



In vitro* haustorium development in roots and root cultures of the hemiparasitic plant *Triphysaria versicolor

Alexey Tomilov, Natalia Tomilova & John I. Yoder*

Department of Vegetable Crops, University of California, Davis, CA 95616, USA (*request for offprints: Fax: +1-530-752-9659; E-mail: jiyoder@ucdavis.edu)

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Abstract

Parasitic plants in the Orobanchaceae invade host plant roots through root organs called haustoria. Parasite roots initiate haustorium development when exposed to specific secondary metabolites that are released into the rhizosphere by host plant roots. While molecular approaches are increasingly being taken to understand the genetic mechanism underlying these events, a limitation has been the lack of a transformation system for parasitic plants. Since the haustorium development occurs in roots of Orobanchaceae, root cultures may be suitable material for transient or stable transformation experiments. To this end, root cultures were obtained from explants, and subsequently calluses, from the hemiparasitic plant *Triphysaria versicolor*. The cultured roots retained their competence to form haustoria when exposed to host roots, host root exudates, or purified haustorium-inducing factors. The root culture haustoria invaded host roots and initiated a vascular continuity between the parasite and host roots. The ontogeny of haustoria development on root cultures was indistinguishable from that on seedlings roots. Root cultures should provide useful material for molecular studies of haustorium development.

Abbreviations: 6-BA – 6-benzylaminopurine; DMBQ – 2,6-dimethoxybenzoquinone; IAA – indole-3-acetic acid

Introduction

Exudates released by plant roots are rich in molecules that affect the growth and development of other organisms in the rhizosphere. While the effects of plant root exudates on microbial development in the rhizosphere has received considerable attention, less is known about how plant exudates affects the growth of neighboring plants (Lynch, 1990). This is due in large part to the difficulty of rapidly assaying phenotypes associated with plant–plant interactions.

The most obvious and robust phenotypes are exhibited by parasitic plants in the Orobanchaceae (Musselman, 1980; Press and Graves, 1995). These plants use secondary metabolites produced by host plant roots as signal molecules to induce germination as well as developmental programs specific for parasitism (Estabrook and Yoder, 1998; Keyes et al., 2001). One such program, haustorium development,

marks the transition from autotrophic to heterotrophic growth. Haustoria are globular shaped organs that develop on parasite roots and function in host attachment and invasion. Once a haustorium has invaded a host root, a vascular connection is established through which the parasites robs host nutrient reserves (Riopel and Timko, 1995). The effects on the host plants can be dramatic, leading to stunting or death of the host, and parasitic weeds, such as *Striga* and *Orobanche*, can be devastating agricultural pests (Parker and Riches, 1993). By understanding how haustorium development is triggered by host root exudates, it may be possible to develop novel strategies against parasitic weeds. Insights into the genetic control of parasitic plant–host plant interactions will also provide a more refined understanding of chemical signaling between non-parasitic plants as well.

The Orobanchaceae includes both obligate and facultative parasites that were previously placed in the

Scrophulariaceae (Olmstead et al., 2001). Haustorium development in these plants can be monitored *in vitro* by applying host exudates or purified haustorial inducing factors, such as 2,6-dimethoxybenzoquinone (DMBQ) to aseptic parasite seedlings (Riopel and Musselman, 1979; Baird and Riopel, 1984; Chang and Lynn, 1986). The first morphological event associated with haustorium development is the cessation of root tip elongation, occurring within minutes of exposure to exudates. In *Striga*, this period is associated with a decrease in DNA replication (O'Malley and Lynn, 2000). Within a few hours of exposure to host root exudates, there is a proliferation of haustorial hairs in the region just distal to the parasite root tip. Haustorial hairs are epidermal in origin and function to attach the parasite to the host root (Baird and Riopel, 1985). Around the same time cortical cells underlying the proliferating hair begin an isodiametric expansion that continues for about 24 h. In the absence of host contact, the radical tip of *Striga* and other obligate parasites is transformed into a primary haustorium and there is no further root development until host tissues have been penetrated. In contrast, haustorium development in facultative parasites does not transform the tip meristem and root growth continues distal to the haustorium (Kuijt, 1969).

