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**RESEARCH PAPER**

# *In vitro* infection of host roots by differentiated calli of the parasitic plant *Orobanche*

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## Abstract

**Root parasites of the genus *Orobanche* are serious weeds in agriculture. An aseptic infection system of host roots using calli of three *Orobanche* species was developed for the study of host–parasite interaction. The response of calli to various hormonal combinations was studied, because a requirement for infection is the differentiation of root-like protrusions, which are capable of producing haustorial connections to the host. Infectious root-like protrusions develop under the influence of 0.5–1.0 mg $l^{-1}$ IAA, and under the combination of 0.2 mg $l^{-1}$ NAA with 5.0 mg $l^{-1}$ kinetin. These protocols produced root protrusions with pad-like structures that resembled attachment organs of *Orobanche* seedlings, and proved effective in parasitizing host roots. Direct contact with the medium inhibited haustorium development and prevented infection. To overcome this problem, certain root portions were isolated from the medium by inserting thin glass plates underneath. Calli were then placed on the raised root portions and successfully infected the roots and developed young *Orobanche* tubercles with vascular system that directly connected to the host.**

Key words: Aseptic infection, *Brassica*, callus induction, germination, *Orobanche*, red clover, tomato.

## Introduction

Parasitic plants of the genus *Orobanche* (broomrapes) connect to dicotyledonous host plants using a special intrusive multicellular organ, the haustorium, and deprive water and nutrients from them. They are holoparasitic, devoid of leaves and totally dependent on their hosts. Survival of the parasite depends on its ability to establish contact with a host and develop an haustorium.

Some *Orobanche* species are serious weeds that cause heavy direct damage to many important dicotyledonous crops such as tomato, sunflower, and legumes (Parker and Riches, 1993). *O. aegyptiaca* and *O. ramosa* are important scourges of vegetable and field crops in large parts of Asia and the Middle-East. In China, there are about 20 *Orobanche* species, including the economically important *O. aegyptiaca* and *O. ramosa*, which have the widest host range (Zhang and Jiang, 1994). The potential impact of the latter species on oilseed rape (*Brassica napus*) was recently reported in France, where this crop is heavily damaged by *O. ramosa* (Gibot-Leclerc *et al*., 2001). *O. minor* infestations are currently damaging clover in the United States (Colquhoun *et al*., 2001). This species is also found in Japan and in other Asian, European, and African countries. Each broomrape plant produces thousands of tiny seeds that remain viable in the soil for many years, allowing a rapid increase of the parasite seed bank in agricultural soil (Lopez-Granados and Garcia-Torres, 1993; Joel *et al*., 1995). Effective control of these weedy species is extremely difficult and infection often results in a significant reduction of crop yields (Joel, 2000). Many countries therefore declared *Orobanche* species as quarantine weeds, which should be handled under strictly isolated conditions.

Normal development of the parasite starts with germination that comes in response to the reception of a chemical stimulus from host roots (Yokota *et al*., 1998; Yoneyama

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*et al*., 2001; Sato *et al*., 2003). But the seed responds to stimuli only after being 'conditioned', i.e. exposed to water and suitable temperatures for several days (Joel *et al*., 1995). The germinated seedling infects host roots by developing an haustorium that penetrates the host root and serves as a physiological bridge between the two organisms. The young parasite then develops a tubercle, with adventitious roots and a shoot.

Seeds of *Orobanche* are often contaminated with micro-organisms, which interfere with biological studies (Joel and Losner-Goshen, 1994). The study of the biochemical and physiological features of the parasite and of host–parasite interaction requires the development of an *in vitro* infection system, free of micro-organism contamination, with full regeneration of the parasite. Nevertheless, growing these parasitic plants *in vitro* is difficult, because of their dependence on a connection to hosts for normal development, and because of their specific germination requirements.

*In vitro* infection using *Orobanche* seedlings is possible (Losner-Goshen *et al*., 1996), but results in low rates of success, perhaps due to the high genetic diversity in the natural seed populations. A more homogenous parasite source may be obtained from calli that are propagated *in vitro*. The main objective of the present study was, therefore, to develop an efficient system for the infection of host roots with *Orobanche* calli that results in the full regeneration of *Orobanche* plants. Plant regeneration procedures also have potential applications in the development of gene transfer systems for molecular studies of the parasite.

Calli of different kinds have previously been induced from seedlings of *O. aegyptiaca* (Ben-Hod *et al*., 1991*a*) and *O. ramosa* (Batchvarova *et al*., 1999), but all previous methods for the infection of host roots with *Orobanche* calli have only shown limited success and low efficacy. Infection of intact tomato roots *in vitro* with calli of *O. aegyptiaca* was achieved when the *Orobanche* calli were transferred from the enriched culture medium on which they were raised to a substrate containing only mineral salts and vitamins (Ben-Hod *et al*., 1991*b*). Such intact host plants with their roots grown in Petri dishes were, however, hard to keep free from micro-organism contamination, because the tomato shoots were allowed to develop outside the sterile plates.

