Uptake and Continued Metabolic Activity of *Azotobacter* Within Fungal Protoplasts

Abstract. *Uptake of vegetative cells of* Azotobacter vinelandii *into protoplasts of the mycorrhizal fungus* Rhizopogon *sp. can be induced by treatment with polyethylene glycol (molecular weight, 6000). An L-form of the bacteria has been selected for within the differentiated fungal mycelium which is capable of acetylene reduction and nitrogen fixation, as confirmed by nitrogen-15 assays; this allows the fungus to grow on media lacking any combined nitrogen. The fungus grows and reduces acetylene on concentrations of antibiotics that prevent the growth and activity of free-living* Azotobacter. *Electron microscopy has revealed modified mitochondrial forms or included bacterial L-forms surrounded by an extra fungal membrane within the hyphae of the modified strains. Poly-β-hydroxybutyric acid, a storage product of* Azotobacter *cysts, has also been identified in the hyphae. This would appear to be the first report of the transgenosis for acetylene reduction activity and nitrogen fixation into a eukaryote cell.*

A culture of *Rhizopogon* sp. isolated from forest litter, shown to be mycorrhizal with the roots of *Pinus radiata* by the Forestry Research Institute (Rotorua) and this laboratory, was grown on Hagem's medium (*1*) and subcultured every 10 days. Protoplasts were prepared from the younger parts of the mycelium by using an enzyme preparation from *Trichoderma viride* (*2*). At first the protoplasts were highly cytoplasmic, but after 2 hours in 0.5*M* $MgSO_4$ they became vacuolate. A suspension of a culture of *Azotobacter vinelandii* grown on Ashby's medium (*3*) was introduced to the protoplasts in the presence of 20 percent (weight to volume) polyethylene glycol (PEG) (molecular weight, 6000) (*4*). Polyethylene glycol caused the aggregation of the bacteria around the fungal spheroplasts and it was hoped that some uptake would occur (Fig. 1B). The protoplast bacterial mixture was washed free of PEG after 10 minutes, and samples taken after thorough washing with 0.5*M* $MgSO_4$ indicated that most of the free bacteria had been washed away, but any remaining free *Azotobacter* were lysed with a lysozyme wash (500 μg/ml).

The protoplasts were plated onto Hagem's medium, lacking both combined nitrogen and malt extract (*5*) and containing 400 μg of penicillin per milliliter. Previous tests had indicated that this concentration of penicillin prevented the growth of *Azotobacter* without affecting fungal protoplast regeneration. After 48 hours, individual fungal colonies, apparently originating from single spheroplasts, could be seen growing on control plates with combined nitrogen, but on media lacking nitrogen the untreated fungal spheroplasts did not regenerate. During the course of several experiments five colonies of the *Azotobacter*-treated protoplasts did regenerate and were isolated—a rate of one colony per 1.2×10^6 protoplasts. They were subcultured 96 hours after plating out and again subjected to a lysozyme wash, this time in the absence of $MgSO_4$, to lyse any adhering bacteria. Portions of the fungal mycelium were used to inoculate liquid Ashby's medium, to favor the growth of any contaminating *Azotobacter*. Samples were taken 72 hours after inoculation and plated on Ashby's medium solidified with 1.2 percent agar. No colonies of *Azotobacter* were found and no acetylene reduction could be detected from either of the two contaminating organisms found. Noninoculated controls also gave negative results.

On nondeficient Hagem's medium the modified *Rhizopogon* grew as a thick mat of hyphae on the surface of the agar, the colonies looking identical to the control fungus. On nitrogen-deficient medium the modified fungus grew as a less dense mat and tended to grow into the agar medium. This latter fact made it almost impossible to weigh the mycelium and give measurements of acetylene reduction per milligram of the fungus. Because of this problem the nitrogen fixation has been expressed in terms of standard 5-mm-diameter disks of medium sampled by using a sterile cork-borer.

Acetylene reduction activity was assayed at 25°C by using a Perkin-Elmer gas chromatograph, with a gas phase in the assay vials of 10 percent acetylene and 90 percent air. The assay was linear over the assay period. A 0.2-ml sample of the acetylene-ethylene gas phase from the 15-ml assay vial was used in each de-