We are studying haustorium development in the facultative parasite *Triphysaria*, an annual wildflower common throughout the pacific coast (Hickman, 1993). The genus *Triphysaria* includes both self-compatible and self-incompatible species. *Triphysaria* is a generalist parasite with a broad host range that includes both monocots and dicots (Albrecht et al., 1999; Yoder, 2001). Unlike its close relatives *Striga* and *Orobancha*, *Triphysaria* is of no agricultural significance. These traits combine to make *Triphysaria* a useful root parasite for genetic studies (Yoder, 1998).

Genomic approaches are being taken to dissect the haustorium signaling pathway. About 2000 transcripts expressed in *Triphysaria versicolor* root tips during early haustorium development have been sequenced and annotated by homology comparisons with public databases (Matvienko et al., 2001a). The transcriptional regulation and biochemical activities of some transcripts suggest that they may function in haustorium development (Matvienko et al., 2001b).

A genetic transformation system is needed to assay the role of potential haustoria genes in a parasite but, to our knowledge, none exist. Previous studies demonstrated that roots cultures obtained from the parasite

Striga remain competent to form haustoria when exposed to host factors (Wolf and Timko, 1991). We describe here the conditions for establishing and maintaining *Triphysaria* roots cultures. Similar to those from *Striga*, *Triphysaria* root cultures formed functional, invasive haustoria in the presence of host roots and host root factors. Such root culture should be useful for transient and stable transformation systems.

Materials and methods

Materials

T. versicolor seeds were collected from mature plants growing in a grassland cow pasture near Napa California. Maize seeds (324IR) were obtained from Pioneer Hi – Bred International (Johnston, IA).

Three growth media were used in these experiments: MS (Murashige and Skoog, 1962), Hoagland's (Hoagland and Arnon, 1938), and B5 (Gamborg et al., 1968). All media was had a pH of 6.1 and supplemented with 2% glucose and micronutrients (Jamison and Yoder, 2001).

The chemicals 2,6-dimethoxybenzoquinone (DMBQ) was purchased from Pfaltz and Bauer, Inc. (Waterbury, CT), and indole-3-acetic acid (IAA) and 6-benzylaminopurine (6-BA) were purchased from Sigma (St. Louis, MO).

T. versicolor root and callus cultures

About 10 aseptic seedlings of *T. versicolor* were inoculated into 50 ml liquid Hoagland's medium supplemented with 2% (w/v) glucose and 22.8 μ M IAA. The medium was then shaken at 40 rpm at 25 °C under fluorescent lights on a 16 h cycle. After 30 days the roots were cut from the seedlings and transferred into fresh Hoagland's medium with various concentrations of IAA.

As described in results, we determined that an auxin stimulation regime applied monthly optimized root culture proliferation. The auxin stimulation involved sub-culturing roots in medium containing 22.8 μ M IAA for 2–3 days and then transferring to medium without auxin for an additional thirty days. Root cultures have been maintained and increased for over 6 months by this regime.

Calluses were induced by placing root cultures onto Hoagland's medium solidified with 1% Phytagar

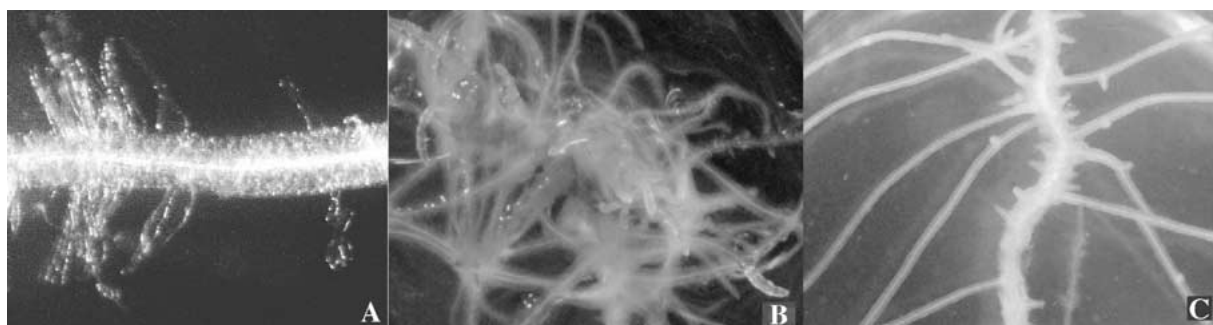


Figure 1. Morphology of *T. versicolor* roots after cultivation in different media. (A) Abnormal root hair formation in B5 medium with 22.8 μM IAA after 40 days of cultivation; (B) morphologically normal root cultures developing lateral roots on Hoagland's medium with IAA after 30 days; (C) root cultures after 60 days in Hoagland's medium with IAA.

