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The brighter side of soils: Quantum dots track organic nitrogen through fungi and plants

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Abstract. Soil microorganisms mediate many nutrient transformations that are central in terrestrial cycling of carbon and nitrogen. However, uptake of organic nutrients by microorganisms is difficult to study in natural systems. We assessed quantum dots (fluorescent nanoscale semiconductors) as a new tool to observe uptake and translocation of organic nitrogen by fungi and plants. We conjugated quantum dots to the amino groups of glycine, arginine, and chitosan and incubated them with *Penicillium* fungi (a saprotroph) and annual bluegrass (*Poa annua*) inoculated with arbuscular mycorrhizal fungi. As experimental controls, we incubated fungi and bluegrass samples with substrate-free quantum dots as well as unbound quantum dot substrate mixtures. *Penicillium* fungi, annual bluegrass, and arbuscular mycorrhizal fungi all showed uptake and translocation of quantum dot-labeled organic nitrogen, but no uptake of quantum dot controls. Additionally, we observed quantum dot-labeled organic nitrogen within soil hyphae, plant roots, and plant shoots using field imaging techniques. This experiment is one of the first to demonstrate direct uptake of organic nitrogen by arbuscular mycorrhizal fungi.

Key words: amino acids; arbuscular mycorrhizal fungi; chitosan; fluorescent labels; nitrogen cycle; organic nitrogen; Penicillium; *quantum dots*.

Introduction

Microbial communities mediate many of the soil processes that provide nutrients to plants and that release trace gases to the atmosphere. Thus, understanding how these microbial communities function is essential to understanding ecosystems as a whole. Ecosystem ecologists have traditionally approached soils as a black box in which nutrient transformations are conducted by an uncharacterized community of microorganisms (Tiedje et al. 1999). In many cases, this framework has been appropriate; it has allowed investigators to construct nutrient budgets of ecosystems and to document patterns in soil characteristics. However, we must possess more mechanistic information about the functioning of microorganisms in ecosystems to adequately predict nutrient dynamics under a range of environmental conditions (Treseder and Allen 2000). Historically, detailed investigations have been hampered by limitations inherent in resolving the spatial dynamics of microbial processes taking place in a complex, fragile, and opaque environment.

One recent subject of debate is the extent to which plants are able to directly consume organic nitrogen (ON). Traditionally, ON was thought to undergo a transformation into inorganic N before being taken up by plants (Liebig 1843). This theory assumes N mineralization is the limiting process for plant growth in most ecosystems (Vitousek 1997). However, isotope labeling has provided evidence that plants can take up ON, at least as simple forms such as amino acids (Turnbull et al. 1995, Lipson and Monson 1998, Näsholm et al. 1998, Lipson and Näsholm 2001, Miller and Cramer 2005, Rains and Bledsoe 2007). It is unclear, however, whether ON molecules are directly assimilated by plant roots or plants acquire ON indirectly via mycorrhizal fungi or other microbial symbionts.

Arbuscular mycorrhizal fungi (AMF; see Plate 1) are common worldwide and form symbioses with ~80% of plant families (Newman and Reddell 1987, Smith and Read 1997). They mine the soil for nutrients and translocate a portion to their host plants in exchange for carbon (Smith and Read 1997). The prevailing paradigm is that AMF specialize in the capture of inorganic nutrients such as phosphate, ammonium, and nitrate (Read 1991, Smith and Smith 1997). Uptake of ON by AMF has been challenging to investigate in natural systems because of technical difficulties in tracing the flow of organically derived nutrients from the soil into hyphae.

One promising technique to investigate the flow of nutrients between plants, microorganisms, and soil in field conditions is the use of quantum dots. Quantum dots (QDs) are nanoscale semiconductors that fluoresce in different colors depending on their size (Chan and Nie 1998, Wong and Stucky 2001, Reiss et al. 2002, Alivisatos et al. 2005). They are typically composed of a cadmium selenide core wrapped in a zinc sulfide shell,

