# Regeneration of Pathogenic *Aphanomyces cochlioides* and *A. euteiches* from Protoplasts.

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**ABSTRACT**

**Enzyme mixtures were tested for the digestion of cultured *Aphanomyces cochlioides* and *A. euteiches* mycelia to promote the formation of protoplasts. Cell wall-digesting enzymes at 0.1% (w/v) in osmoticum were sufficient to convert the mycelia to protoplasts within 2 hr, similar to digestion conditions for other oomycetes. Protoplast integrity was maintained upon embedding in molten agar containing 1M mannitol. Within 4 days post-plating on potato dextrose agar, 10 to 20% of the embedded protoplasts of both fungal species formed germ tubes that subsequently formed mycelial colonies. Fungal isolates derived from regenerated protoplasts of *A. cochlioides* and *A. euteiches* retained the ability to induce black root disease in sugarbeet seedlings and water soaking in pea seedlings, respectively. The generalized protocol for production and regeneration of protoplasts for *Aphanomyces* species may be of use in the development of a gene transfer protocol for this important crop pathogen.**

**Additional key words:** sugarbeet, legume, oomycete, fungus.

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Damping off and root rot diseases caused by the oomycetes *Aphanomyces cochlioides* Drechsler and *A. euteiches* Drechsler (Drechsler, 1929) can be significant impediments to sugarbeet and legume production, respectively (Parke and Grau, 1992). Yield losses due to infection are a consequence of decreased stand establishment and rotting of adult roots and disease development is favored by high moisture and temperature. Although a seed coating that includes the fungicide Tachigaren (hymexazol) can improve sugarbeet seedling establishment (Brantner, et al., 1997), adult plants may not be protected by this treatment later in the growing season. Genetic resistance against *A. cochlioides* was identified in sugarbeet several decades ago (Bockstahler and Reece, 1948), but it is poorly characterized and has been incorporated into elite parents for hybrid sugarbeet production only to a limited extent. Variation in pathogenicity (Delwiche, et al., 1987, Malvick and Percich, 1998) of *A. euteiches* and in the susceptibility of pea accessions (Davis, et al., 1995) to this pathogen has been reported.

Both *A. cochlioides and A. euteiches* require high soil moisture for their infectivity (Parke and Grau, 1992). The resting stage of the fungus (oospore) can survive in the soil for many years. Under appropriate conditions, the oospore germinates and fungal hyphae grow within the soil. Zoosporangia are then formed which liberate numerous zoospores; the zoospore is the infectious entity in the pathogenesis of sugarbeet and pea by *A. cochlioides* and *A. euteiches*, respectively (Papavizas and Ayers, 1974).

Although ultrastructural observations have been made regarding the infection of sugarbeet and pea by *Aphanomyces* species (Papavizas and Ayers, 1974), little is known about the genetics or biochemistry of the interaction of these fungi with their hosts. In recent years, investigation into the genetic and biochemical mechanisms that determine compatible and incompatible interactions between plants and fungi has been impacted highly by molecular genetics, in particular by studies involving gene transfer technologies (for reviews, see De Wit, 1997, Kombrink and Somssich, 1995, and Annis and Goodwin, 1997). Thus, the determination of toxin (Knogge, 1996) and degradative enzyme (Annis and Goodwin, 1997) activities produced by fungi and necessary for either pathogenicity or high pathogen virulence has come about by integrating biochemistry with pathogen transformation. Examples of this include the discovery of the importance of the phytotoxin cercosporin in the infection of tobacco and soybean by *Cercospora nicotianae* and *C. kikuchii*, repectively (Upchurch et al., 1991), and investigations into the cell wall degrading activities conferring high virulence to maize of *Cochlioibolus*

*carbonum*(Walton, 1994). Moreover, the elucidation of the molecular basis for classic gene-for-gene interactions in host-pathogen genetics utilized gene transfer technologies in both the plant host and fungal pathogen (DeWit, 1997). Where gene transfer to the pathogen has been used in these studies, the procedures typically have employed the use of fungal protoplasts as an integral step in the transformation procedure.

