Bioenerg. Res.
DOI 10.1007/s12155-015-9636-8

A Novel Delivery System for the Root Symbiotic Fungus, *Sebacina vermifera*, and Consequent Biomass Enhancement of Low Lignin COMT Switchgrass Lines

Prasun Ray[1] · **Takako Ishiga**[1] · **Stephen R. Decker**[2] · **Geoffrey B. Turner**[2] · **Kelly D. Craven**[1]

Abstract *Sebacina vermifera* (MAFF-305830) is a mycorrhizal fungus originally isolated from the roots of orchids that we have previously shown to be tremendously beneficial in enhancing biomass yield and drought tolerance in switchgrass, an important bioenergy crop for cellulosic ethanol production in the United States. Towards this end, we have developed a bentonite clay particle-based delivery system for mass production and dissemination of *S. vermifera* for large-scale field trials. A greenhouse-based experiment was conducted to evaluate this novel delivery method for biomass enhancement of wild type and transgenic, low lignin (COMT down-regulated) switchgrass lines compared to an efficient in vitro colonization method. *S. vermifera* colonization enhanced plant biomass regardless of delivery method, although the percentage of fungal biomass in planta increased with the clay-based delivery system. Further, we found that release of some clay minerals in solution was enhanced in the presence of *S. vermifera*, while others were seemingly reduced. Intriguingly, the presence of *S. vermifera* has little or no impact on cell wall composition, including lignification. This research is the first report documenting the development of a bentonite clay particle-based delivery system for mass production of any symbiotic microbe and suggests that *S. vermifera* can be packaged with a mineral composite and effectively delivered to a target host plant.

Keywords Switchgrass · Mycorrhizae · *Sebacina* · COMT

Introduction

The Sebacinales belong to a taxonomically, ecologically, and physiologically diverse group of fungi in the Basidiomycota. While historically recognized as orchid mycorrhizae, recent DNA studies have demonstrated both their pandemic distribution and the broad spectrum of mycorrhizal types they form [1]. Indeed, ecological studies using PCR-based detection methods have found Sebacinales fungi in field specimens of bryophytes (moss), pteridophytes (fern), and all families of herbaceous angiosperms (flowering plants) from temperate, subtropical, and tropical regions. These natural host plants include, among others, liverworts, wheat, maize, and *Arabidopsis thaliana*, a genetic model plant traditionally viewed as non-mycorrhizal [2–4]. *Sebacina vermifera* (MAFF-305830) was first isolated from the Australian orchid *Cyrtostylis reniformis* [5]. Research from our laboratory on this strain clearly indicates its plant-growth-promoting abilities in non-orchid host plants. *S. vermifera* colonization enhanced seed germination and biomass production of the native warm-season grass, switchgrass, even under drought conditions [6, 7].

Switchgrass (*Panicum virgatum* L.) has been identified as a bioenergy crop in the United States for cellulosic ethanol production [8]. In addition to high biomass potential and a perennial growth habit, this North American prairie grass has broad adaptability and requires minimal nutritional inputs, and thus can be cultivated on marginal lands unsuitable for more input-demanding agronomic crop plants. Indeed, field studies have demonstrated that switchgrass grown and managed as a

Electronic supplementary material The online version of this article (doi:10.1007/s12155-015-9636-8) contains supplementary material, which is available to authorized users.

✉ Kelly D. Craven
kdcraven@noble.org

[1] Plant Biology Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

[2] Biosciences Center, National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA

Published online: 12 June 2015

biomass crop produces 540 % more renewable energy than energy consumed in its production and has significant environmental benefits [9–11]. Still, the processing of cellulose into fermentable simple sugars has remained the key economical factor limiting the utility of cellulosic bioenergy feed stocks [12, 13]. Among other things, this recalcitrance is linked to the inaccessibility of cellulosic microfibrils to enzymatic activity due to the presence of the plant polymer lignin and reduction of lignin or alteration of its linkages has been shown to reduce cell wall recalcitrance.