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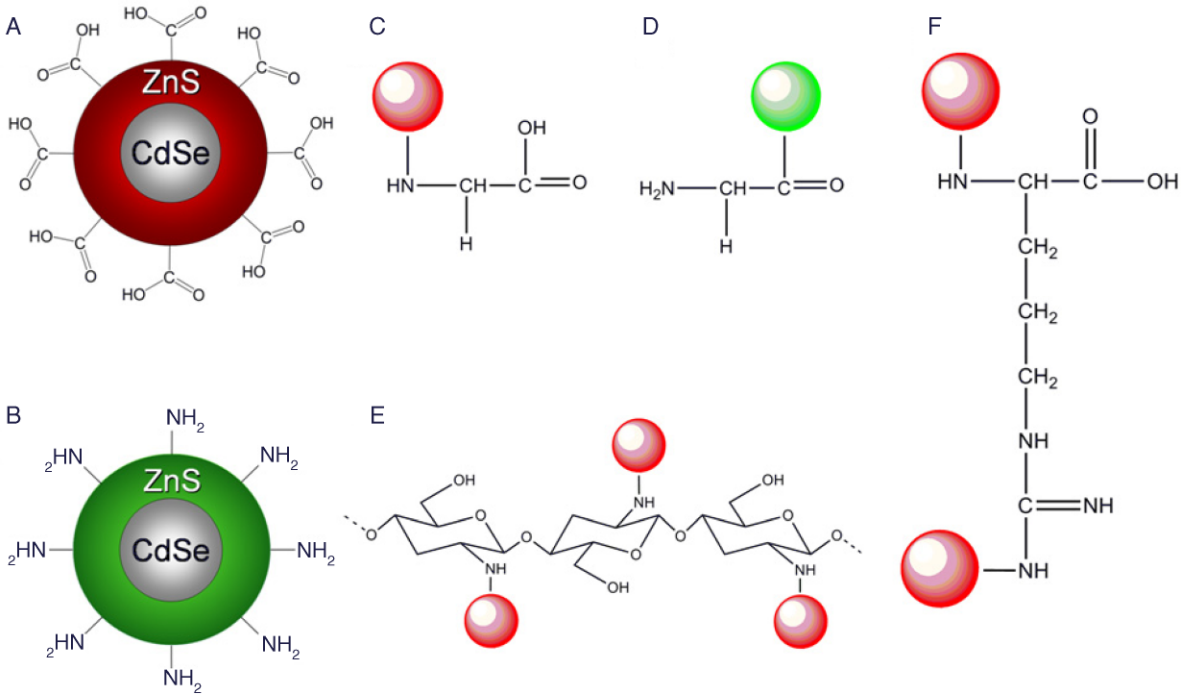

FIG. 1. Schematic diagram of a quantum dot and configurations of quantum dot conjugates. (A, B) The polymer coating contains receptor molecules that allow the quantum dot to form strong covalent bonds with carboxyl or amino groups of organic compounds. (A) For quantum dots that bind to amino groups of organic compounds, the receptor molecules are carboxyl groups. (B) For quantum dots that bind to carboxyl groups, the receptor molecules are amino groups. (C) Amino-bound glycine, (D) carboxyl-bound glycine, (E) amino-bound chitosan, and (F) amino-bound arginine. This study only used amino-bound substrates.

and they can be enclosed by protective polymer coatings (Fig. 1; Dubertret et al. 2002). Polymer-coated QDs are commercially available and range in diameter from 2 nm (Northern Nanotechnologies, Toronto, Ontario, Canada) up to 20 nm (Invitrogen, Carlsbad, California, USA). There are many advantages of QDs over traditional labels and tags. Quantum dots have broad absorption spectra with very narrow emission peaks, meaning multiple colors can be assessed on the same sample by using the same light source. Since QDs are resistant to metabolic and chemical degradation and are not susceptible to photobleaching, they are effective tracers for long-term studies (Alivisatos et al. 2005, Michalet et al. 2005). Kloepfer et al. (2005) found that QD-labeled bacteria (*Bacillus subtilis*) retained QD fluorescence and electron density, even after storage for one year in rich medium. In addition, QDs can be bound to amino or carboxyl groups within organic compounds (Fig. 1). In this way, the movement of these compounds through soils or organisms can be imaged and tracked (Dubertret et al. 2002, Kloepfer et al. 2005). Previously, most quantum dot work focused on biomedical applications (Gao et al. 2004, Alivisatos et al. 2005, Garon et al. 2007, Qian et al. 2007). To our knowledge, this study is the first to apply quantum dot technology to ecological questions.

We assessed QDs as a tool to observe the translocation of ON in laboratory and soil-based systems. Specifically, our objectives were to use QDs to track ON uptake (labile and recalcitrant) by a model group of non-mycorrhizal fungi, *Penicillium solitum*, and by AMF-colonized plants. By testing this technique on *Penicillium* fungi, we assessed uptake of QD substrates in a known ON-acquiring saprotroph as a proof of method. We then extended this method to AMF to examine ON uptake by this group. We used glycine and arginine (common amino acids) to represent labile ON and chitosan (deacylated form of chitin) to represent recalcitrant ON.

METHODS

Conjugation of quantum dots.—Quantum dot conjugation was performed by following a modified Kloepfer et al. (2005) method. Commercial red (565-nm emission) carboxyl QDs (Invitrogen) were conjugated with the amino group of reagent-grade glycine or arginine by using the binding activator 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The reaction was performed in a 10-mmol/L borate buffer (pH 7.4). Each milliliter of reaction solution contained 3.33 mmol/L substrate, 0.1 μmol/L QDs, and 2 mg EDC. The conjugation reaction was performed on a gentle shaker for 3 h in the dark. The quantum dot conjugates underwent dialysis against 2 L of sterile water and were then centrifuged to remove any sedimented material.