Table 1. Root cultures using different aged seedlings for inoculum

Age of inoculated seedling	Light (16 h) ^a			Dark (24 h)		
	Explants with roots (%)	Av. no. of roots per explant	Root length (cm)	Explants with roots (%)	Av. no. of roots per explant	Root length (cm)
1 day	100.0 \pm 0.0	41.2 \pm 1.5	2.5 \pm 0.4	30.0 \pm 5.8	1.9 \pm 0.1	0.0 \pm 0.0
14 days	100.0 \pm 0.0	77.4 \pm 1.6	3.3 \pm 0.2	100.0 \pm 0.0	35.3 \pm 2.8	0.4 \pm 0.0

^a The percent of plants with lateral roots and the length of the roots were calculated 20 days after inoculation. Results are averages of three experiments with 10 explants each.

Table 2. Auxin effects of root culture propagation

Age of inoculated seedling ^b	Auxin concentration (μM)	Light (16 h) ^a		Dark (24 h)	
		Increase ^c (%)	Root initiation ^d	Increase ^e (%)	Root initiation
1 day	22.8	532 \pm 149	– ^f	646	– ^f
	0.228	594 \pm 16	+	181	–
	0	554 \pm 136	+	95	–
	Step-down: ^g 22.8–0	736 \pm 309	+++	489	–
	Step-down: 22.8–0.228	563 \pm 129	++	500	–
14 days	22.8	438 \pm 44	– ^f	579	– ^f
	0.228	245 \pm 27	+	387	–
	0	285 \pm 77	+	564	–
	Step-down: 22.8–0	481 \pm 97	+++	1016	–
	Step-down: 22.8–0.228	394 \pm 77	++	1031	–

^a Root cultures were evaluated for weight and morphology 30 days after sub-culture in the auxin concentration shown.

^b See Table 1.

^c Percent increase was calculated by dividing the change in fresh weight after 30 days by the initial fresh weight and multiplying by 100. Results are averages of three experiments with 10 explants each.

^d Root initiation was evaluated visually.

^e Results are from one dark experiment with 10 explants.

^f Abnormal root morphology and primarily callus formation.

^g In the step-down experiments, root cultures were inoculated into 22.8 μM auxin for 2 days and then transferred to media with less auxin for 28 days.

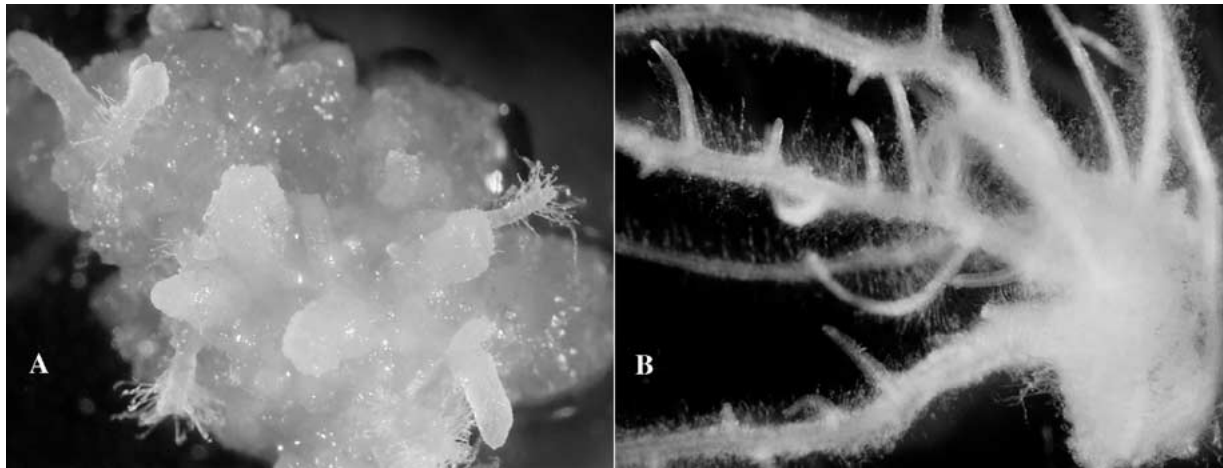


Figure 2. *T. versicolor* callus cultures and differentiation of roots. (A) Root differentiation by *T. versicolor* callus in Hoagland's medium with IAA; (B) roots derived from callus in (A) after 1 month.

containing various concentrations of NAA and 6-BA. The plates were incubated under the conditions described above. Roots began to differentiate from these calluses after about 6 weeks.