Genetic improvement of switchgrass to reduce this intrinsic recalcitrance to fermentative bioprocessing would have a profound positive impact on the nascent bioenergy industry. Fu et al. [14] have shown that down-regulation of the caffeic acid 3-omethyltransferase (COMT) gene in the lignin pathway leads to the generation of transgenic switchgrass plants with a normal growth phenotype but with reduced lignin content, altered lignin composition, increased saccharification efficiency, and increased ethanol yield compared with wild-type controls. Moreover, the transgenic plants require much lower cellulase enzyme levels to obtain ethanol yields equivalent to controls. Thus, these transgenic switchgrass lines appear to be a promising approach for developing improved cultivars of biofuel crops.

Considering their proven beneficial influence on plant growth [6, 7] and their apparent ubiquity [4], Sebacinales fungi should be considered as a previously hidden, but amenable and effective microbial tool for enhancing plant productivity and stress tolerance.

Bentonite is a low-cost mineral form of calcium-montmorillonite, a naturally occurring clay mineral composite, that has been traditionally used in agriculture as a soil preservative, to improve water and nutrient management of plants, as well as carrier for bio-inoculants [15, 16]. Thus, we considered both whether this type of particle was an effective carrier of *S. vermifera* and whether the components of the particle itself might improve plant performance. This research describes the development of a carrier-based delivery method for *S. vermifera* by impregnating the fungus onto bentonite clay particle. We demonstrate that this technique is both efficient in colonization and transfer, and gives equivalent if not better fitness benefits as other, more time-intensive strategies. Further, we explore whether low lignin transgenic lines benefit similarly to wild-type plants upon inoculation with this mycorrhizal fungus.

Materials and Methods

Preparation of Inoculum Using Bentonite Clay as a Carrier

S. vermifera strain used in this study (MAFF-305830) was obtained from the National Institute of Agro-biological Sciences, Tsukuba, Japan.

The clay particles were thoroughly sieved using mesh size 10 (2 mm) to get a uniform particle size. One-liter media bottles were filled with 400 ml of sterile clay particles by volume and subsequently sterilized by autoclave two times with a gap of 2 days. Thereafter, 150 ml of Modified Melin Norkan's (MMN) broth [17] pH 7 was added to each bottle and sterilized one more time by autoclave before inoculating with the seed culture. Each bottle was inoculated with 50 ml of a 4-week-old *S. vermifera* liquid culture (MMN) prepared in 250-ml Erlenmeyer flasks. An equivalent amount of MMN broth was added to a control set of bottles. Both the control and the inoculated bottles were incubated in a slanted, stationary position at 24 °C for 8 weeks. The bottles were shaken once per week for uniform distribution of the seed culture.

Assessment of the Viability of *S. vermifera* Coated onto Clay Particles

After incubation, the clay particles coated with or without *S. vermifera* were air dried overnight at room temperature under a laminar hood chamber. For assessment of the viability, air-dried clay particles coated with or without *S. vermifera* were plated on MMN agar plates and incubated for 4 weeks at 24 °C (Fig. 1a, b). After incubation, furthermore, clay particles were directly visualized under a Hitachi TM3000 tabletop scanning electron microscope (Hitachi, Japan) (Fig. 1e, f).

Bioleaching of Macronutrients from Clay Particles by *S. vermifera*

Clay particles coated with or without *S. vermifera* and an absolute control (untreated with MMN broth) were soaked in sterile water and incubated at 24 °C for 14 days either without agitation or gently shaking in triplicates. After incubation, water (1 ml) was collected, filtered through a 0.22-μm filter, and analyzed for cations and anions using ion chromatography (Fig. 2). Chromatographic separation was achieved on a Dionex ICS-5000 IC system (Thermo Fisher Scientific, USA) using a Dionex CS12A, Ion Pac (2×250 mm) analytical column for cations or a Dionex AS11, Ion Pac (2×250 mm) analytical column for anions. Ions were eluted using gradient elution at a flow rate of 0.3 ml/min and detected by suppressed conductivity. Column temperature was maintained at 30 °C and injection volume was 25 μl.