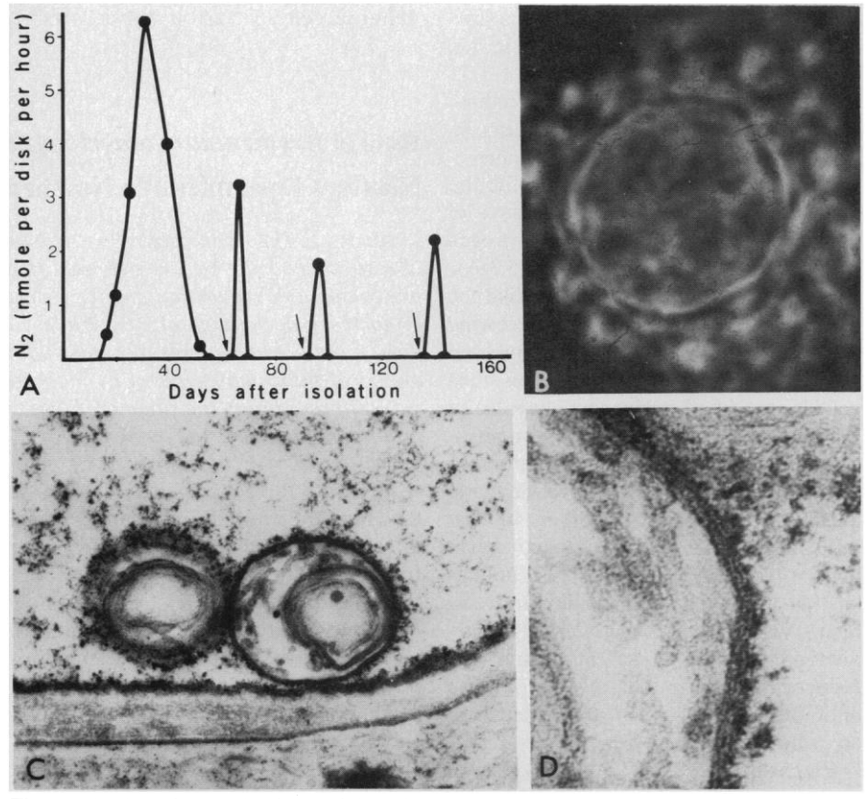

Fig. 1. (A) Acetylene reduction activity of one of the modified fungal strains. Activity was maintained for at least 145 days. Arrows indicate transfer to fresh nitrogen-deficient medium. (B) Fluorescence micrograph of fluorescein-stained *Azotobacter* cells closely surrounding an unstained fungal spheroplast. The fluorescing bodies within the spheroplast indicate possible bacterial uptake (× 950). (C) A pair of either modified mitochondria or bacterial L-forms within the fungal hypha of a strain capable of acetylene reduction. They are always circular in cross section and surrounded by polyribosomes (× 3400). (D) Double outer membrane surrounding the modified mitochondria, or bacterial L-forms, within active strains of the fungus. If these bodies represent bacterial L-forms the outer membrane is presumably of fungal origin (× 5700).

termination, and parallel ethylene controls were always determined.

During the first 9 days in culture the modified fungus did not reduce acetylene. Acetylene reduction was detected 17 days after isolation and the activity peaked on day 29 to 30 (Fig. 1A). After that time there was a gradual decrease in activity, until on day 55 there was no activity. Upon transference to fresh nitrogen-deficient Hagem's medium 61 days after isolation there was further activity, which lasted 6 to 7 days and peaked on day 3. This reappearance of acetylene reduction was repeated after 93 days in culture. After 138 days, protoplasts were again produced by enzymic digestion of the cell walls. The protoplasts were plated on nitrogen-deficient Hagem's medium and acetylene reduction was detected 72 hours later. The unmodified *Rhizopogon* mycelium at no time either reduced acetylene or produced detectable ethylene during the course of these experiments, and the modified form did not produce ethylene in the absence of acetylene.

Azotobacter on nitrogen-deficient Hagem's medium had a peak of acetylene reduction activity 5 days after plating, whereas no activity was detected in the fungus until 17 days after isolation.

The optimum *p*H for acetylene reduction by *Azotobacter* was 8, whereas in the fungus optimum reduction occurred at *p*H 4, near the optimum *p*H for the growth of *Rhizopogon* (3.8). Assays of ^{15}N have confirmed fixation of nitrogen with an acetylene/nitrogen ratio of 5.3 (*6*).

The modified fungal strains, unlike the control fungus, were capable of growing on media free from available combined nitrogen, including media based on silica gel. The modified strains were capable of growth and acetylene reduction on nitrogen-free media containing either streptomycin sulfate (50 μg/ml), tetracycline (50 μg/ml), or benzyl penicillin (400 μg/ml). *Azotobacter* neither reduced acetylene nor grew on these media. It is unlikely that lysozyme and penicillin treatments would enable *Azotobacter* to survive outside the fungal mycelium and reduce acetylene in the presence of these antibiotics, especially in the absence of an osmoticum.

Electron microscopy of the hyphae of the modified strains showed in each case the inclusion of spherical bodies 0.5 μm in diameter (Fig. 1C). Such bodies were absent from the wild-type fungus. The bodies enclosed a series of lamellar structures and vesicles characteristic of vegetative cells of *Azotobacter* but lacked any trace of a cell wall. We consider that these bodies represent either modified mitochondria, possibly indicating the location of the bacterial genetic information after transgenosis (*7*), or L-forms of the bacteria, the loss of the cell wall being due to either digestion by the fungus or the action of the various antibiotics used to decontaminate the cultures. The L-forms are enclosed by an extra membrane (Fig. 1D), whereas in a study of chloroplast uptake by fungal protoplasts (*8*) no extra membrane was found.