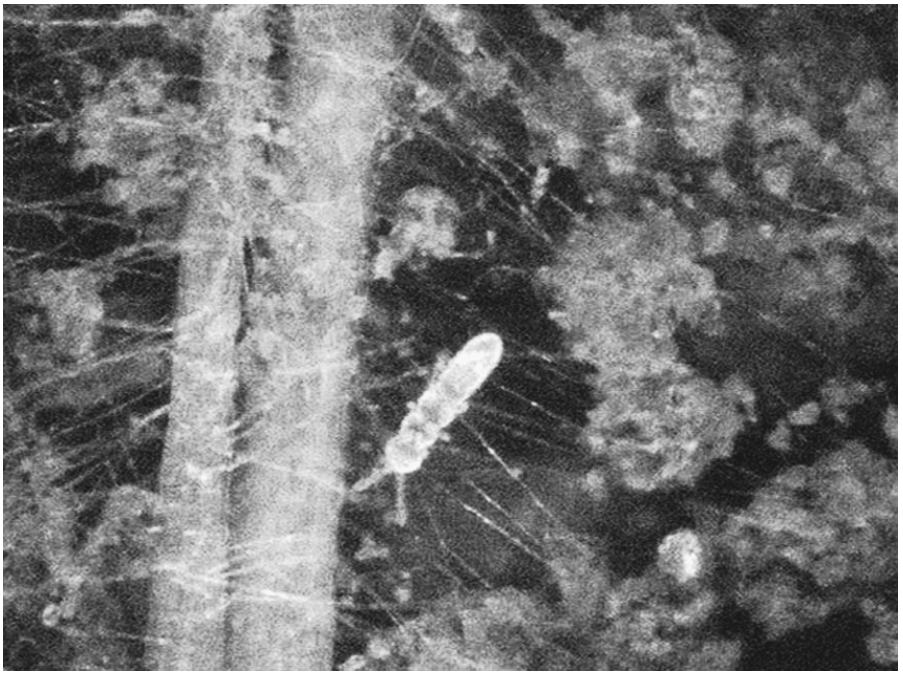

PLATE 1. Arbuscular mycorrhizal hyphae form web-like structures as they emanate from fine roots in a boreal forest in Alaska (USA). A springtail (Collembola) can be seen crawling among the hyphae. Photo credit: K. K. Treseder.

Quantum dot conjugation of chitosan (MP Biomedical, Solon, Ohio, USA) was performed in the same manner as for glycine and arginine except each milliliter of reaction solution contained 5.8 mg chitosan, 0.1 μmol/L QDs, and 2.2 mg EDC. The QDs used in this study were <15 nm in diameter (Invitrogen).

Uptake of QD-ON by Penicillium solitum.—To assess uptake of QD-labeled substrates, we tested a common saprotrophic fungus, *Penicillium solitum*. *Penicillium solitum* was grown from spores in liquid modified Melin-Norkrans (MMN) media (Marx 1969). After 24 h, hyphae were transferred with 250 μL MMN into microcentrifuge tubes. The tubes were incubated with either 250 μL (0.1 μmol/L) of QD-glycine in a time series that ranged 2, 6, or 24 h; 250 μL (0.1 μmol/L) of QD-arginine, 24 h; 2.65 mg QD-chitosan in 250 μL sterile water, 5 or 24 h; a QD control of 250 μL (0.1 μmol/L) QDs containing no substrate, 2, 4, 6, or 24 h; or QD controls of 250 μL (0.1 μmol/L) QDs and substrate (glycine, arginine, or chitosan) lacking binding reagent, 2, 4, 6, or 24 h. After incubation, the hyphae were centrifuged and washed with 1% saline to remove unbound QDs. All cultures and incubations were performed in the dark. Additionally, all preceding steps were performed under sterile conditions. Quantum dots were excited using an Argon/2 laser (458 or 488 nm; Carl Zeiss, Jena, Germany), and fungal uptake was observed with a Zeiss LSM 510 META confocal microscope (Carl Zeiss). The detector was set to 565 ± 10 nm and was calibrated to display no fluorescence of unlabeled material. This approach reduced the incidence of autofluorescence.

Uptake of QD-ON by arbuscular mycorrhizal fungi and Poa annua.—To test uptake of QD-ON substrates by AMF-colonized plants, annual bluegrass (*Poa annua*) was grown from seed in 1:1 autoclaved sand : vermiculite. Seedlings were cultivated in an 11-L planting pot inoculated with a mixture of AMF (Mycorrhizal Applications, Grants Pass, Oregon, USA). After 30 d of growth, mycorrhizal plants were transferred to sterile 10-mL capped test tubes containing 3.5 g of autoclaved 1:1 sand : vermiculite and 1.6 mL sterile water. Each tube received one plant. The plants were incubated with 500 μL QD-glycine solution, QD-chitosan, or QD control for either 4 or 24 h. After incubation, the plants were removed and gently washed with 1% saline to help remove sand particles and unbound QDs. Confocal laser scans were performed as described in *Uptake of QD-ON by* Penicillium solitum.