In vitro parasitism assays

T. versicolor seeds were surface sterilized in 50% (v/v) bleach (sodium hypochlorite 2.13% (w/v) final) and 0.1% (v/v) Triton X-100 (Sigma, St. Louis) and then rinsed in four to six volumes of sterile water. Approximately 200 seeds were placed in round Petri dishes (100 mm × 25 mm) containing 0.25× Hoagland's nutrient medium, 1% (w/v) sucrose and 0.5% (w/v) Phytagar (Gibco-BRL Life Technologies, Rockville, MD). The plates were sealed with parafilm, placed at 4 °C for 2 days to stimulate germination, and then transferred to a 16 °C growth chamber under a 12 h light regime. Germination occurred 7–10 days later.

Parasitism of maize roots was monitored *in vitro*. Surface sterilized maize kernels were plated onto solid medium (0.25× Hoagland's medium, 1% (w/v) sucrose and 1% (w/v) Phytagar) in square Petri plates. The plates were wrapped with micropore tape (3 M, Health Care, St. Paul) and grown near vertical at 25 °C for a week. Aseptic *T. versicolor* seedlings, or cuttings from root cultures, were placed on top of the maize seedlings ensuring that the host and parasite roots were in physical contact. Plates containing the inoculated maize seedlings were then returned to a vertical position in a plastic case maintained at high humidity with wet paper towels. Haustorium development, host

attachment and host invasion were monitored using a dissecting microscope.

In order to visualize vascular development within haustoria, parasitized maize roots were cut on either side of an invasion site and placed into freshly prepared FAA (63% (v/v) ethanol, 5% (v/v) acetic acid, 2% (v/v) formalin) at 4 °C for at least 24 h. Tissue samples were then transferred into aqueous 75% (v/v) lactic acid and heated in the autoclave for 20 min at 120 °C. Xylem vessels were observed using an Axioskop Routine microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) and photographed with a Sensys Air-Cooled CCD camera (Photometrics Ltd., Tucson, Arizona).

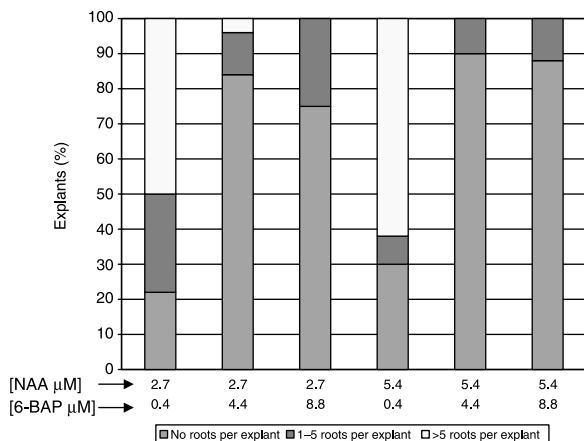


Figure 3. Proportion of calluses producing roots in different media. Calluses were propagated in Hoagland's medium containing either 0.4, 4.4, or 8.8 μM 6-BA, and 2.7 or 5.4 μM NAA. The percentage of calluses producing either no roots, one to five roots, or more than five roots is shown for each hormone concentration.