No cyst formation was observed in the fungal hyphae but the finding of frequent pairs of the modified mitochondria or L-forms in thin section suggests that division does occur. Poly-β-hydroxybutyric acid, the storage product of *Azotobacter* cysts, was found in the modified though not in the wild-type fungal hyphae. It was membrane-bound but occasionally merely included in the fungal cytoplasm.

These results strongly indicate the possibility of directed transgenosis for nitrogen-fixing ability to eukaryotic cells, although the mechanism behind its operation is obscure. An interesting parallel to these findings exists in the report of a forced symbiotic association between cultured cells of carrot and cells of *A. vinelandii* (*9*). The location of the bacterial genetic information and whether the modified fungus is still capable of a mycorrhizal relationship with *P. radiata* remain to be determined.

KENNETH L. GILES
HEATHER WHITEHEAD
Plant Physiology Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand

References and Notes

1. Hagem's medium consists of 9.4 m*M* NH_4Cl, 3.4 m*M* KH_2PO_4, 2 m*M* $MgSO_4 \cdot 7H_2O$, 1 ml of 1 percent $FeCl_3$, 5 g of glucose, 5 g of malt extract, and 1.5 percent agar per liter; the *p*H is 4.7.
2. O. M. H. de Vries and J. G. H. Wessels, *Antonie van Leeuwenhoek J. Microbiol. Serol.* **39**, 397 (1973).
3. Ashby's medium consists of 3.2 m*M* K_2HPO_4, 2 m*M* $MgSO_4 \cdot 7H_4O$, 0.1 m*M* $FeSO_4 \cdot 7H_4O$, 0.1 m*M* $MnSO_4 \cdot 2H_2O$, 0.04 m*M* Na_2MoO_4, 1.7 m*M* NaCl, 5 m*M* $CaCO_3$, 5 g of glucose, and 1.2 percent agar per liter; the *p*H is 8.0.
4. H. T. Bonnett and T. Erikssen, *Planta* **120**, 71 (1974).
5. Here KCl replaced NH_4Cl, and the malt extract was replaced by nicotinic acid (0.5 mg/liter), pyridoxine-HCl (0.1 mg/liter), and thiamine-HCl (0.1 mg/liter).
6. K. L. Giles and H. C. M. Whitehead, *Cytobios*, in press.
7. C. H. Doy, P. M. Gresshoff, B. G. Rolfe, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 723 (1973).
8. I. K. Vasil and K. L. Giles, *Science* **190**, 680 (1975).
9. P. S. Carlson and R. S. Chaleff, *Nature (London)* **252**, 393 (1974).

26 November 1975; revised 28 April 1976

Role of *Erythronium americanum* Ker. in Energy Flow and Nutrient Dynamics of a Northern Hardwood Forest Ecosystem

Abstract. *The aboveground activity of the spring herb,* Erythronium americanum, *is restricted to the period between snowmelt and forest canopy development. Its phenology and production capacity closely adapt the species to this temporal niche in northern deciduous forests. While* E. americanum *has a minor effect on energy flow, it may reduce losses of potassium and nitrogen from the ecosystem during the period of maximum removal by incorporating these elements in accumulating biomass. Later, during the summer, these nutrients are made available when the aboveground, nonperennating tissues decay.*

Intensive biogeochemical studies of the Hubbard Brook Experimental Forest in central New Hampshire (*1*) provide a framework for evaluating the contribution of individual species to energy flow and nutrient dynamics in the northern hardwood forest ecosystem. Deciduous trees constitute > 97 percent of the living biomass and exert a primary influence on ecosystem functions (*2*). However, deciduous trees are summer-green species and their most active period is from late May to mid-October. Vernal photosynthetic herbs, primarily *Erythronium americanum* Ker. (Liliaceae), are abundant in the ecosystem and are active during the period between snowmelt and development of the overstory forest canopy. This is a time of high light intensity at the forest floor, warm and moist soil conditions, and increased nutrient availability. During this period, *Erythronium* is the dominant autotroph. This study was initiated to evaluate its role in ecosystem energetics and biogeochemical cycling (*3*).

In 1972 and 1973, phenology and biomass accumulation of the *Erythronium* population were measured on a 13.2-ha watershed. This watershed (W6) serves as a reference ecosystem for the Hubbard Brook study and its biogeochemistry and ecology are extensively known (*1, 4, 5*). For biomass estimates, the watershed was divided into five elevational strata. Average weights per plant and per plant part were obtained from harvests within each stratum and were combined