Field-applicable imaging.—To test the imaging capabilities of QD-ON substrates under more natural conditions, we used a minirhizotron to detect QD fluorescence in soils. Eight grams of *Poa annua* seed was cultivated in an 11-L planting pot of autoclaved 1:1 sand : vermiculite mix. The pot was inoculated with a multispecies mix of AMF (Mycorrhizal Applications) and fitted with a horizontal minirhizotron tube. Five glass applicator tubes were installed along the minirhizotron tube so that the ends of the applicators rested ~2 mm above the minirhizotron tube. The pot was watered twice per day on a misting bench in a 23°C

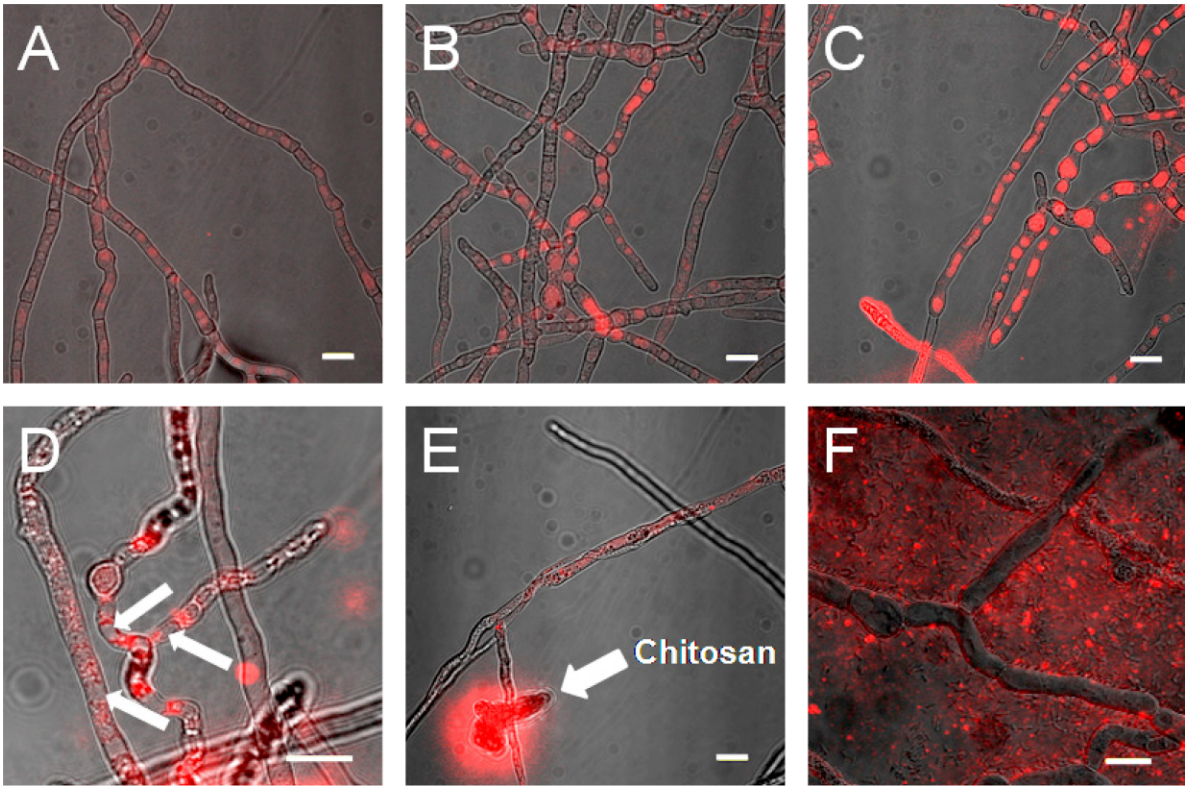

FIG. 2. Superimposed (white light and fluorescence) confocal laser scans of *Penicillium solitum* uptake of quantum dot–organic nitrogen (QD–ON) conjugates. (A) At 2 h of incubation with QD-glycine, fungal hyphae showed evidence of uptake. (B) At 6 h, less labeled glycine was seen in solution and more within the hyphae. (C) After 24 h very little labeled glycine was seen outside of the hyphae. (D) After 24 h, QD-arginine fluorescence appeared in the cytoplasm but not in vesicles (arrows). (E) Uptake of QD-labeled chitosan after 5 h of incubation. (F) Quantum dot control (quantum dots unbound to glycine) after 24 h of incubation showed no signs of uptake. The scale bar is 10 μm.

greenhouse. After 30 d of growth, 25 mL (0.1 μmol/L) of QD-glycine was injected into one applicator tube above an area of observable hyphal growth. Uptake was observed using a BTC-100X minirhizotron camera (Bartz Technology, Santa Barbara, California, USA) fitted with a UV light source.

To further test QDs as a non-destructive method that could be applied in the field, we used a handheld UV light source and digital camera to observe unmagnified QD fluorescence in plant shoot tissue. Orange excitation (585-nm emission) carboxyl QDs were bound to the primary amino group of glycine. *Poa annua* seedlings were grown in 1:1 autoclaved sand : vermiculite. After 20 d, seedlings were transferred to sterile microcentrifuge tubes containing 1 mL QD-glycine (1 μmol/L) solution in liquid MMN media. To maintain the media at a consistent volume during the experiment, liquid MMN was added as needed. After 14 d the seedlings were harvested and thoroughly rinsed with 1% saline solution to remove any QDs unassociated with the plants. The QD conjugates were excited using a 4-V portable handheld UV light, and photos were taken with a Canon 7.1 megapixel digital camera (Canon, Lake Success, New York, USA) without zoom or magnification.