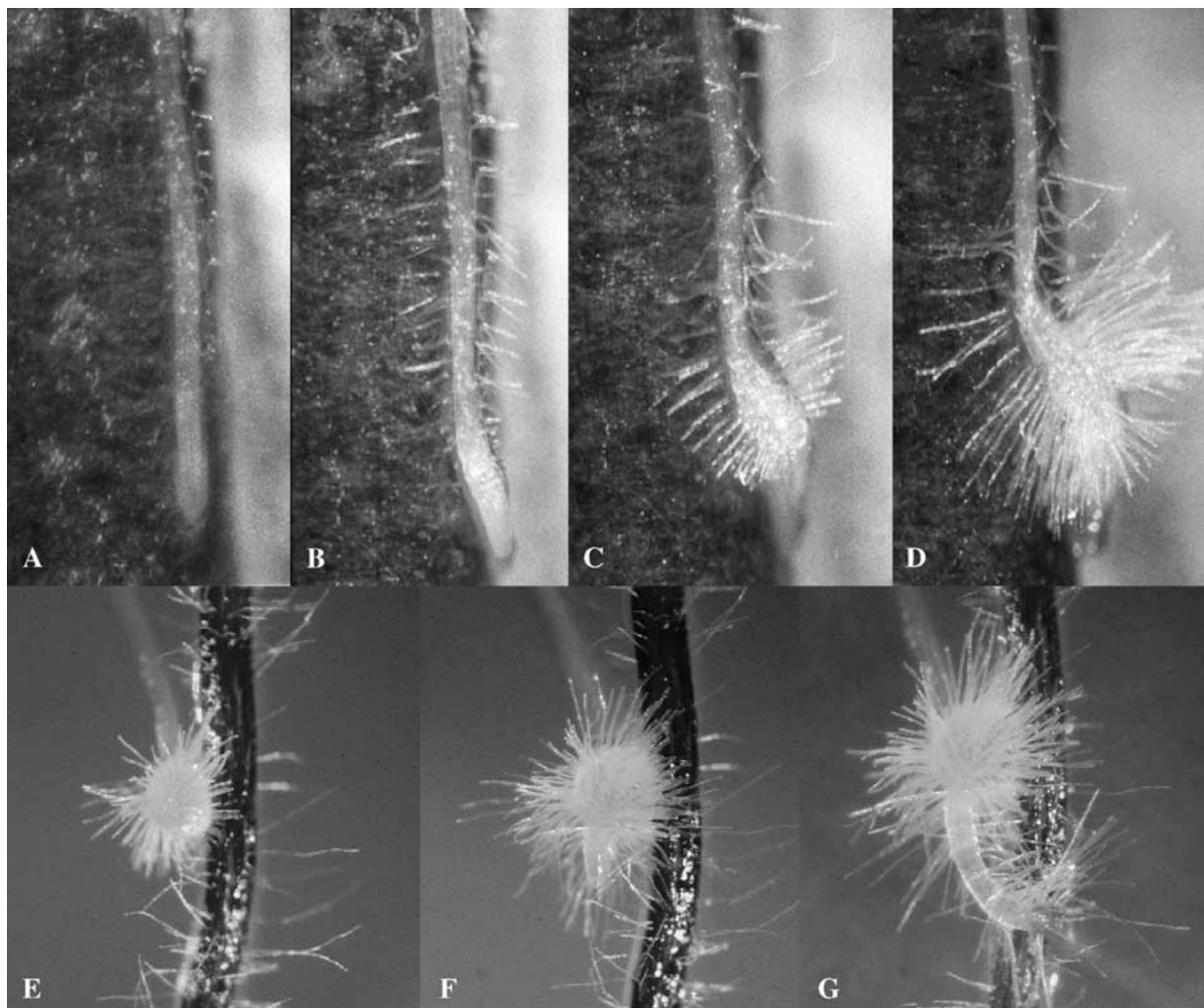


Figure 4. Maize root invasion by *T. versicolor* seedlings and root cultures. Panels A, B, C and D show the timecourse of haustoria formation on a *T. versicolor* seedling in contact with a maize root. For each panel the *T. versicolor* root is on the left and the maize root on the right. Pictures were taken immediately after adding the *T. versicolor* to maize (A), and 6 h (B), 12 h (C), and 24 h (D) later. Panels E, F, and G show the invasion of maize by *T. versicolor* root cultures. Panel E was taken 24 h after adding the *T. versicolor* root culture to the maize root. The haustoria is morphologically similar to that of panel D. Panel F is 2 days and panel G is 9 days after adding the root culture to maize. After 9 days the root culture develops normally distal to the haustorium, typical of secondary haustoria.