We are interested in developing an efficient gene transfer protocol for *A. cochlioides* in order that the molecular basis for disease induction in sugarbeet might be more critically investigated. The objective of the present study was to define digestion and culture conditions for *Aphanomyces* species that would permit the efficient and routine isolation of viable, regenerable protoplasts. Additional experiments were performed to ensure that protoplasting does not impair the regenerated isolates for infection of sugarbeet plants. Results presented here constitute the first report, to our knowledge, of the successful production and regeneration of protoplasts of a member of the genus *Aphanomyces*.

## MATERIALS AND METHODS

### Culture Media and Fungal Strains

Potato dextrose agar (PDA), corn meal agar (CMA), potato dextrose broth (PDB), potassium chloride, D-mannitol, and Trizma™ base were purchased from Sigma Chemical (St. Louis, MO). Culture media were prepared according to standard recipes, unless otherwise indicated. Oatmeal broth was prepared according to Parke and Grau (1992). Agarose was LE-grade from FMC Bioproducts (Rockland, MD). Novozyme 234 (1,000 β-glucanase units per gram) was purchased from Interspex Technologies (Foster City, CA) and Cellulysin™ cellulase (12,000 units per gram) was purchased from CalBiochem (San Diego, CA).

Strains of *A. cochlioides* and *A. euteiches* were a generous gift from C. Windels (U. of Minnesota—Crookston). Single zoospore isolates of *A. cochlioides* [19-1(z)] and *A. euteiches* [MM174 (z)] were used and cultures were maintained on CMA or PDA plates in the dark at 22°C with weekly subculturing. For storage of 6 months or longer, cultures were maintained at 22°C in oatmeal broth where abundant oospores were produced.

### Protoplast Preparation and Embedding

Single agar plugs of ~5 $mm^3$ were cut from the edge of 7 day-old fungal cultures on PDA and were individually inoculated to flasks containing 100 ml of PDB. The stationary liquid cultures were maintained at 22°C in the dark for 5 days. Media was decanted from one half of the

flasks and was replaced by 100 ml of 0.1X PDB; these cultures were placed in a rotating shaker (30 rpm) at 22°C for 2 additional days.

For harvesting, mycelia were transferred to glass culture tubes and were centrifuged for 10 min at 100 X g at room temperature. As much of the culture media as was possible was removed from each pelleted mycelium and the mat was resuspended in 10 ml of 0.6 M potassium chloride. After centrifugation of the mycelia for 10 min at 100 X g, the supernatant was discarded and each mycelium again was resuspended in 10 ml of 0.6 M potassium chloride and centrifuged at 100 X g for 10 min. After removal of the second potassium chloride rinse from the mycelial mat, 5 ml of 0.6 M potassium chloride was added and the pellet was resuspended. In two independant experiments using five individual cultures per experiment, the mean wet mass per culture of mycelia produced in the 1X PDB treatment was 0.30 g (sd = 0.05) and that for the mycelia produced in the 0.1XPDB treatment was 0.65 g (sd = 0.05). Each mycelium was treated individually for protoplasting.

Five milliliters of a filter-sterilized digestion solution containing various combinations of cell wall-degrading enzymes dissolved in 0.6 M potassium chloride were added to each mycelial suspension. Final concentrations of enzymes were 1% (w/v) Novozyme 234, 1% (w/v) Cellulase, a mixture of 1% (w/v) Novozyme 234 + 0.1% (w/v) Cellulase, or a mixture of 0.1% (w/v) Novozyme 234 + 0.1% (w/v) Cellulase (Table 1). Mixtures of mycelia and digestion solution were transferred to standard 15 cm Petri dishes and were sealed with Parafilm (American National Can Co., Greenwich, CT). Digests were incubated at 30°C with gentle rotation (30 rpm). Each treatment was perfomed in duplicate within each experiment and three replicates of the experiment were performed. The release of protoplasts from digested mycelia was monitored using an inverted light microscope (Olympus Model #IMT-2).