RESULTS

Uptake of QD-ON by Penicillium solitum.—We used *Penicillium solitum* to test the uptake and storage of unconjugated QDs, QD-glycine, QD-arginine, and QD-chitosan, by a common, well-studied saprotrophic fungus. Within 2 h of incubation with QD-glycine, fluorescence became apparent in the vacuole compartments of the hyphae (Fig. 2A). Uptake continued throughout the 24-h duration of the experiment, and QD concentrations in hyphae intensified with time (Fig. 2B, C). Uptake was also evident using QD-arginine, but for the 24-h duration of the experiment, the label appeared to remain outside the vacuoles and instead stayed within the cytoplasm (Fig. 2D). The *P. solitum* fungi were able to acquire QD-chitosan (Fig. 2E). Fluorescence was observed in the cytoplasm of the hyphae within 5 h of incubation. Additionally, neither of the QD controls displayed any sign of uptake or surface binding to the hyphal walls (Fig. 2F).

Uptake of QD-ON by arbuscular mycorrhizal fungi and Poa annua.—We used individuals of *Poa annua* inocu-

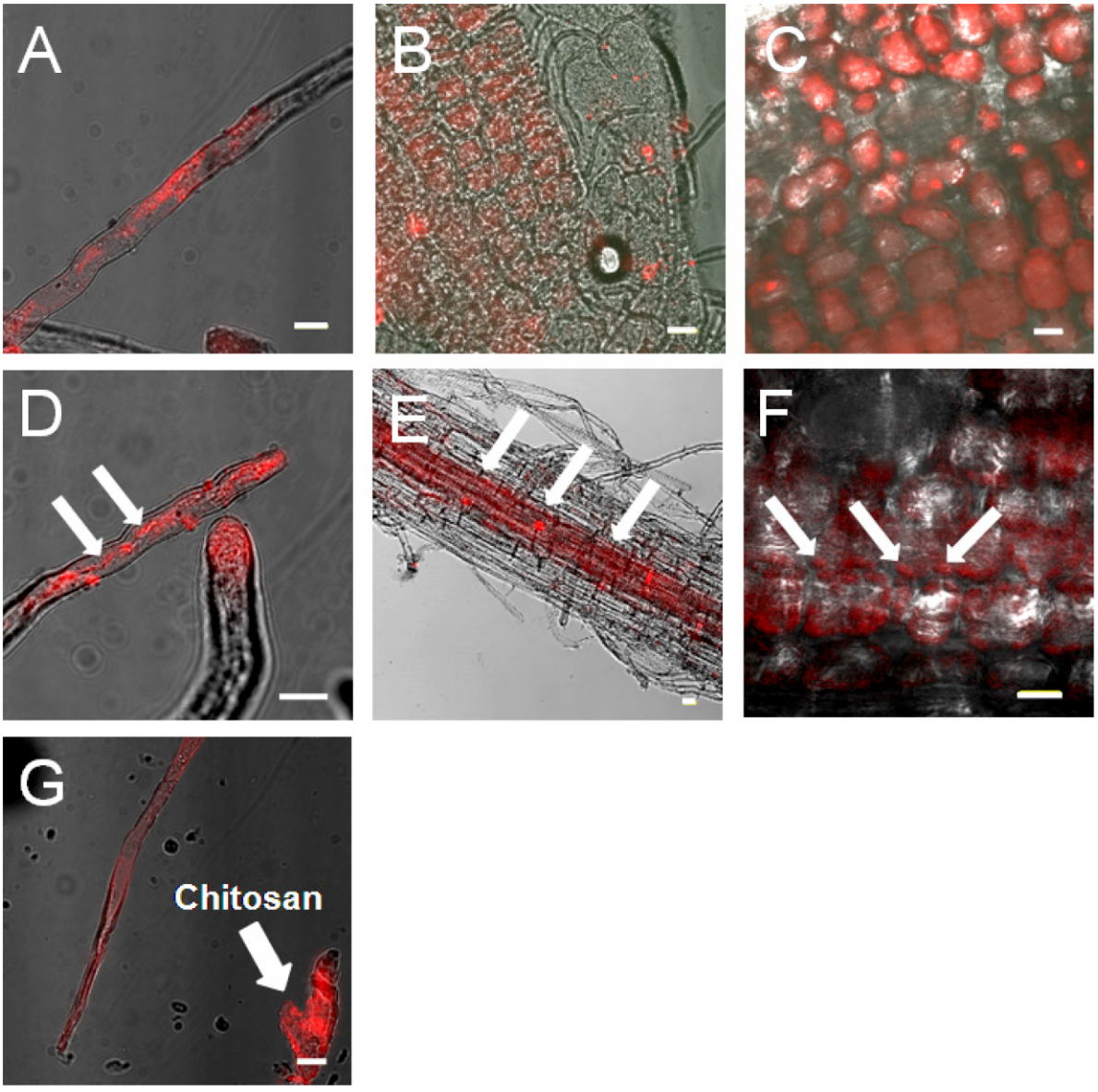

FIG. 3. Confocal light and emission scans of quantum dot–organic nitrogen (QD–ON) conjugates after incubation with the roots and mycorrhizal colonists of annual bluegrass (*Poa annua*). Fluorescence 4 h after incubation with QD-glycine: (A) from arbuscular mycorrhizal fungi (AMF) hyphae, (B) into the plant root, and (C) up to the plant shoot. Fluorescence 24 h after incubation with QD-glycine: (D) internal fluorescence of AMF vacuoles (arrows), (E) vascular tissue of the plant root (arrows), and (F) chloroplasts in the shoot cells (arrows). (G) Fluorescence in AMF after 5 h of incubation with QD-chitosan. The scale bar is 10 μm.