The present paper describes the development of a completely aseptic infection by calli of three *Orobanche* species and on roots of a variety of host plants from different families. This new and simple aseptic 'Cover Glass Infection System' (CGIS) can now be used as a model *in vitro* system for the study of root parasites. The paper also describes, for the first time, *in vitro* culture of *O. minor* that belongs to a distinct section in the genus *Orobanche* (Parker and Riches, 1993) and differs significantly from *O. aegyptiaca* and *O. ramosa*.

## Materials and methods

### Plant material

Seeds of *Orobanche minor* Sm. were collected from mature plants that parasitized red clover grown in an experimental field of Utsunomiya University, Japan. Seeds of *O. aegyptiaca* Pers. were collected from plants grown on tomato in Western Galilee, Israel. Seeds of *O. ramosa* L. were collected from plants parasitizing tomato around the Gezira area of central Sudan. All seed lots were tested for germinability by standard germination test in Petri dishes after Linke (2001).

Seeds of commercial varieties of *Brassica napus* L. (cv. Jian 6 and Shuang 72X) and *B. rapa* L. (cv. Youbai) were harvested in Hangzhou, China in 2001. *B. juncea* Coss. Seeds (cv. Ames 24521) were supplied by the North Central Regional Plant Introduction Station of USDA-ARS, USA. Seeds of tomato (*Lycopersicon esculentum* Mill.) cv. 2888 were purchased in Japan from the local market. Seeds of red clover (*Trifolium pratense* L.) cv. Hamidori were purchased from local markets. All seeds, upon receipt in Utsunomiya University, Japan, were stored in the dark at 4 °C until use.

### Seed sterilization

Various methods for surface-sterilization of *Orobanche* seeds have previously been examined (Kumar, 1977; Ben-Hod *et al*., 1991*a*; Losner-Goshen *et al*., 1996; Batchvarova *et al*., 1999; Westwood, 2000; Goldwasser and Yoder, 2001; Portnoy and Joel, 2001). In this study the double surface-sterilization of *Orobanche* seeds by sequential immersions in 70% ethanol for 2 min and 1% sodium hypochlorite containing 0.1% Tween-20 for 15 min (10 min for *O. aegyptiaca* seeds) with constant agitation was, in most cases, sufficient to prevent contamination. Host seeds were surface-sterilized in a similar way. For *O. aegyptiaca* seeds this surface-sterilizing procedure was repeated after 1 d, because in some cases contamination persisted after the first treatment. This second treatment replaced the use of the antibiotic cocktail that was used by Ben-Hod *et al*. (1991*b*) to overcome the same problem.

Sodium hypochlorite (5%) and Tween-20 were obtained from Kanto Chemical Co. Ltd., Japan. All water used was deionized Millipore water (Milli-Q SP Reagent Water System, USA), autoclaved before use. Similarly, items such as filter paper were all sterilized by autoclaving.

### Seed conditioning and germination

Without conditioning and stimulation the observed germination rates (and consequently callus production) were extremely low, far from reaching the germination percentages that were obtained for the same seed lots in standard germination tests.

*Orobanche* seeds, approximately 20 each, were sown on a 5 mm disc of glass fibre filter paper (Advantec GC90, Japan). Twenty such discs were then placed in a sterile 5 cm Petri dish lined with two layers of filter paper wetted with 1.5 ml of distilled water. The Petri dishes were sealed with Parafilm and wrapped with aluminium foil to provide absolute darkness. These were then placed in dark, controlled growth chambers at 23 °C for seed 'conditioning'. After 7 d of 'conditioning', the seeds were blotted to remove excessive water. Each five discs were then transferred to a separate new 5 cm Petri dish containing two layers of filter paper wetted with 1.2 ml of $10^{-6}$ M $GR_{24}$ or with distilled water (for control). $GR_{24}$ is an artificial stimulant (Johnson *et al*., 1981), which is used to promote germination of conditioned *Orobanche* seeds. The Petri dishes were incubated under the same conditions as for the 'conditioning', and the initial germination was examined 7 d after the $GR_{24}$ treatment. All experiments were replicated at least three times. In these standard germination tests, which were conducted on

glass-fibre paper rather than solid medium, the mean germination percentage of *O. ramosa* reached 60.5–72.0%, *O. minor* 65.8–76.6%, and *O. aegyptiaca* reached 80.0–90.6%.

$GR_{24}$ was provided by Professor B Zwanenburg from the University of Nijmegen, the Netherlands. It was stored dry in the dark at –20 °C before use. Stock solutions ($10^{-3}$ M) were prepared by dissolving in a few drops of acetone and adding distilled water to the desired concentration.

### *Growing* Orobanche in vitro

For callus production, conditioned *Orobanche* seeds and conditioned seeds that were treated for 1–2 d with $GR_{24}$ on filter paper as described above were thoroughly washed and transferred onto solid culture media of different composition in 5 cm Petri dishes, where germination took place and callus formation was induced.