Evaluation of Switchgrass Colonization Efficiency of the Inoculum

Clay particles coated with or without *S. vermifera* were mixed with sterile sand in a ratio of 2:1 and filled into standard 5-ml pipette tips. In vitro germinated sterile seedlings of switchgrass (*P. virgatum* L var. Alamo) were placed singly on top of the clay in the 5-ml tips. The tips were then placed in 5 ml

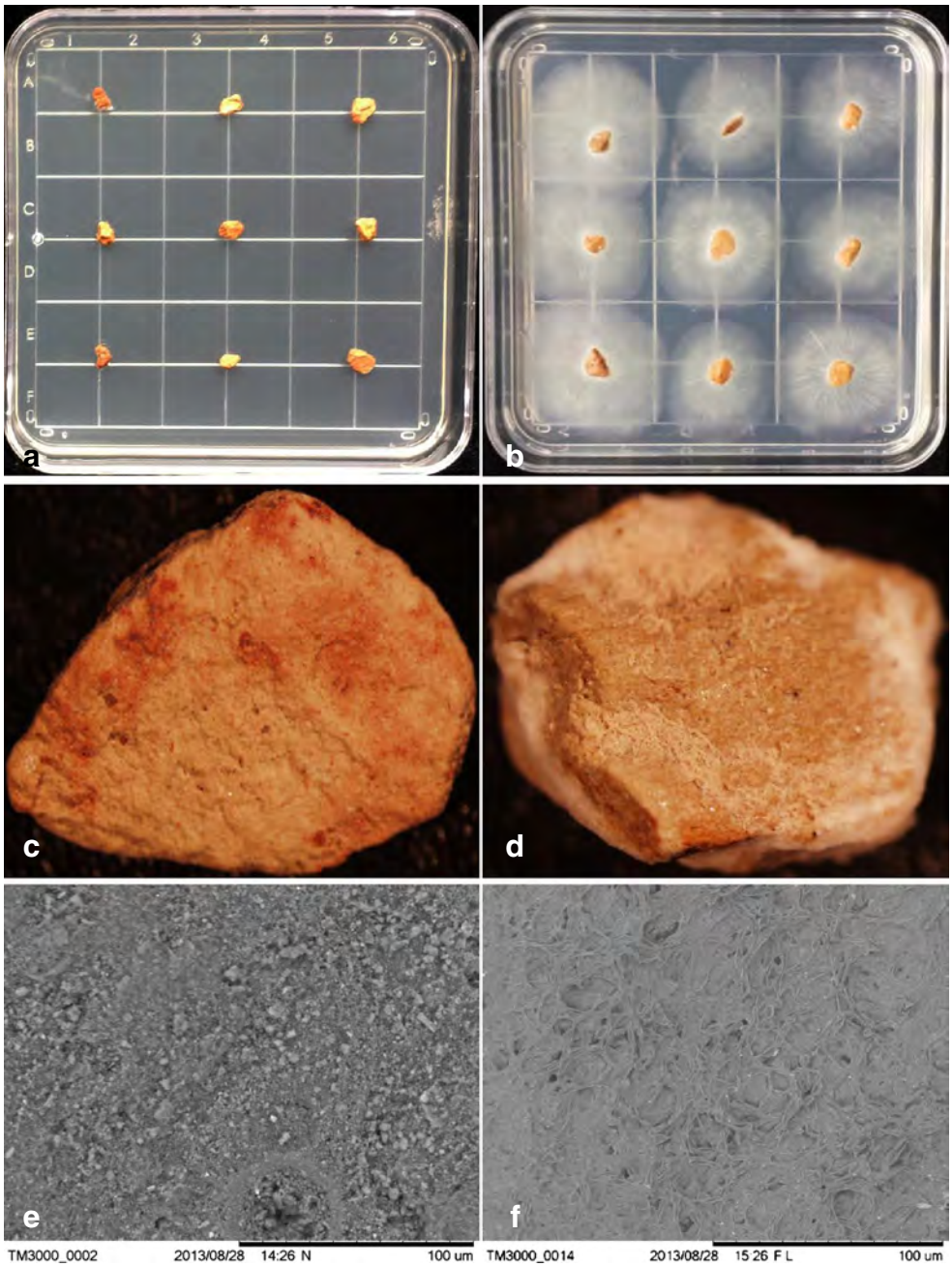


Fig. 1 Bentonite clay based carrier. *Left panel* (**a**, **c**, **e**): uncoated; *right panel* (**b**, **d**, **f**): coated with *S. vermifera* (MAFF305830). **a**, **b** Viability assay; **c**, **d** blow-up image of individual clay particle; **e**, **f** SEM of the surface of the clay particle