lated with AMF to track the movement of QD-glycine, QD-chitosan, and QD controls to hyphal, root, and shoot structures. The QD-glycine labels were first observed in AM hyphae (Fig. 3A), plant root cells (Fig. 3B), and plant shoot cells (Fig. 3C) within 4 h of incubation. After 24 h of incubation, QD-glycine had collected in specific structures within each tissue type: tube-shaped vacuoles within AM hyphae (Fig. 3D), vascular tissues within roots (Fig. 3E), and chloroplasts within shoot cells (Fig. 3F). The AM hyphae contained QD-chitosan within 5 h of incubation (Fig. 3G), but no QD fluorescence was observed in the plant roots or shoots during the 24-h QD-chitosan treatment (data not shown). Moreover, no uptake or transportation of any QD control was seen in the fungi, plant roots, or plant shoots (data not shown).

Field-applicable imaging.—We observed fluorescence of QD-glycine within plant material and fungal hyphae by using imaging techniques that can be applied in field settings. *Poa annua* fluoresced with QD-glycine in both roots and shoots during a 14-d incubation period (Fig. 4A–D). We were able to excite the QD-glycine with a

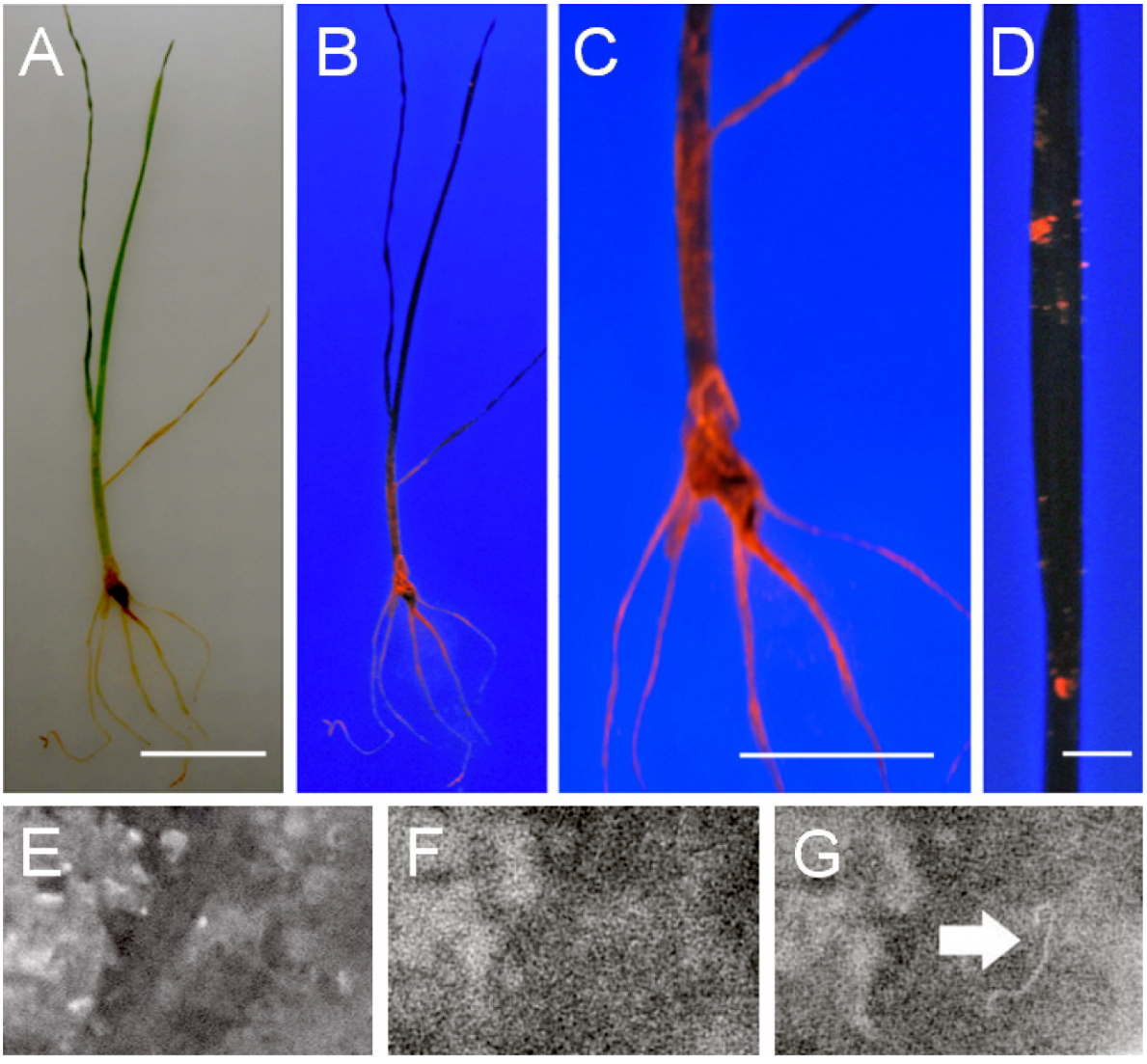

FIG. 4. Fluorescence of quantum dot (QD)-glycine in a plant and fungi using field imaging techniques. (A–D) Unmagnified digital images of a *Poa annua* individual incubated with orange excitation QD-glycine: (A) white light view; (B) UV view; (C) root detail; and (D) blade detail. Orange fluorescence indicates presence of QDs. Scale bars are (A, B) 1 cm, (C) 5 mm, and (D) 500 μm. (E–G) Minirhizotron images of fungal hyphae uptake of QD-glycine: (E) white light image pre-QD injection; (F) UV image pre-QD injection; and (G) 2 h after injection of QD-glycine, UV minirhizotron images showed hyphal uptake (arrow) of the labeled glycine. The hypha is ~2 mm long.

handheld UV light source and observe movement of the label with the naked human eye. Additionally, we used a minirhizotron camera (4-μm resolution) to track the flux of QD-glycine from soil to fungal hyphae in a greenhouse experiment. The hyphae were only slightly visible before QD-glycine injection, but fluoresced 2 h after injection (Fig. 4E, F).

DISCUSSION

Our results suggest that the QD-labeled ON is taken up and transported to particular structures in fungi and plants, depending on the type of ON compound conjugated to the QD. In natural settings, proteinaceous materials (e.g., amino acids, peptides, and proteins) comprise 40% of soil nitrogen and aminosugars comprise 5–6% (Schulten and Schnitzer 1998). As reviewed by Rentsch et al. (2007) and documented by Paungfoo-Lonhienne et al. (2008), saprotrophic and ectomycorrhizal fungi are known to directly acquire amino acids, peptide chains, and proteins, and plants contain transporters for amino acids, peptides, and proteins. Additionally, transportation of chitin and other polysaccharides is not well studied. Previous studies have shown that *Penicillium* fungi take up and compartmentalize intact amino acids such as glycine and arginine (Kitamoto et al. 1988, Hillenga et al. 1996) and are