Chemical induction of haustorium development

Maize root exudates were prepared from seedlings aseptically grown in agar. Maize kernels were surface sterilized in 50% bleach and 0.2% triton for 40 min followed by extensive washing in sterile water. The seeds were then placed in Magenta boxes (four seeds per box) containing 0.6% (w/v) Phytagar and grown at 25 °C under a 16 h fluorescent light regime. After 14 days, plants were removed and the medium was scraped from the magenta boxes.

The medium was then centrifuged at 10,000 rpm for 30 min to pellet the agar. The supernatant was

filtered and lyophilized in a vacuum freeze dryer. The exudate was rehydrated with sterile water (1 ml per g fresh root weight) and stored at 4 °C.

T. versicolor seedlings and root culture explants were exposed to maize root exudates or DMBQ. About 10 seedlings were placed parallel to one another on the surface of solidified 0.25× Hoagland's medium and incubated in a near vertical position for 1 week to facilitate the growth of root tips along the surface of the medium. Root cultures were similarly arranged on agar medium but were induced immediately after plating. Haustorium induction was achieved by adding 2 ml of either maize root exudates or 10 μM DMBQ to

the *T. versicolor* roots. Control explants were treated with sterile water. Plates were kept horizontal for 2 h to allow absorption of the liquid inducer into the agar medium and then returned to the vertical orientation for 24 h at 25 °C. The number of haustoria and was determined using a dissecting microscope.

Results and discussion

T. versicolor root and callus cultures

T. versicolor roots were excised from 14-day-old plants and inoculated into 24-well trays containing different media supplemented with 22.8 µM IAA (Wolf and Timko, 1991). The trays were shaken at either 16 or 25 °C for 20 days. The highest percentage of lateral roots obtained per explant, 92% ± 13, was obtained in Hoagland's medium at 25 °C. These conditions also resulted in cultures with the most typical root morphology (Figure 1). All subsequent experiments were done in Hoagland's medium.

We next tested the effect of light and seedling age on root culture development (Table 1). *T. versicolor* seedlings inoculated 14 days after germination yielded more root cultures than seedlings inoculated 1 day after germination. Overall, the frequency of lateral root initiation and subsequent root growth was greater in the light than dark.

The growth of root cultures in different concentrations of auxin was evaluated (Table 2). We also investigated the effect of placing the cultures for 2 days in high levels of auxin (22.8 µM IAA), followed by cultivation in low or no auxin (Table 2). The rationale of this auxin step-down regime was to initiate lateral root development in high auxin while optimizing subsequent growth in low auxin conditions. The step-down protocol proved quite effective. The best conditions for root culture propagation were to incubate sub-cultured roots 2 days in 22.8 µM auxin before transfer to medium without auxin. This cycle was repeated monthly.

Callus was induced from the root cultures by plating on solid Hoagland's medium containing different concentrations of the growth regulators 6-BA and NAA. Optimal callus formation was obtained in 5.4 µM NAA and 0.44 µM 6-BA.

After 6 weeks in medium, roots began to differentiate from callus (Figure 2). As shown in Figure 3, optimal root differentiation was obtained in 0.4 µM 6-BA at either 2.7 or 5.4 µM NAA. No shoots were observed at any concentration of 6-BA and NAA.

Roots that differentiated from callus were themselves used as inoculum for liquid root cultures. These root cultures were morphologically indistinguishable from those initiated from seedlings.

In vitro parasitism of maize

The parasitism of maize was monitored *in vitro* by placing *T. versicolor* root cultures, or radicals of *T. versicolor* seedlings, onto aseptically growing maize roots. Haustoria developed near the root tips of both

Table 3. Haustorium development in root cultures added to maize roots

Culture conditions ^a	Exp no.	Roots with haustoria ^b (%)	Average (%)
Step-down to no IAA	1	17	28 ± 10
	2	30	
	3	36	
Step-down to 0.228 µM IAA	1	75	70 ± 7
	2	73	
	3	62	

^a Root cultures obtained from the IAA step-down procedure (22.8 µM IAA for 2 days followed by either 0.228 µM or no IAA for 28 days) were placed onto aseptic maize roots.

^b The percentage of roots that developed haustoria in three independent experiments was determined after 2 weeks.