Better callus induction was obtained in all three *Orobanche* species with the B5 culture medium (Gamborg *et al*., 1968), compared with the MS medium (Murashige and Skoog, 1962). Therefore the reported results given below were taken mainly from experiments with B5. Additional constituents in the medium were 3.6% potato dextrose agar (PDA), vitamin mixtures (70 mg $l^{-1}$ myo-inositol, 0.1 mg $l^{-1}$ thiamine, 0.5 mg $l^{-1}$ pyridoxine, and 0.5 mg $l^{-1}$ nicotinic acid), 3% sucrose, 600 mg $l^{-1}$ casein hydrolysate, 5% sterile coconut water, and 8 mg $l^{-1}$ $GA_3$. In addition, various combinations of the following hormones, 0.2–5.0 mg $l^{-1}$ each, were also added: naphthylacetic acid (NAA), 3-indoleacetic acid (IAA), with 6-benzylaminopurine (BAP) or kinetin (KIN). The pH was adjusted to 5.8 (B5) or 5.6 (1/2 MS) with NaOH.

$GA_3$ was filter-sterilized by passing through a 0.2 µm syringe filter; the other constituents (except for sterile coconut water) were sterilized by autoclaving at 120 °C for 15 min. The B5 and MS basal salts and other chemicals were obtained from Wako Pure Chemicals Ltd., Japan; from Sigma Co.; or from ICN Biomedicals Inc., USA.

Analysis of variance was calculated for the percentage of callus formation, and statistical inferences were made based on Duncan's multiple range tests between the means of the results for the different treatments within each *Orobanche* species, performed at the 0.05 level of probability.

### In vitro *infection*

The development of *in vitro* connections between the parasitic calli and host plants was investigated in rectangle sterile Petri dishes (2×10×15 cm). This Petri dish system kept the intact plants free from micro-organism contamination, contrary to the above-mentioned systems.

15–20 sterilized seeds of the host plants were sown *in vitro* on solid half-strength MS medium supplemented with 1.8% agar, 2% sucrose, and vitamin mixtures. The Petri dishes were sealed with Parafilm and placed at the angle of approximately 45° from horizontal. The lower part of each Petri dish was wrapped with aluminium foil, so that rooting took place in relative darkness. The shoots of the host plants that were kept inside the upper part of the Petri dish were subjected to a lighting regime of 14/10 h (light/dark, 60 µmol $m^{-2}$ $s^{-1}$) at 23 °C.

In the new *in vitro* infection system, a harder medium was used containing 1.8% agar, and the Petri dishes were placed at an angle of 45° rather than horizontal, which allowed the downward growth of the host roots on the surface and successfully prevented them from growing into the medium.

After the host developed a well-defined root system, thin glass plates (*c*. 2 cm wide square or round microscope cover slips) were inserted between the host roots and the medium at the elongation region of the roots. Then calli of *Orobanche* were placed on these raised root regions. The system was examined under the dissecting microscope every 3 d, and broomrape infection was followed and documented. All procedures were carried out under strictly aseptic conditions. The total enclosure of the host prevented contamination with micro-organisms, and did not seem to affect host–parasite association.

### *Anatomical examination*

Germination and callus formation were followed under the dissecting microscope at least twice every week. For anatomical examination, seedlings and calli of various kinds were fixed in FAA (2% formaldehyde, 5% acetic acid, 60% ethanol in water) or in glutaraldehyde (5% in phosphate buffer), dehydrated in an ethanol series, and embedded in the resin LR White (Agar Scientific, England), sectioning and staining procedures followed Joel and Losner-Goshen (1994). The sections were examined under the light microscope. In addition, root portions carrying the connection zone of *Orobanche* infection were cleared with lactic acid and stained with phloroglucin–HCl, and then examined and photographed under the microscope.

## Results and discussion

### *Germination*

Both conditioning and stimulation by $GR_{24}$ were needed in order to achieve, within a week, a relatively high percentage germination of surface-sterilized *Orobanche* seeds on the solid media *in vitro*. Seed germination started 5–7 d after transferring $GR_{24}$-treated *Orobanche* seeds onto solid culture media and ranged 44–56% in *O. ramosa*, 56–74% in *O. minor*, and 73–88% in *O. aegyptiaca*.

For callus production, the optimal duration of $GR_{24}$ treatment after seed conditioning was 1–2 d (see below). Longer or shorter $GR_{24}$ treatments resulted in negligible induction of calli. Thus, for example, after treating conditioned *O. minor* seeds with $GR_{24}$ for only 12 h, germination was as low as 24–33% and no more than 10% of the seeds eventually developed into calli. Although a very high germination rate (75–82%) was reached after 5 d of $GR_{24}$ treatment, the resulting seedlings were much elongated and were not sufficient for callus induction, and, consequently, the percentage of callus formation ranged from zero to only 7.6%. Similar results were observed with *O. ramosa* and *O. aegyptiaca*. The influence of germination stimulants on seedling development in root parasites like *Orobanche* and *Striga* has so far not been explored.

### *Callus induction*

Neither the B5 nor the MS media alone could support the development of calli, and, therefore, more constituents, including plant hormones, were added to the culture media. GA was previously found to be particularly important for *Orobanche* callus production (Ben-Hod *et al*., 1991*a*; Batchvarova *et al*., 1999) and was therefore added to almost all media. Some of the more successful media that were tested for callus production are listed in Table 1. The germination of $GR_{24}$ treated seeds on these media was sufficient for initiating the development of calli. Callus formation began 5–10 d after seed germination.