tip holder boxes, filled partly with 300 ml sterile water. Separate tip boxes were maintained for clay particles coated with or without *S. vermifera* and maintained in the growth chamber for 8 weeks (Fig. 3a). After 8 weeks, plants were harvested and *g*DNA was extracted from roots using QIAGEN DNeasy® Plant mini kit according to the manufacturer's instructions. Fungal colonization was validated by PCR using *Sebacina* specific primers, ITS3Seb and NL4 (Table S5). A subset of colonized root samples were stained with WGA-AF® 488 (WGA, 10 μg/ml; Life Technologies, Carlsbad, CA) and propidium iodide (PI; 10 μg/ml; Biotium, Hayward, CA) for fungal and plant cell wall staining following standard protocol and imaged using a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica, Wetzlar, Germany) (Fig. 3b).

Greenhouse-Based Evaluation of the Inoculum Using Low Lignin Switchgrass Lines

A greenhouse-based experiment was conducted to evaluate the efficiency of the clay particle-based inoculum for biomass enhancement of low lignin (COMT down-regulated) switchgrass lines. For this study, low lignin (COMT down-regulated) and corresponding wild-type (null segregant) plant materials

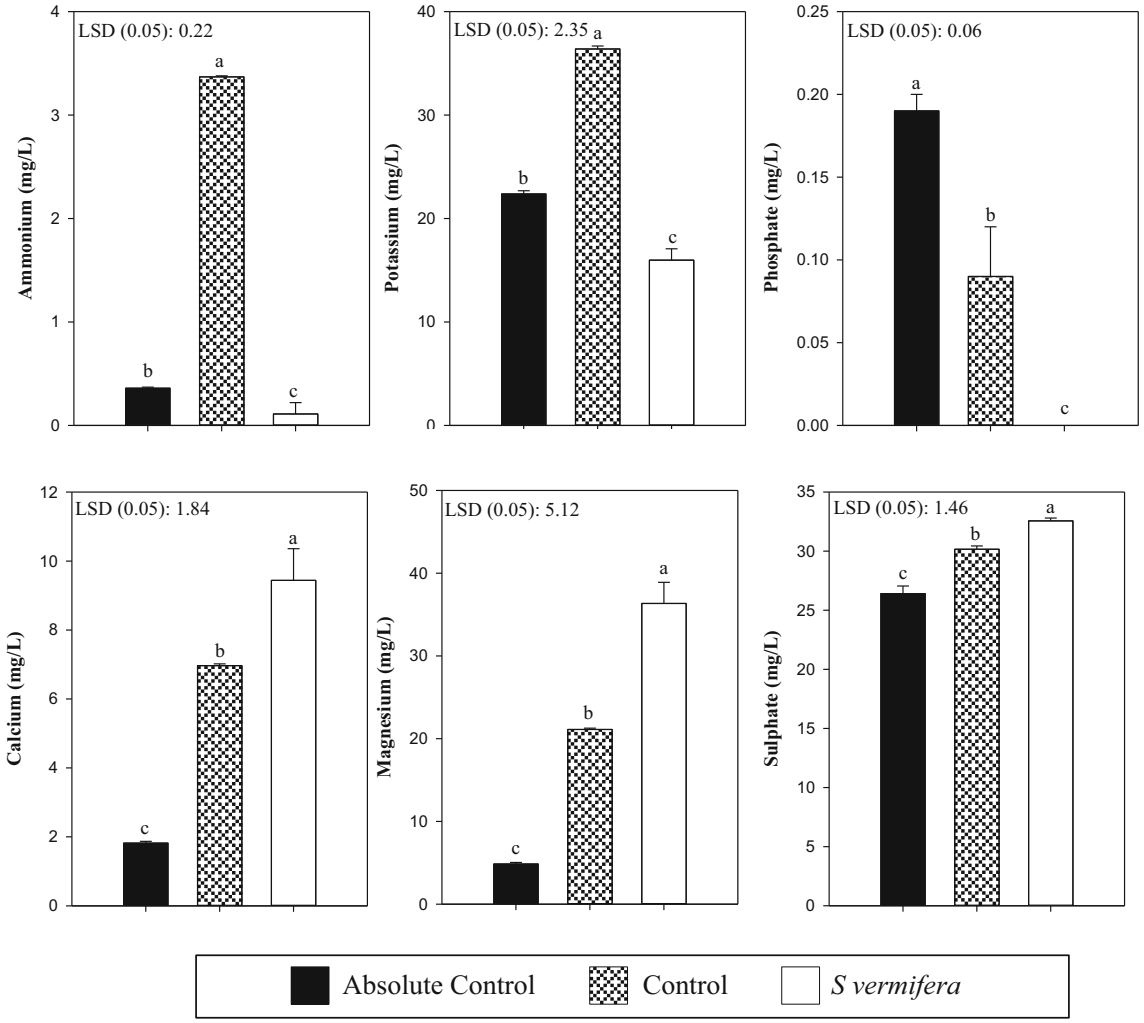


Fig. 2 Bioleaching of macronutrients from bentonite clay coated with or without *S. vermifera* (MAFF305830) with respect to control and absolute control. *Bars* with *different letter* indicate significant difference between treatments according to Duncan's multiple range test at $p \leq 0.05$

were procured from Dr. Zeng-Yu Wang's lab at Samuel Roberts Noble Foundation and clonally propagated in vitro in hormone-free, one-half strength MS media containing 0.3 % phytagel, amended with 1 % sucrose following standard tissue culture method. Plants were maintained in a growth chamber at 26 °C under a 17:9 h photoperiod.