When abundant protoplasts were observed after about 2 hr, the mixture was transferred to a polypropylene culture tube (Falcon #2059) by filtration through a 105 micron mesh polypropylene membrane and was centrifuged for 10 min at 50 X g at room temperature. As much of the enzyme solution was removed as possible and the pelleted cells were resuspended in 3 ml of a solution of 0.7 M potassium chloride and 10 mM calcium chloride. The cells were incubated at room temperature for 10 min and were centrifuged at 50 X g for 5 min. The supernatant was removed and the protoplasts were resuspended to a final volume of 1 ml

**Table 1.** Yield of *A. cochlioides* protoplasts after the digestion of mycelia with different enzymes.

| | Digestion Mix (all in 0.6M KCl) | | | |
|---|---|---|---|---|
| Culture Conditions[†] | 1% Novozyme[‡] | 1% Cellulase[‡] | 1% Novozyme + 0.1% Cellulase[‡] | 0.1% Novozyme + 0.1% Cellulase[§] |
| 1X PDB | <1,000 | <100 | <1,000 | $0.3 \times 10^6$ |
| 0.1X PDB | <1,000 | <100 | <1,000 | $1.4 \times 10^6$ |

[†] mycelia were cultured for 5 days in PDB. For the 0.1X PDB treatment, mycelia were cultured for 5 days in PDB followed by 2 days in 0.1X PDB.

[‡] estimates for yield for these treatments were made by concentration of the total preparation of protoplasts produced in these digests into a volume of ~0.1 ml.

[§] numbers of protoplasts are expressed as a mean protoplast number (standard deviation = $0.26 \times 10^6$) per gram of wet mycelia over three independent experiments.

in MTC (0.8 M mannitol, 10 mM TRIS-HCl, 10 mM $CaCl_2$, pH 7.5). Protoplast yield was estimated using a dual-chambered haemocytometer (Reichert-Jung, Germany).

For embedding of protoplast suspensions, 0.1 to 0.5 ml of the cell suspensions were pipetted into a culture tube containing 10 ml of 42°C molten solution of 1M mannitol and either 0.5X PDA or 1.0% agarose that included 0.5X PDB (Yang, et al., 1993). The protoplast/agar suspension was gently mixed and poured into petri dishes containing PDA that had been pre-warmed to 37°C. After the agar hardened, plates were sealed with Parafilm and transferred to a dark incubator maintained at 22°C.

**Regeneration and Pathogenicity Assays**

Embedded protoplasts were monitored daily for evidence of regeneration using an inverted light microscope. Agar sections containing isolated, regenerating protoplasts were transferred to fresh PDA plates using a sterile dissecting needle. Hyphal tips from the resulting colonies were transferred to PDA and CMA plates for further culturing. All cultures were maintained at 22°C in the dark.

Seed of sugarbeet (*B. vulgaris* cv 'Ultramono') and pea (*Pisum sativum* cv 'Wando') were surface sterilized and embedded in agar supplemented with Gambourgs B5 medium (Sigma Chemical, Inc.) in square petri dishes according to the method of Keijer et al. (1997). Petri dishes containing seeds were incubated horizontally at 25°C under fluorescent lights that delivered a 14 hr photperiod until germination (about 4 to 6 days post-plating). At 5 days post-seeding, agar plugs cored from the the edge of mycelial mats of one week old cultures of *A. cochlioides* and *A. euteiches* were placed between the seedlings. Three isolates each of *A. cochlioides* and *A. euteiches* that were derived from protoplasts were tested for induction of disease in sugarbeet and pea, respectively. At 7 days post-inoculation, seedlings were examined for disease symptoms.