**Table 1.** *Effect of plant growth regulators on* in vitro *callus formation from seedlings of* O. ramosa, O. minor, *and* O. aegyptiaca[a]

| Culture medium[b] | Growth regulators (mg $l^{-1}$) | % Callus formation[c] (±SE) | Description of callus at developing and maintaining stages | Success of *in vitro* infection[d] |
|---|---|---|---|---|
| *O. ramosa* | | | | |
| MS-0 | 8.0 $GA_3$ | 34±2 c | Hard, yellow to brown, healthy-looking shoots, some protrusions | – |
| B5-0 | 8.0 $GA_3$ | 24±3 d | Soft, white to brown, few amorphous protrusions | – |
| B5-3 | 1.0 IAA, 8.0 $GA_3$ | 41±2 bc | Hard, white to brown, many root-like protrusions with haustorium-like structures | ++ |
| B5-3a | 0.5 IAA, 8.0 $GA_3$ | 38±2 bc | Hard, white to brown, many root-like protrusions with haustorium-like structures | ++ |
| B5-4 | 2.0 NAA, 8.0 $GA_3$ | 30±3 cd | Hard, yellow to brown, slow-growing, some root-like protrusions | + |
| B5-5 | 2.0 NAA, 0.5 BAP, 8.0 $GA_3$ | 55±2 a | Hard, yellow to brown, many root-like protrusions with haustorium-like structures, drops of secretion | ++ |
| B5-6 | 0.2 NAA, 5.0 KIN, 8.0 $GA_3$ | 44±4 b | Hard, yellow to brown, large, some root-like protrusions | + |
| *O. aegyptiaca* | | | | |
| B5-0 | 8.0 $GA_3$ | 19±2 c | Soft, yellow to brown, large, few amorphous protrusions | – |
| B5-3 | 1.0 IAA, 8.0 $GA_3$ | 56±3 a | Hard, white to brown, large, many root-like protrusions with attachment organs | ++ |
| B5-3a | 0.5 IAA, 8.0 $GA_3$ | 47±4 b | Hard, white to brown, some root-like protrusions | + |
| B5-4 | 2.0 NAA, 8.0 $GA_3$ | 18±3 c | Hard, yellow to brown, some root-like protrusions | + |
| B5-5 | 2.0 NAA, 0.5 BAP, 8.0 $GA_3$ | 58±2 a | Hard, yellow to brown, large, many root-like protrusions | ++ |
| B5-5b | 2.0 NAA, 0.2 BAP, 8.0 $GA_3$ | 44±5 b | Hard, yellow to brown, many root-like protrusions | + |
| B5-6 | 0.2 NAA, 5.0 KIN, 8.0 $GA_3$ | 43±2 b | Hard, white to yellow, large, some root-like protrusions | + |
| *O. minor* | | | | |
| B5-0 | 8.0 $GA_3$ | 20±2 d | Soft, yellow to brown, few amorphous protrusions | – |
| B5-3 | 1.0 IAA, 8.0 $GA_3$ | 38±2 c | Hard, yellow to brown, large, some root-like protrusions | + |
| B5-3a | 0.5 IAA, 8.0 $GA_3$ | 48±3 b | Hard, yellow to brown, many root-like protrusions | + |
| B5-4 | 2.0 NAA, 8.0 $GA_3$ | 32±3 c | Soft, white to yellow, large, few amorphous protrusions | – |
| B5-5 | 2.0 NAA, 0.5 BAP, 8.0 $GA_3$ | 50±4 b | Soft, white to yellow, large, few amorphous protrusions | – |
| B5-5a | 2.0 NAA, 0.5 BAP | 24±2 d | Soft, white to yellow, large, few amorphous protrusions | – |
| B5-6 | 0.2 NAA, 5.0 KIN, 8.0 $GA_3$ | 62±3 a | Hard, yellow to brown, fast-growing, many root-like protrusions and shoots, drops of secretion | ++ |

[a] The conditioned *Orobanche* seeds were treated with $10^{-6}$ M $GR_{24}$ for 1 d before transfer to solid culture media.
[b] All media contained B5 or MS as basal salts. Other constituents and their concentrations are shown in Materials and methods.
[c] Callus formation was recorded approximately 4 weeks after germination. For each species, means followed by the same small letter are not significantly different at the 0.05 level of probability.
[d] (+, ++) Moderately and most suitable for *in vitro* infection, respectively; (–), not suitable for *in vitro* infection.

There was a variation in the percentage of callus formation, which was not necessarily related to the percentage of germination.