capable of breaking down and absorbing more recalcitrant forms of ON such as the polysaccharide chitin (Binod et al. 2007). *Penicillium* fungi compartmentalize nutrients and amino acids in two main locations: in vacuoles where they can be stored and in cytoplasm where they can be quickly metabolized or transformed (Griffin 1994). Vacuoles of *Penicillium* hyphae can contain up to 51% of total cellular glycine and up to 98% of total cellular arginine (Kitamoto et al. 1988, Roos et al. 1997). We observed QD-chitosan in cytoplasm (Fig. 2E) and QD-glycine in vacuoles (Fig. 2A–C), but QD-arginine was observed in cytoplasm and not vacuoles (Fig. 2D). The lack of QD-arginine in vacuoles is most likely the result of QD-arginine size after conjugation. Glycine contains one amino terminal as a potential QD binding site (Fig. 1C); however, arginine contains two (Fig. 1F). Therefore, it is possible for each arginine molecule to bind with more than one QD during conjugation. The QD-arginine (theoretically twice as large as QD-glycine) may be small enough to pass through the cell wall, but too large to pass into the vacuole.

Plant roots can acquire amino acids individually or as peptides and proteins via endocytosis and various amino acid, peptide, and protein transporters (Fischer et al. 1998, Rentsch et al. 2007, Paungfoo-Lonhienne et al. 2008). Absorbed proteinaceous material can then be transferred to vascular tissue (phloem and xylem), where it is transported to and from the shoot or transformed into other molecules (Rentsch et al. 2007). In the shoot, amino acids and peptides are exchanged between vascular tissue and mesophyll cells where they are metabolized and assimilated by chloroplasts (Miflin and Lea 1977). Subsequently, chloroplasts contain the largest concentration of amino acids in the shoot (Riens et al. 1991). In our experiment we visually confirmed similar transfers using QD-glycine. We observed the movement of QD-glycine from root cells (Fig. 3B) to vascular tissue (Fig. 3E) and from mesophyll cells (Fig. 3C) to chloroplasts (Fig. 3F) within a 24-h period. If the bound substrates or polymer coatings were removed from QDs by plant or microbial processes, we would expect QD fluorescence in the main storage sites of cadmium (QD core) or zinc (QD shell) within plants. Plants typically store cadmium in the apoplast (free space outside the plasma membrane) and zinc in the vacuoles of epidermal and subepidermal cells (cells lacking chloroplasts (Vazquez et al. 1992, 1994, Sarret et al. 2002). However, in our study we observed QD fluorescence in vacuoles of mesophyll cells and in chloroplasts, areas expected to contain amino acids individually or as peptide chains.