## RESULTS AND DISCUSSION

The ability to form regenerable protoplasts has been instrumental in the development of cell fusion and gene transfer protocols for plant pathogenic fungi (Hargreaves and Turner, 1992). With the desire of applying this technology to the study of black root disease in sugarbeet, we investigated the possibility that protocols for the production and regeneration of protoplasts for other fungi (Hargreaves and Turner, 1992) might also succeed with *A. cochlioides*. The generation of protoplasts for the application of gene transfer technology has been the method of choice

for other oomycete pathogens of agricultural importance. Although zoospores are in effect protoplasts as they emerge from zoosporangia, this emergence is typically asynchronous, prohibiting the consistent and routine recovery of large quantities of protoplasts. Protoplasting of mycelia of *A. euteiches* also was performed during the course of these experiments to ascertain the generality of the protocol for *Aphanomyces* fungi. Mycelia of *A. cochlioides* and *A. euteiches* were subjected to digestion with cell wall-degrading enzymes commonly used in the production of fungal protoplasts, with an emphasis on conditions successfully used on oomycetes (Bailey, et al., 1991, Judelson, et al., 1991, Judelson et al., 1993).

Incubation of *A. cochlioides* mycelia in 0.1% cellulase + 0.1% Novozyme 234 in an osmoticum of 0.6M KCl led to the routine production of protoplasts on the order of $10^6$ protoplasts per gram of fresh weight of mycelia (Table 1 and Figure 1), which is in agreement with the amounts of protoplasts prepared from other oomycete fungi. Typically, $10^6$ to $10^7$ protoplasts per gram of mycelia are released from fungal hyphae when using optimized protoplasting procedures (Hargreaves and Turner, 1992). Similar results were obtained for the production of protoplasts from *A. euteiches*, although the mycelial mass was somewhat greater than that for *A. cochlioides* after the same time period of culturing. Few protoplasts were released from mycelia when digested with cellulase alone or Novozyme 234 alone (Table 1), indicating a need for their combined use for efficient protoplast formation.

Approximately 1% of the protoplasts embedded in 0.5X PDA in the presence of nutrients and 1.0 M mannitol began to regenerate after 2 days. By 4 days post-embedding, 10 to 20% of the plated protoplasts had formed hyphal tubes (Figure 2) and developed into colonies. A similar frequency of protoplast regeneration has been documented for oomycete and true fungi (Bailey et al., 1991, Judelson et al., 1991). No significant difference was observed in regeneration frequencies between protoplasts embedded in 0.5X PDA as compared to 1% agarose containing PDB. All colonies that were transferred to plates of PDA or CMA continued to grow with a phenotype characteristic of the progenitor isolate. Thus, the protoplasting procedure did not appear to alter the growth habit of the *A. cochlioides* isolates on culture media. Similar observations were made with *A. euteiches*, although the regeneration of 10 to 20% of the embedded protoplasts occured within 1 to 2 days post-plating (data not shown).

Isolates of *A. cochlioides* and *A. euteiches* that were derived from single protoplasts were examined for maintainence of virulence to sugarbeet or pea seedlings, respectively, or alteration in the ability to form characteristic spore types. As shown in Figure 3, isolates derived