At the very beginning of their development the young calli of all three *Orobanche* species were white and had a typical globular shape as shown in Fig. 1A. However, with further development, calli of two main types were induced and maintained, with different growth rates and colours, depending on the combination of plant growth regulators in the medium (Table 1). Some calli were fast growing, but remained unorganized, carrying only amorphous protuberances. These calli were white or yellow, fragile and soft in consistency (Fig. 1B). Other calli were slow growing, but soon showed much differentiation, usually carrying root-like protuberances. These calli were hard and compact, either yellow, white or brown (Fig. 1C). These two types were previously described for *O. aegyptiaca* by Ben-Hod *et al*. (1991*a*) and for *O. ramosa* by Batchvarova *et al*. (1999). Successful induction of *O. minor* calli has, however, never been described before. Callus differentiation needed between 17 d and 30 d and there were no consistent differences between species and culture media.

Shoot meristem initiated at the distal end of *O. ramosa* callus on MS culture medium containing $GA_3$. Three weeks later, very conspicuous and healthy miniature shoots developed without contact with any host (Fig. 1D). These shoots have further grown and maintained after being transferred to new media, but did not develop to the flowering stage.

$GA_3$ increased callus formation. This was also true in the presence of other plant hormones (compare B5-5 and B5-5a). IAA and kinetin were also helpful in promoting callus production. Kinetin and 2,4-D induced the development of the rapid growing undifferentiated callus (data

