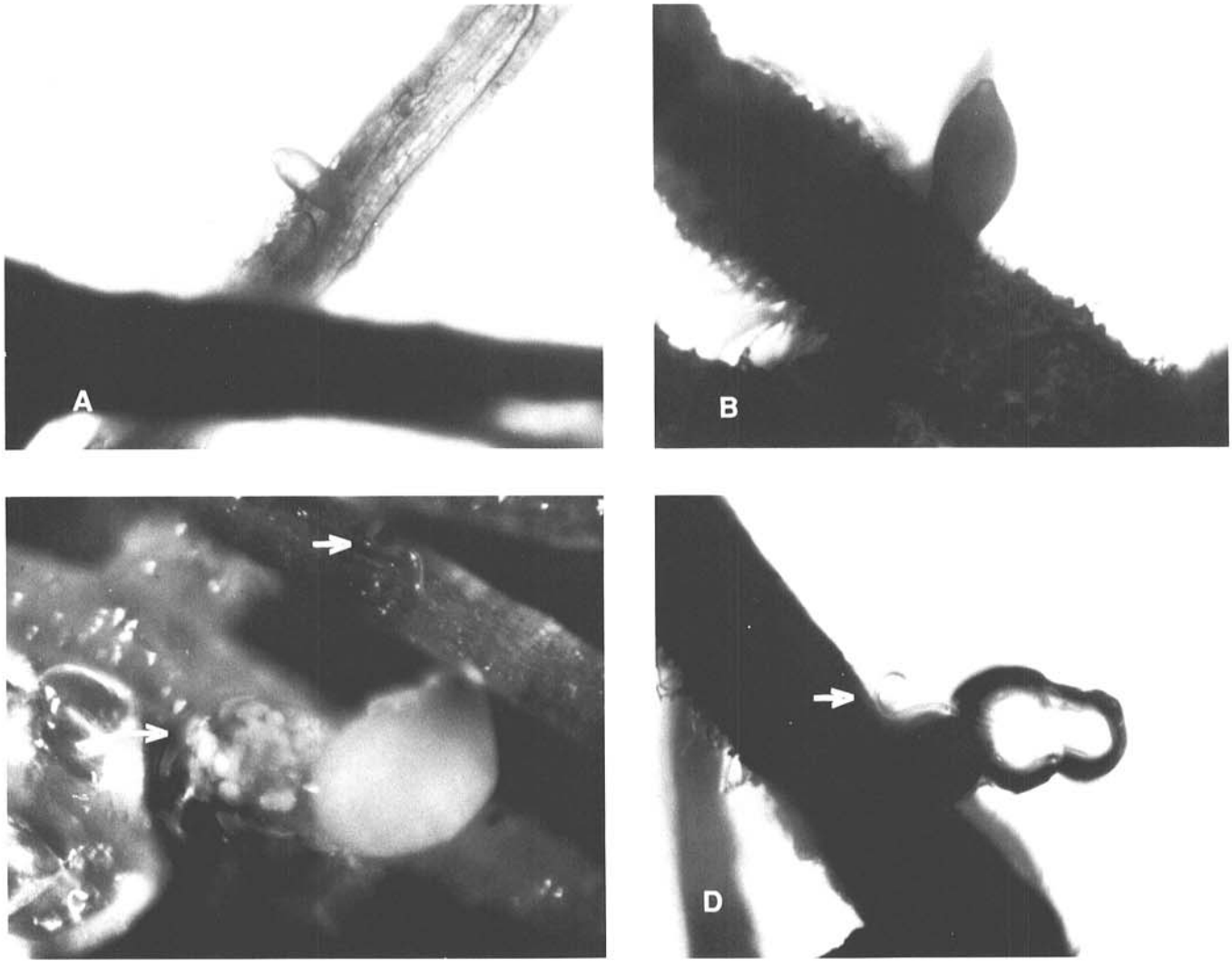

Fig. 4. Propagation of *Heterodera glycines* on transformed roots of soybean cultivar Williams 82. **A,** Female feeding 14 days after inoculation with second-stage juveniles. **B,** Female 21 days after inoculation. **C,** Emergence of second-stage juvenile from cyst and juvenile (top arrow) probing root surface. **D,** Migrating juvenile (arrow).

roots (14,15). After an additional 3 wk, new second-generation cysts were observed, indicating that the nematode could complete its entire life cycle in transformed root cultures (Fig. 4A).

Hairy root cultures may provide some advantages over normal root explants for monoxenic culture of *H. glycines*. First, transformed roots grow indefinitely in tissue culture obviating the need to periodically reestablish new root explants from germinating seedlings. Furthermore, because the transformed roots are clonal in origin, established hairy root cultures should assure a uniformity in genetic background. Second, hairy root cultures may enhance reproductive capacity of the nematode. Such was the case for the propagation of *M. javanica* on cultured tomato hairy roots (33). This increase in reproduction was ascribed to the large numbers of lateral roots produced by the transformed tissues (33). Root branching also is characteristic of soybean hairy root cultures (Fig. 1). Third, since the *A. rhizogenes* system provides a way to insert new genes into differentiated tissues, novel genes conferring nematode resistance or the biosynthesis of potential control compounds could be engineered into the soybean genome and directly tested for their efficacy in conferring resistance to *H. glycines*. Finally, a simple method to axenically cultivate the soybean cyst nematode could be of considerable value in the study of the molecular biology and genetics of *H. glycines*.

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