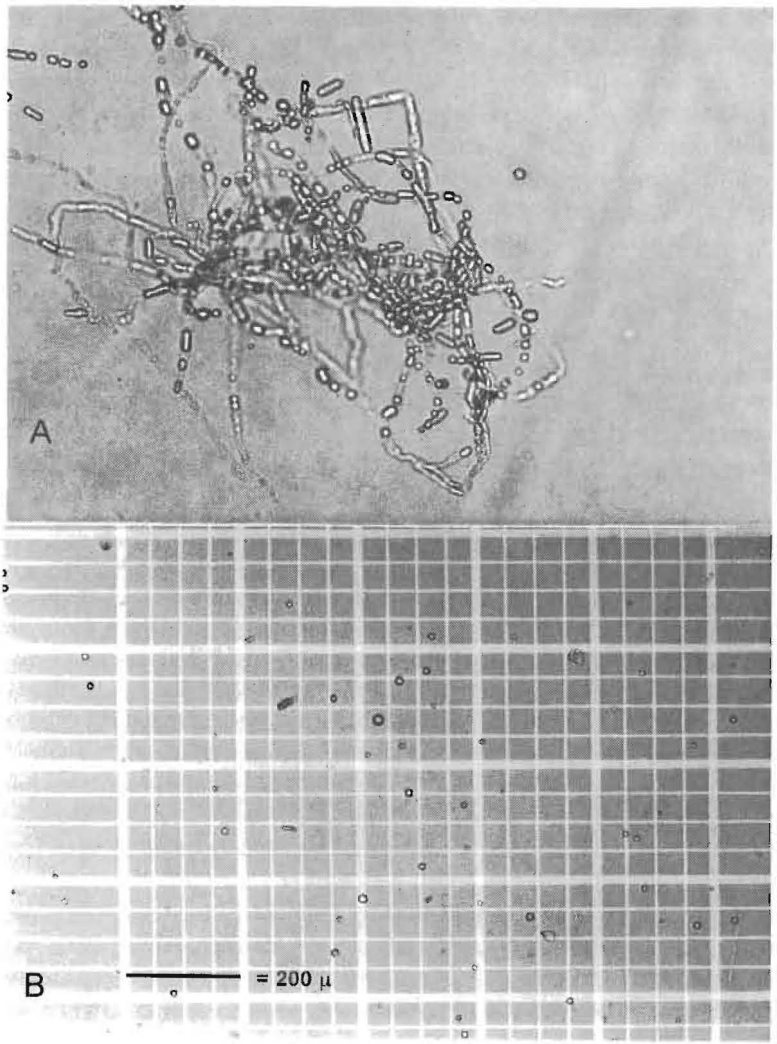


**Figure 1**. Production of protoplasts of *A. cochlioides*. In A, the digestion has proceeded for 1 hr and only infrequent round protoplasts are observed. Mature protoplasts (B) are shown on the grid of a haemocytometer (bar = 200 µ).

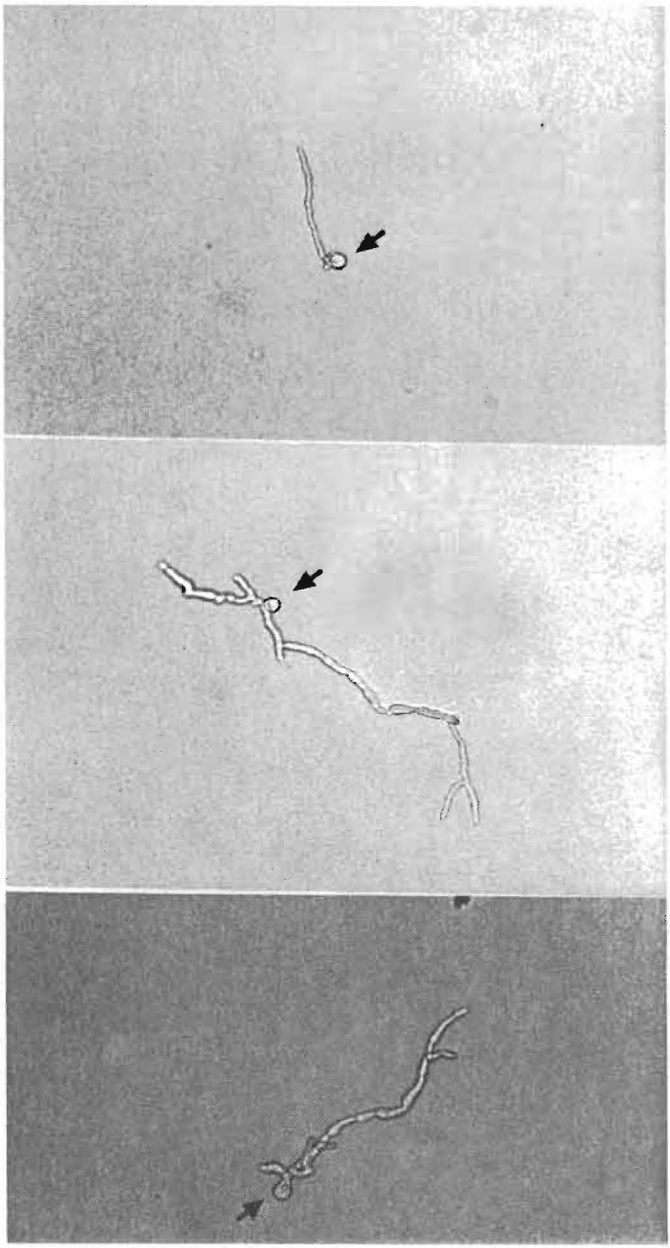

**Figure 2.** Regeneration of protoplasts of *A. cochlioides.* Arrows point to the protoplasts that originated the hyphae shown (photographed at 3 days post-plating).