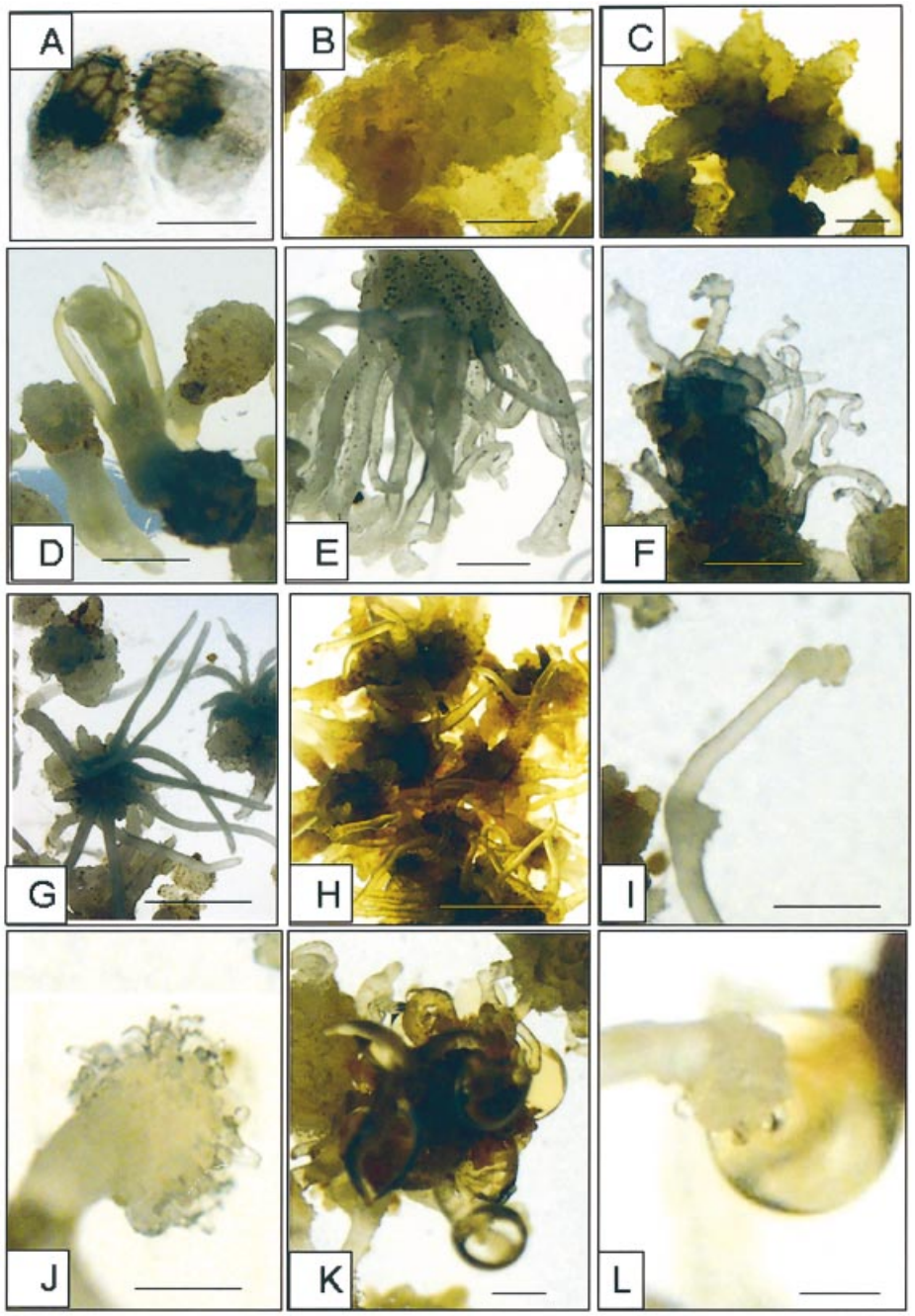


**Fig. 1.** (A) The initial stage of callus development in *O. ramosa.* The radicle emerging out of the root is white, globular and composed of callus tissue. Bar=0.3 mm. (B) Soft, fast-growing and undifferentiated callus of *O. ramosa*, grown on medium B5-0. Bar=0.5 mm. (C) Harder, slow-growing differentiated callus of *O. minor* with thick protrusions (medium B5-3). Bar=0.5 mm. (D) Typical young shoot at the distal end of *O. ramosa* callus developing on MS culture medium (medium MS-0). Bar=0.25 mm. (E, F) *O. ramosa* calli with thin root-like protrusions, grown on media B5-3 and B5-5, respectively. Bar=0.4 mm. (G) *O. aegyptiaca* calli on medium B5-6, with thin root-like protrusions. Bar=0.5 mm. (H) *O. minor* calli on medium B5-3a with thin root-like protrusions. Bar=0.5 mm. (I) Root-like protrusions with terminal and lateral attachment organs carrying typical haustorial papillae (medium B5-5). Bar=0.5 mm. (J) Haustorial papillae on an attachment organ at the apex of a root-like structure (medium B5-5). Bar=0.2 mm. (K, L) Drops of secretion at the tip of root protrusions in *O. ramosa* (medium B5-5). Bar=0.2 mm.

not shown), while IAA induced the formation of hard and differentiated callus with many root-like protrusions (Table 1; Fig. 1E, H).

Whereas *O. ramosa* and *O. aegyptiaca* developed highly differentiated calli in the presence of NAA (with or without BAP) (Fig. 1F), *O. minor* developed only undif-

ferentiated calli. Interestingly, when both NAA and kinetin were present in the medium the resulting callus was highly differentiated also in *O. minor*, with well organized root-like protrusions (Fig. 1G). Similar results with NAA were reported for *O. aegyptiaca* by Ben-Hod *et al*. (1991*a*).

This is the first report of high percentage of callus formation. Most of the calli were maintained successfully after being subcultured every 3 months into fresh media. The protocol allows the production of large amounts of calli for the study of biological and chemical features of the parasite and of host–parasite interaction.

### In vitro *infection*

*In vitro* infection on host roots was achieved with the harder and slow-growing *Orobanche* calli, which developed many root-like protrusions with terminal and lateral attachment organs. These attachment organs carried typical haustorial papillae (Fig. 1I, J), thus resembling the attachment organs of *Orobanche* seedlings (Joel and Losner-Goshen, 1994). The attachment organs seem to be the main characteristic, which allows these calli to successfully infect host roots (see below).

Drops of secretion of different colours were observed at the tip of the root-like protrusions (Fig. 1K, L). Their occurrence was rather stable and consistent, being more frequent in some treatments. Similar secretions were also reported for *O. aegyptiaca* calli by Ben-Hod *et al*. (1991*a*). Secreted material has been collected from different calli, and chemical identification of these secretions is now under way. Using the new 'Cover Glass Infection System' (CGIS) it was possible to obtain *in vitro* parasitism by all three *Orobanche* species on intact host roots. The cover glass system allowed infection with *O. ramosa* and *O. aegyptiaca* on the root systems of *Brassica napus*, *B. juncea*, and tomato (Fig. 2B, C). Similarly, calli of *O. minor* successfully infected red clover roots (Fig. 2A). Calli with many root-like protrusions and attachment organs from media B5-3 and B5-5 were the most successful for *in vitro* infection with *O. ramosa* and *O. aegyptiaca*, while those from medium B5-6 were the most successful for *in vitro* infection with *O. minor*.

The presence of the glass plates prevented direct contact of the calli with the sucrose-containing nutrient medium, which supported the root system of the host plant, at the same time it allowed close contact of calli with host roots. Sucrose was previously shown to inhibit haustorium formation (Ben-Hod *et al*., 1991*b*).

The root-like protrusions with attachment organs developed haustoria that penetrated adjacent host roots (Fig. 3C). On top of glass plates the connection to host roots was usually successful, and a tubercle developed at this infection site (Fig. 2D, E). In most experiments, more than ten successful infections were observed in each rectangular Petri dish.