For inoculation using bentonite clay, a uniform hole of 5 cm depth was made in the center of D25L single cell root trainers (5 cm in diameter by 25 cm in height; Stuewe & Sons, Inc., Oregon, USA) filled with non-sterile Metromix-350 medium (Scotts-Sierra Horticultural Products, Marysville, OH) using a 14-ml polypropylene round bottom tube (BD Falcon, New Jersey, USA). Clonally propagated low lignin (COMT down-regulated) and wild-type plants were directly transplanted into this hole. Thereafter, each hole was filled with one half tablespoon of bentonite clay coated with *S. vermifera* or the control clay particles lacking the fungus (Fig. S1a–d). Transplantation for colonization with bentonite clay was done in parallel with transplantation of in vitro colonized plants. Both sets were maintained in root trainers for 30 days for initial establishment and subsequently transplanted to 3-gal (11.34 l) pots filled with Metromix-350 medium.

Methodology of In Vitro Colonization

In vitro colonization was performed using 175-ml plant containers (5 cm in diameter by 11 cm in height) with lids. The containers were filled with 50 ml of M (minimal) medium pH 5.5 containing 0.3 % phytagel amended with 1 % sucrose. Clonally propagated switchgrass plants were grown in this media. For the purpose of in vitro colonization, a seed culture of *S. vermifera* was prepared by grinding the mycelia in liquid M media. Individual plants were inoculated with 20 μl of liquid culture by injecting it into the media with a sterile pipette (Fig. S1e). To maintain uniformity, the media containing the control plants were injected with the same volume of liquid M media. Plants were maintained in a growth chamber at