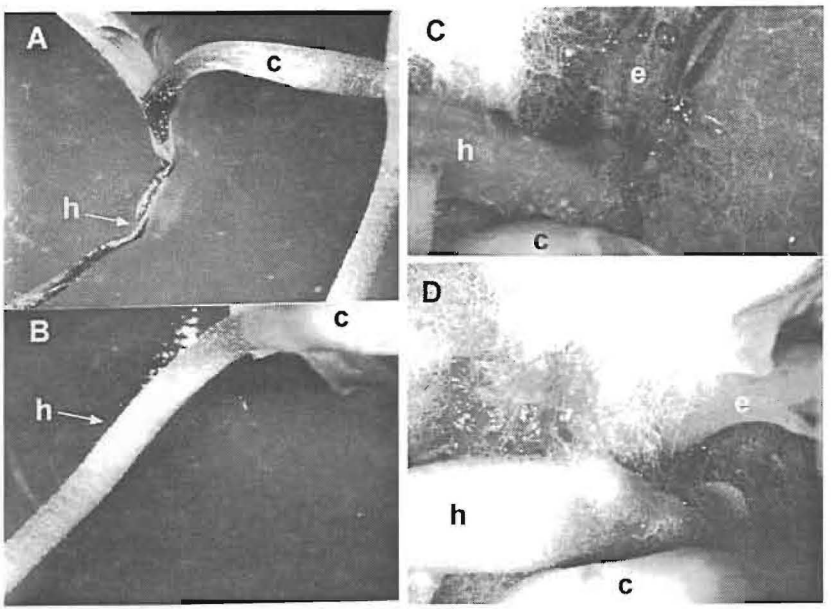


**Figure 3**. Inoculated seedlings of sugarbeet (panels A and B) and pea (panels C and D) with *A. cochlioides* and *A. euteiches* Agar plugs colonized by *A. cochlioides* (panels A and D) or *A. euteiches* (panels B and C) were placed between 7 day old seedlings. Seedlings were photographed at 14 days post-inoculation. Lower case labels denote hypocotyls (h), cotyledons (c), and epicotyls (e). Exudate on the pea epicotyl in panel C reveals the extent of water soaking in the infected tissue.

from single protoplasts of *A. cochlioides* retained the ability to induce blackroot disease in 14 day old sugarbeet seedlings. Water soaking in pea seedlings, however, could not be induced by protoplast-derived isolates of *A. cochlioides* (Fig. 3) as would be expected based on the host range of this pathogen. Likewise, water soaking was induced in 14 day old pea seedlings, but not in sugarbeet seedlings, after inoculation with protoplast-derived isolates of *A. euteiches* (Fig. 3). The host-range effect for the protoplast-derived isolates was the same as that for the progenitor isolates *A. cochlioides* 19-1(z) and *A. euteiches* MM174(z). At 21 days post-inoculation, diseased seedlings were harvested and immersed in distilled water in order to encourage formation of zoosporangia and zoospores. Zoosporangia and zoospores produced from, and oospores produced within, seedling tissue infected with the protoplast-derived isolates were readily observed (data not shown). This was similar to the formation of spores from seedling tissue infected with progenitor isolates *A. cochlioides* 19-1(z) and *A. euteiches* MM174(z).

A procedure for the efficient production of regenerable protoplasts from *A. cochlioides* and *A. euteiches* is described. Regeneration frequencies and retention of pathogenicity in isolates derived from individual protoplasts was consistent with observations made for other oomycete fungal pathogens. Increasing the scale of the protocol described here should enable the production of sufficient protoplasts required for gene transfer experiments. Future work will focus on the transfer of "selectable marker" genes in this fungus using both chemical (Hargreaves and Turner, 1992) and electrotransfer (Kapoor, 1995) methodologies.

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