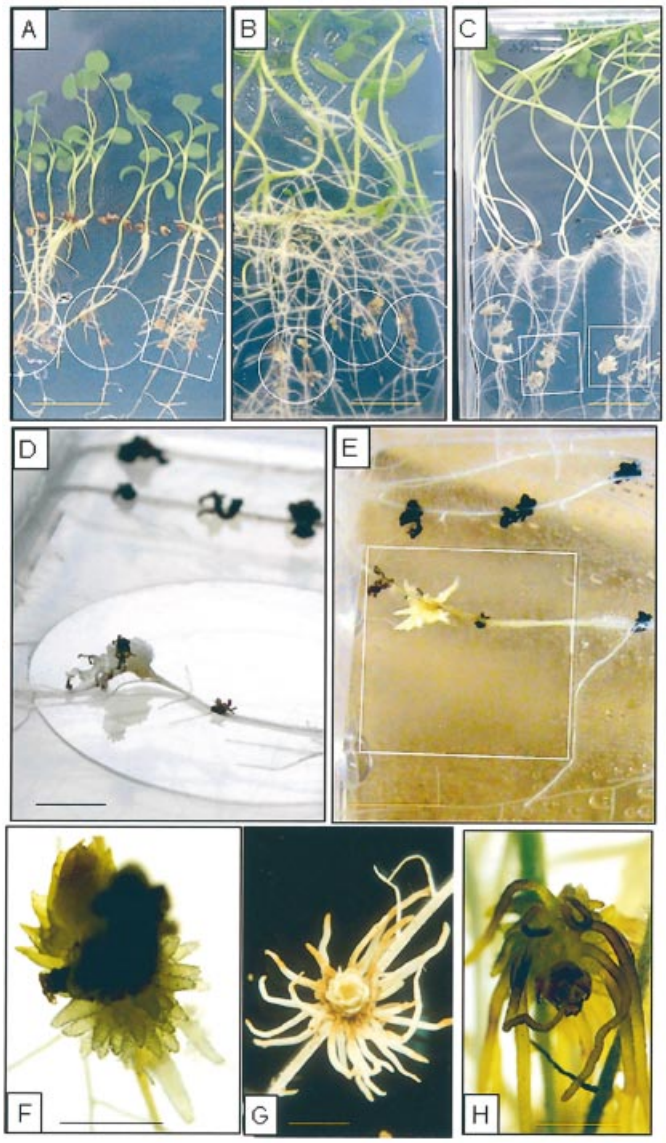


**Fig. 2.** (A–C) The Cover Glass Infection System (CGIS): *in vitro* infection of intact host roots by *Orobanche* calli with root-like protrusions. Square or round micro cover glass was inserted underneath the roots in aseptic rectangular Petri dishes. The location of the cover glass is indicated. Bar=20 mm. (A) *O. minor* calli from medium B5-6 infecting the root systems of red clover cv. Hamidori; (B) *O. aegyptiaca* calli from medium B5-5 infecting roots of tomato cv. 2888; (C) *O. ramosa* calli from medium B5-3 infecting roots of *Brassica napus* cv. Shuang 72X. (D) *O. ramosa* tubercle on a round cover glass connected to root systems of *B. juncea* cv. Ames 24521 (medium B5-5). The remnants of the callus remain on top of the developing tubercles. Bar=4 mm. (E) Tubercle on a square cover glass connected to root of *B. napus* cv. Jian 6 (medium B5-5). The dark calli located outside the cover glass were placed on roots that were directly touching the medium, and therefore degenerated and did not develop tubercles. Bar=8 mm. (F) Mature tubercle of *O. aegyptiaca*, with adventitious roots and a shoot apex, on root of *B. napus* cv. Jian 6 (medium B5-3). The dark body is the remains of the callus from which it developed. Bar=7 mm. (G) *O. aegyptiaca* tubercle grown in soil on a tomato root, showing typical adventitious roots and a shoot apex. Bar=4 mm. (H) Mature tubercle of *O. minor* on root of red clover cv. Hamidori (medium B5-6). The tubercle developed adventitious roots and a shoot apex. Bar=6 mm.