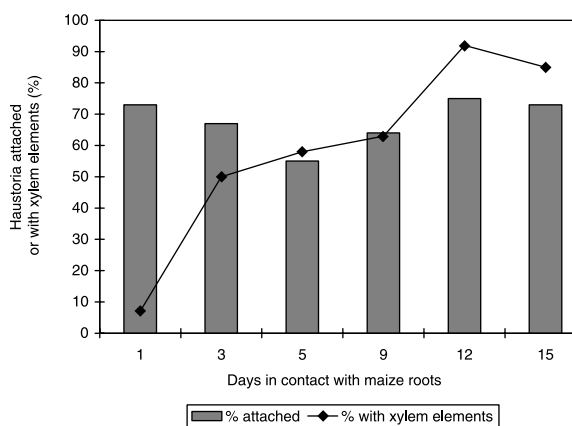


Figure 5. Time course of haustorium attachment and xylem differentiation. *T. versicolor* seedlings were added to maize roots and allowed to remain in contact for up to 15 days. The proportion of haustoria attached to maize was determined by tugging on the *T. versicolor* root at various times; the proportion of attached haustoria is shown as bars on the graph. The proportion of haustoria with xylem elements, shown as the line, was determined by clearing a sample of haustoria with lactic acid as described in Materials and methods.

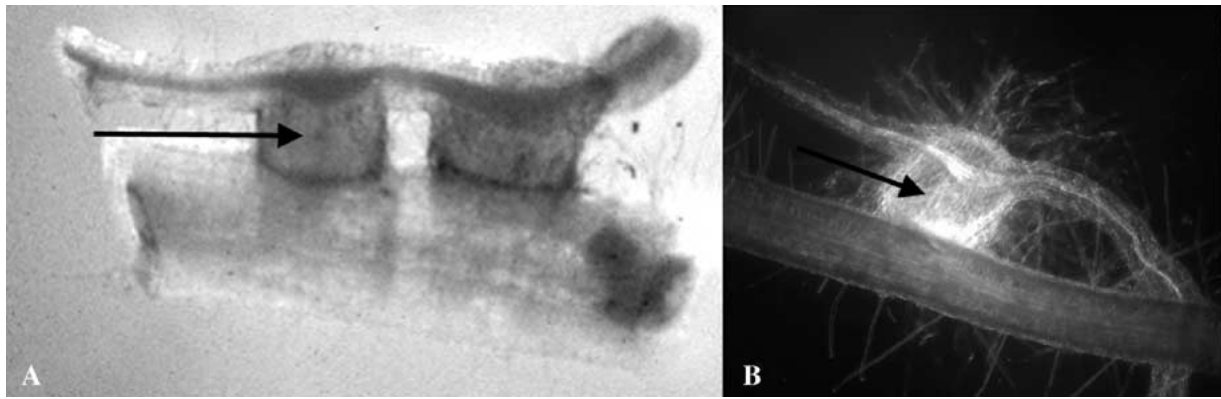


Figure 6. Seedling and root culture haustoria parasitizing maize. (A) Haustoria from intact *T. versicolor* plant attached to maize root. Arrows point to the vascular bridge connecting host and parasite; (B) haustorium from root culture after 3 weeks on maize.