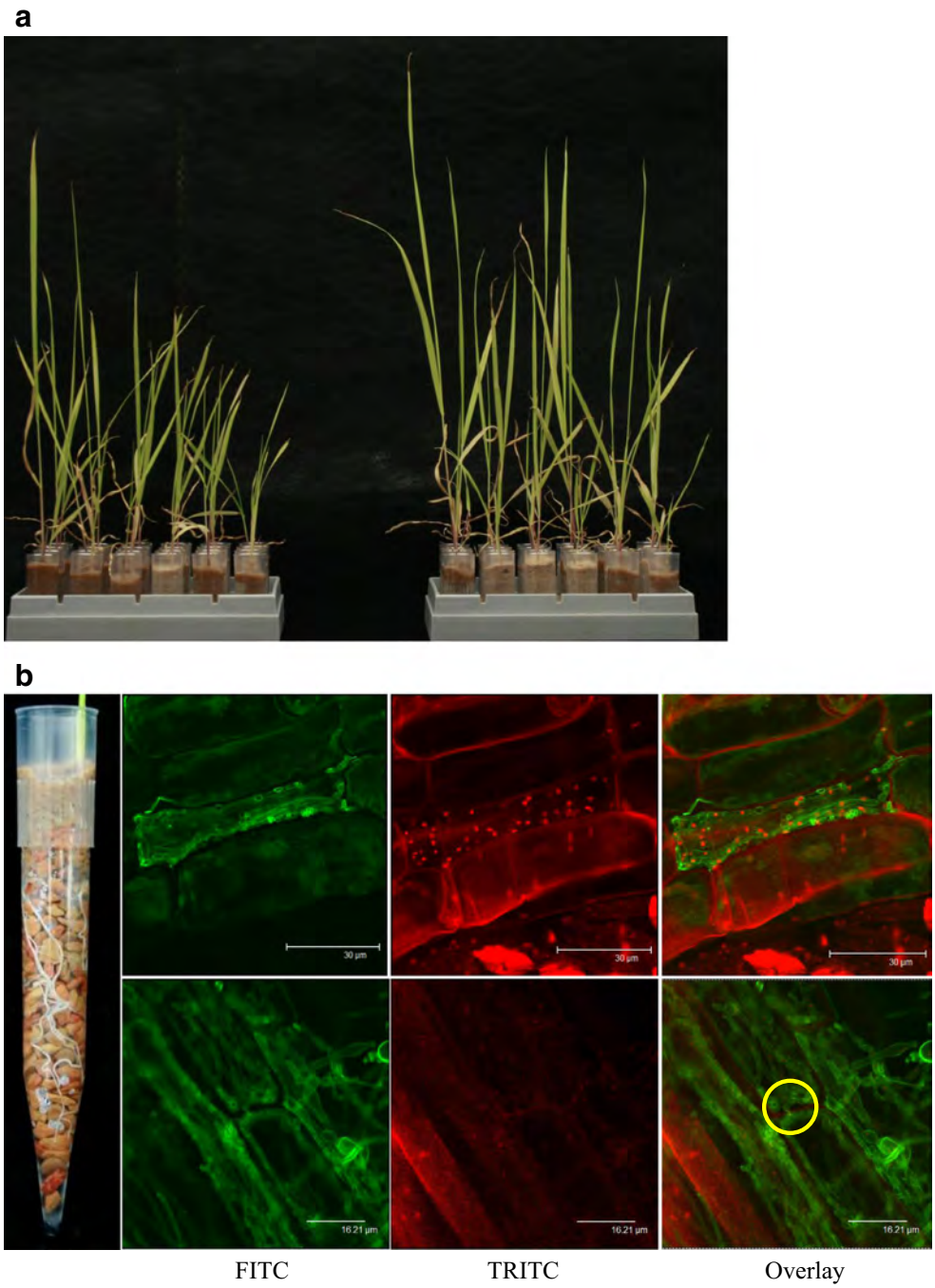


Fig. 3 **a** Evaluation of colonization efficiency of bentonite clay-based inoculum using switchgrass (*P. virgatum* L) as host. *Left box*: control; *right box*: colonized with *S. vermifera* (MAFF-305830). **b** Visualization (2D maximum projection image) of *S. vermifera* (MAFF-305830) colonization of switch grass root cells by confocal microscopy. *Yellow circle*: hyphal constriction. *Far left panel*: root development within 5 ml tip filled with *S. vermifera*-coated clay particle. *FITC* Root cells stained with WGA-AF® 488, *TRITC* root cells counter stained with propidium iodide

26 °C under a 17:9 h photoperiod for 2 weeks. After 2 weeks, the in vitro colonized plants and the corresponding control plants were transplanted into D25L single cell roots trainers filled with Metromix-350 medium and subsequently transferred to the greenhouse. The plants were maintained in the root trainers for 30 days for initial establishment and subsequently transplanted to 3-gal (11.34 l) pots filled with Metromix-350 medium. All plants were maintained in the greenhouse for 90 days under standard conditions (temperature range, 26 to 29 °C) with 50 % relative humidity under a 17:9 h photoperiod. All plants were watered with clear water two to three times per week without any fertilizer supplement.