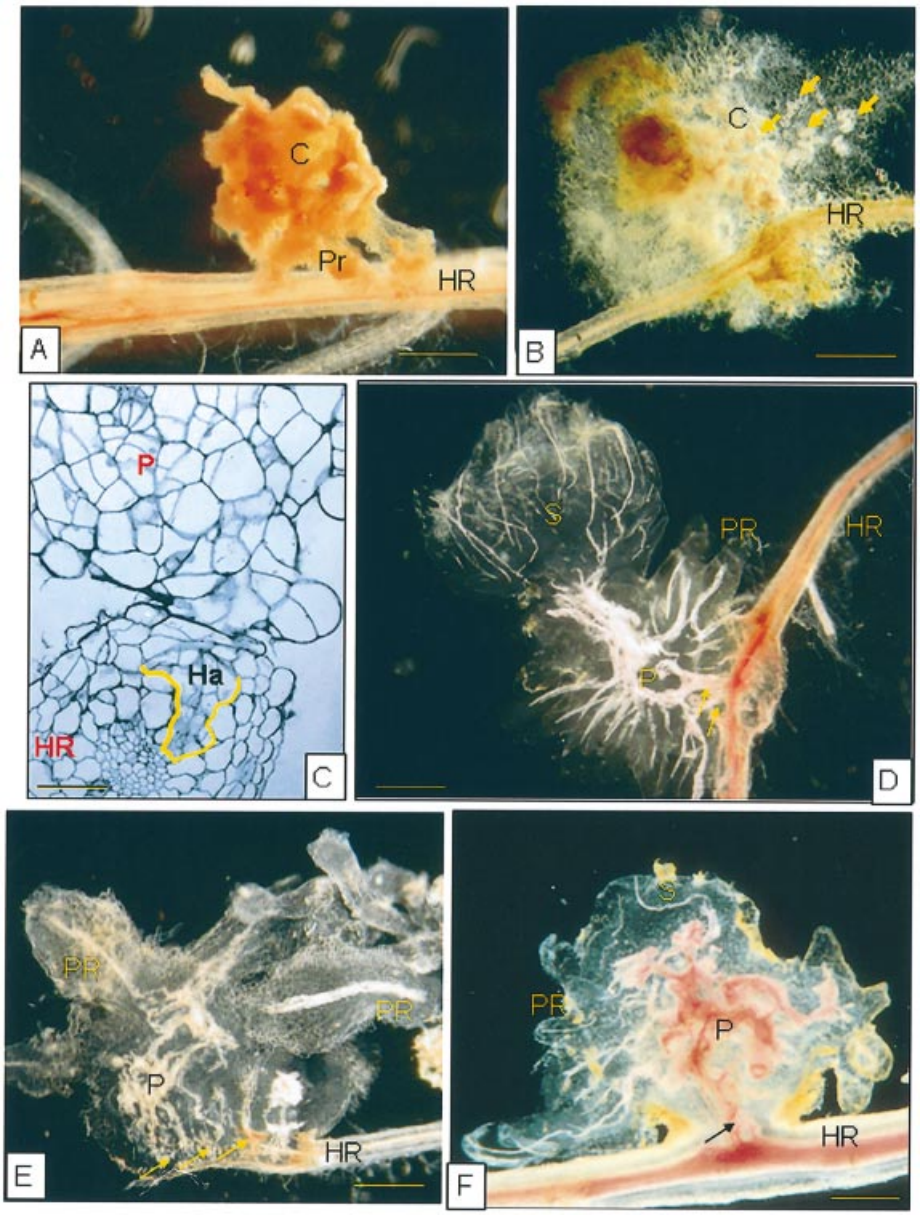


**Fig. 3.** (A, B) Calli that did not develop tubercles even though they were attached to host roots. (A) Whole mount of *O. ramosa* callus on tomato root (medium B5-3a). Bar=2.5 mm. (B) Hand-cut longitudinal section of a callus with scattered vessel element aggregations that are not connected to each other and do not form a continuous vascular system (medium B5-4). Cleared with lactic acid and stained with phloroglucin–HCl. Arrows point to aggregations of vessel elements. Bar=5 mm. (C) Cross-section of the attachment area of a callus protrusion infecting the host root. The margins of the haustorium inside the root are marked (medium B5-5). Bar=0.1 mm. (D–F) Hand-cut longitudinal sections of tubercle cleared with lactic acid and stained with phloroglucin–HCl to show vascular connection between parasite and host. (D) Tubercle that developed from *O. ramosa* callus protrusion (medium B5-5). Bar=1 mm. (E) Tubercle that developed from *O. minor* callus protrusion (medium B5-6). Bar=1 mm. (F) Tubercle that developed *in vivo* on host root from an *O. aegyptiaca* seedling. The vascular system in all tubercles continues in the adventitious roots and in the developing shoot apex. Bar=2 mm. C, callus; Ha, haustorium; P, parasite; PR, adventitious roots; Pr, callus protrusion; HR, host root; S, shoot apex; arrows, the vascular connections of the parasites to central cylinders of host roots.

Without the glass plates, the calli placed on roots located directly on the medium, showed signs of degeneration (browning) and gradually died (Fig. 2D, E). Similarly, the fast-growing unorganized calli of all three *Orobanche* species did not develop any haustorium and showed signs of degeneration (browning) soon after being placed in contact with host roots, either with or without glass plates (data not shown). The tubercles developed from the portion of the root-like protuberances that came in contact with host roots, whereas the rest of the callus died and its remnants remained as dark masses on top of the developing

tubercles (Fig. 2D, F). In this behaviour the callus resembles *Orobanche* seedlings, the proximal portions of which usually degenerate once their distal part develops a tubercle after a successful infection (Joel and Losner-Goshen, 1994). The *in vitro*-grown tubercles carried both a shoot apex and adventitious roots (Fig. 2F, H), and resembled tubercles that developed in soil from the same seed lots (Fig. 2G).

Some calli did not develop real tubercles even though they were attached to the host roots. Inside these calli the xylem was composed of scattered aggregations of vessel elements that were not connected to each other and did not form a continuous vascular system (Fig. 3A, B). However, most *in vitro*-grown tubercles had a continuous vascular system, which extendeded into the shoot apex and into the lateral roots, and was directly connected to the vascular cylinder of the host root (Fig. 3D). This was true for all three species with all examined hosts (Fig. 3D, E). This highly differentiated vascular system looked identical to that of normal *Orobanche* tubercles that develop from seedlings on host roots *in vivo* (Fig. 3F).

*In vitro* infection of tomato roots was previously achieved with *O. aegyptiaca* callus that was transferred from an enriched culture medium on which it was raised to a substrate containing only mineral salts and vitamins (Ben-Hod *et al*., 1991*b*). Losner-Goshen *et al*. (1996) described another aseptic infection method, in which normal *O. aegyptiaca* seedlings (rather than calli) infected tomato root cultures. Both latter methods were complicated and resulted in only very limited infection levels. The present paper is the first to report on a rather simple and effective method for massive development of completely aseptic *in vitro* infection of host roots by *Orobanche* calli. This new aseptic 'Cover Glass Infection System' (CGIS) method may now serve as a model system in the study of *Orobanche* and other parasitic plants *in vitro*.

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