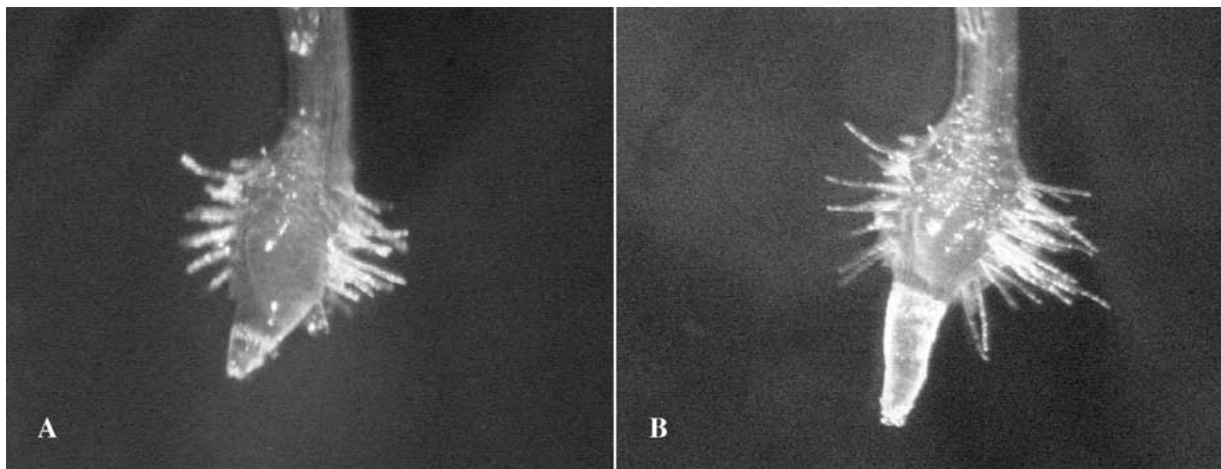


Figure 7. Pre-attachment haustorium formation on root cultures. Haustorial hairs and cortical swelling, hallmarks of pre-attachment haustoria, on root culture tips exposed to 10 μ M DMBQ after 24 h (A) and 48 h (B).

seedlings and root cultures soon after contact with maize roots (Figure 4). Haustoria that developed on seedling roots were morphologically indistinguishable from those that developed on root cultures. Haustorium development was about twice as frequent when the root cultures were maintained in 0.228 μ M IAA compared to medium without auxin (Table 3).

The first observable change associated with haustorium development in both seedlings and cultures was a proliferation of hairs just behind the root tip. These hairs appeared as early as 6 h after contact with the host and continue for about 24 h. We determined the kinetics of *T. versicolor* attachment to maize by gently tugging on the *Triphysaria* seedling at various times after overlaying the roots (Figure 5). By these criteria, about 70% of the haustoria were attached within 24 h

of contact between *Triphysaria* and maize. The proportion of attached haustoria did not increase after 24 h, indicating that if haustoria were competent to attach they did so within 24 h of contact. Neither the frequency of haustorium formation nor the speed of development was affected by the addition of 10 μ M DMBQ to the point of connection (data not shown).

It had been previously shown that cells within the body of the haustorium develop into vessel elements once the haustorium has penetrated the host root and reached the stele (Heide-Jorgensen and Kuijt, 1995). We observed these vessel elements in haustoria on both roots and root cultures after invasion into maize (Figure 6). To determine the time course of xylem development, haustoria were cleared in lactic acid at various times after attachment and examined under a

Table 4. Haustoria induction of root cultures by DMBQ

Culture conditions ^a	Root tips with haustoria relative to (DMBQ) ^b (%)			
	0 μ M	10 ⁻³ μ M	10 ⁻² μ M	10 ⁻¹ μ M
Step-down to no IAA	0	24 \pm 4	49 \pm 5	76 \pm 4
Step-down to 0.228 μ M IAA	0	48 \pm 4	62 \pm 5	84 \pm 5

^a Described in Table 3.

^b Differing concentrations of DMBQ were added to the root tips and the proportion of root tips with haustoria determined 24 h later. Data are from four independent experiments with about 30 root tips in each.

microscope for xylem. As shown in Figure 5, only a few haustoria had developed xylem within 24 h. The proportion of haustoria with xylem increased in a roughly linear mode to about 90% by day 11.

Haustorium development was initiated near the tips of root cultures following treatment of the cultures with DMBQ (Figure 7) or maize roots exudates (data not shown). The haustoria inducing factors present in the exudates were released from maize roots in the absence of parasite contact or damage to the root. These factors were stable at 4 °C for several days. This differed from the observation that either host abrasion or parasite enzymes are required to release factors that trigger haustorium formation in *Striga* (Chang and Lynn, 1986; Kim et al., 1998).

The frequency of haustorium formation in response to DMBQ was dependent on the auxin concentration in which the roots were cultured (Table 4). There were more root tips with haustoria when cultivated in 0.228 μ M IAA than obtained without auxin. This was consistent with our observation that auxin enhances haustorium formation on maize roots (Table 3).

Conclusions

Root cultures generated from *T. versicolor* maintained their ability to develop haustoria and invade host roots similar to intact seedlings. The observation that root cultures maintained in auxin are enhanced in their ability to make haustoria suggests that this hormone acts at some stage in haustorium development. The parasite root cultures may prove to be an amendable system for gene transfer, thereby facilitated the genetic studies of plant parasitism.

Acknowledgements

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