Contrary to the prevailing paradigm, AMF took up labile (QD-glycine) and recalcitrant (QD-chitosan) forms of ON. The QD-chitosan was located within the cytoplasm after 5 h of incubation, similar to *Penicillium solitum*. The QD-glycine was located in tube-shaped vacuoles, complimenting AMF location of phosphate in previous studies (Ashford 2002, Uetake et al. 2002). As shown in Fig. 1A, the red QDs used were encased in a polymer with carboxyl terminals, so even if the ON attached was mineralized it was still bound to a carboxyl group of the QD polymer. Since QDs were taken up only when bound to ON compounds, it appears that ON was acquired directly and actively by AMF.

A few studies have demonstrated that AMF can obtain N derived from organic sources (Cliquet et al. 1997, Hawkins et al. 2000, Hodge et al. 2001, Rains and Bledsoe 2007), but without direct imaging it is difficult to determine whether AMF can take up N in organic form. In a laboratory microcosm, Hodge et al. (2001) placed ^{15}N-labeled litter in soil compartments that could be accessed by AM hyphae but not by plant roots. In doing so, they demonstrated that AMF can acquire N from organic material. However, this approach cannot be easily replicated under field conditions. Mesh-enclosed cores could be installed that allow ingrowth by fungal hyphae but not roots. Unfortunately, though, it is difficult to prevent ON from diffusing to or from the core. Hobbie and Hobbie (2006) recently used natural abundance of ^{15}N in plants and fungi to estimate that ectomycorrhizal fungi are responsible for 61–86% of N uptake by plants in Arctic tundra. However, these calculations require that all pools of available N in the soil display similar ^{15}N signatures. This condition may not be met in many ecosystems.

In one of the few studies of microbial uptake of QDs, Kloepfer et al. (2005) observed QD-adenine uptake by wild-type strains of *Bacillus subtilis*, but not by *ade* and *apt* mutants, which had previously displayed reduced ability to acquire adenine. They interpreted their findings as evidence the QD uptake was governed by processing mechanisms for the adenine. In our study, *Penicillium solitum*, *Poa annua*, and AMF did not take up QDs that were unbound to a substrate, nor did they take up unbound QDs in solution with a substrate but without binding reagent. In this case, it is possible that the fungi and plants were targeting the glycine, arginine, and chitosan for acquisition and then incidentally taking up the conjugated QDs in the process.

We determined that QDs may be used in conjunction with field imaging techniques. Quantum dots of any color will fluoresce under standard UV light. Therefore, QDs are commonly imaged using epifluorescent and confocal microscopy (Voura et al. 2004, Chen et al. 2006). These methods incorporate continuous viewing coupled with light intensity detectors or software, providing quantitative results comparable to fluorescent spectrophotometers (Yezhelyev et al. 2007). Mycorrhizae and plant roots can be imaged in a similar manner using underground minirhizotron cameras. Minirhizotron cameras are commonly fitted with a UV light source and can take photos of 4 μm resolution (Hendrick and Pregitzer 1996). We did not quantitatively examine QD uptake in this study. However, we did use minirhizotron imaging to confirm ON uptake by

fungal hyphae in soil. Moreover, we found that QDs can be used with portable handheld UV lights to observe uptake in grass shoots with the naked eye. This method would allow a researcher to apply QD-labeled nutrients to natural systems and nondestructively determine uptake among plants in real time.

In applying QDs in ecological studies, we must consider two major concerns: artifacts related to QD size and potential toxicity of heavy metals. Currently, most commercial polymer QDs are fairly large after conjugation, roughly the size of small proteins. Quantum dot size can influence uptake dynamics of labeled compounds. Kloepfer et al. (2005) found that QDs larger than 5 nm diameter were not taken up by certain bacterial cells. However, the field of QD technology is rapidly advancing, and some commercial companies now offer polymer QDs as small as 2 nm, equivalent to the size of two nucleic acids (NNT). Smaller QDs could be ideal candidates to address ecological questions comparing multiple microorganisms such as bacteria and fungi. Additionally, heavy-metal toxicity from QDs has been demonstrated in bacteria and human cell lines (e.g., Kloepfer et al. 2005, Cho et al. 2007). However, little is known about QD effects to the environment. Most commercial QDs contain cores of cadmium, a known carcinogen. Although cadmium cores are protected in zinc sulfide and polymer coatings, strict safety precautions must be considered for QD use in environmental studies. As a possible alternative, iron- and silica-based QDs are currently in development (Zheng et al. 2005, Buehler et al. 2006, Lee et al. 2007); since they contain no cadmium, toxicity should be less of a concern. These versions of QDs may be particularly useful for ecological applications.

Our study contributes to the growing body of literature demonstrating that AMF may have a more significant effect on ON dynamics than previously believed (Näsholm et al. 1998, Hawkins et al. 2000, Hodge et al. 2001, Rains and Bledsoe 2007). The QD technique provides a simple, inexpensive, and nondestructive measure of mycorrhizal fungi and root uptake in natural systems. By using this technique to trace the uptake of ON molecules, researchers could improve our knowledge of nutrient acquisition by organisms in general and the role of AMF in plant ON uptake in particular.

Acknowledgments

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