Statistical Analysis

The experimental design consisted of four treatments each within in vitro and bentonite clay methods of colonization and with five replicates per treatment. Plants were arranged in a randomized complete block design. The data were analyzed using analysis of variance (ANOVA) under a two-factor,

factorial design with two main factors: COMT (down-regulated and wild type; factor 1) and *S. vermifera* colonization (present or absent; factor 2). When a significant *F* test was observed, treatment means were compared using least significant difference (LSD) value by Duncan's multiple range test (DMRT) at $p \leq 0.05$ using CoStat statistical software 6.4 (Cohort Berkeley, California) (Tables S1, S2, and S3). The significant main effects, and the interactions (at $p \leq 0.05$), were plotted graphically using SigmaPlot 12.5 (Systat Software, San Jose, California) (Figs. 4a and b, 5a and b, and 6).

Confirmation of Fungal Colonization by PCR

After 90 days, colonization by *S. vermifera* was confirmed by nested PCR using sets of *Sebacina* specific primers (Table S5). *g*DNA was isolated from *S. vermifera*-colonized low lignin (COMT down-regulated) and wild-type switchgrass roots and respective controls. The 3′ region of the 18S (SSU), ITS1 and ITS2, the 5.8S ribosomal subunit, and the 25–28S (LSU) of Sebacinales were amplified by direct PCR using NSSeb1 and NLSeb1.5R. Direct PCR amplified a ~2.2-kb fragment. Subsequently, the primary PCR product was diluted to 1:200 and used as a template for a nested PCR using ITS3Seb and ITS3Seb-R primers covering the 5.8S coding sequence and highly variable ITS2 region of *S. vermifera* ribosomal DNA (rDNA). In this nested PCR, a ~300-bp fragment was indicative as positive proof of *Sebacina* colonization (Fig. S2).

Real-Time Quantitative PCR for Fungal Biomass Analysis

Fungal biomass was analyzed by a real-time quantitative PCR method adopted from Charlton et al. [18] and modified for the current study. To determine the biomass of *S. vermifera* 90 dpi in planta, *g*DNA was isolated from *S. vermifera* colonized and non-colonized COMT and wild-type plants and quantified using a Nanodrop ND-1000 (Wilmington, Delaware, USA). A standard curve of switchgrass *g*DNA was generated by quantitative PCR of *g*DNA samples from non-colonized switchgrass diluted to a concentration range of 0.1 to 5 ng/μl. A standard curve was also established for the fungus by quantitative PCR of *S. vermifera* *g*DNA at concentrations of 0.01 to 0.5 ng/μl. The amounts of fungal and plant *g*DNA in *S. vermifera* colonized plants were then determined by quantitative PCR using the generated standard curves as references. Quantitative PCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) following standard protocol. The primer pairs ITS3Seb and ITS3Seb-R and PvTef-F1 and PvTef-R1 (Table S5) were used to amplify *S. vermifera* and switchgrass gDNA, respectively. For each treatment, five plants were harvested and analyzed by quantitative PCR with three technical repeats for each primer combination. The fungal biomass in planta was calculated as a percentage of the *S. vermifera* DNA relative to switchgrass DNA using the formula (Sv/Pv)×100, where Sv and Pv are the DNA quantities of *S. vermifera* and switchgrass in the sample (Fig. 6, Table S4) [19].

Fig. 4 **a** Biomass of low lignin (COMT down-regulated) and wild-type switchgrass either colonized in vitro or using bentonite clay as carrier 90 days after infection. *Bars* with *different letter* indicate significant difference between treatments at $p \leq 0.05$. **b** Biomass of low lignin (COMT down-regulated) and wild-type switchgrass either colonized in vitro or using bentonite clay as carrier 90 days after infection. *Bars* with *different letter* indicate significant difference between treatments at $p \leq 0.05$

Estimation of Plant Biomass

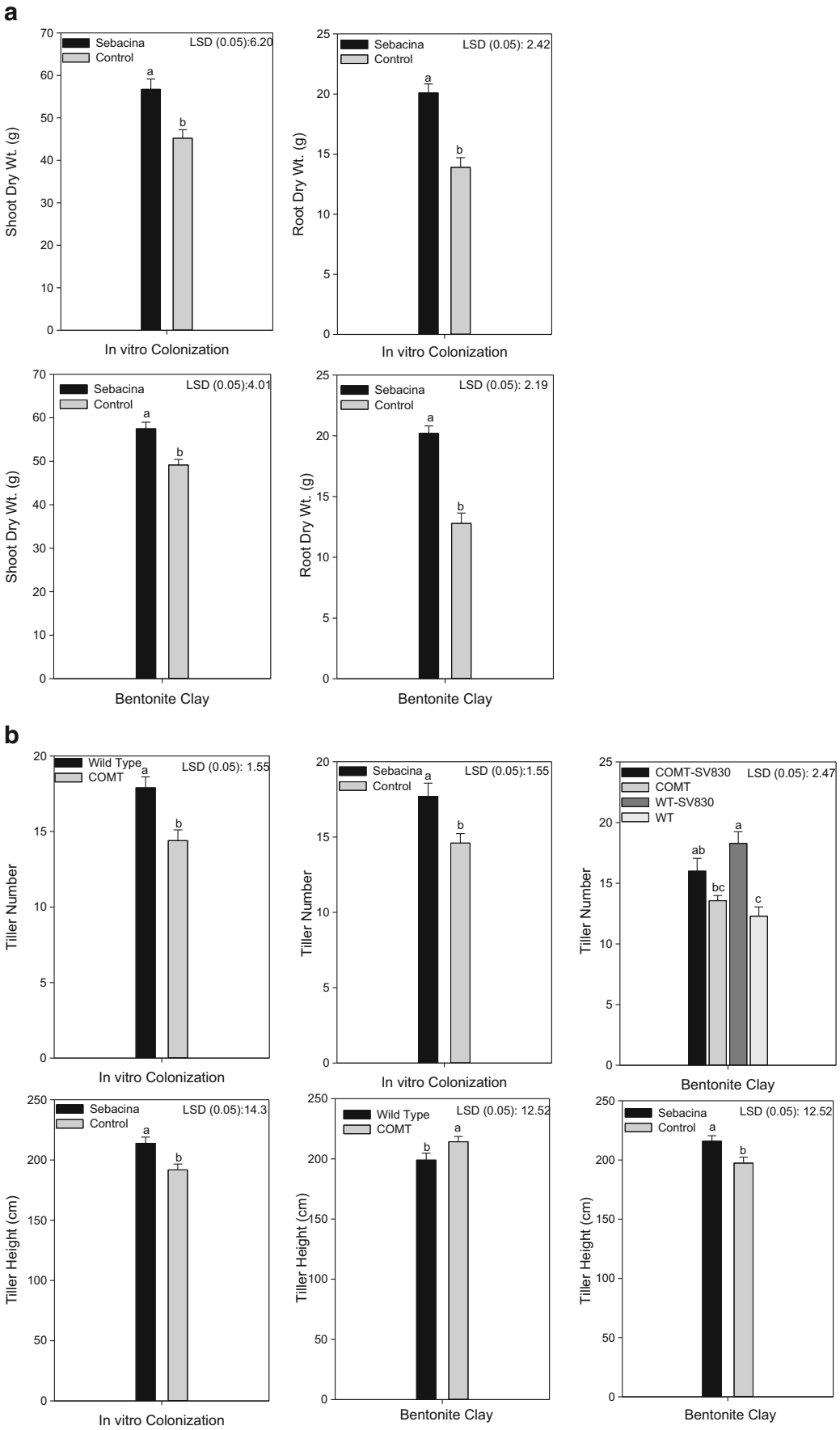
After 90 days, tiller number and tiller height were measured and thereafter plants were completely harvested for measurement of shoot and root dry weight (Figs. 4a, b and 5a, b).

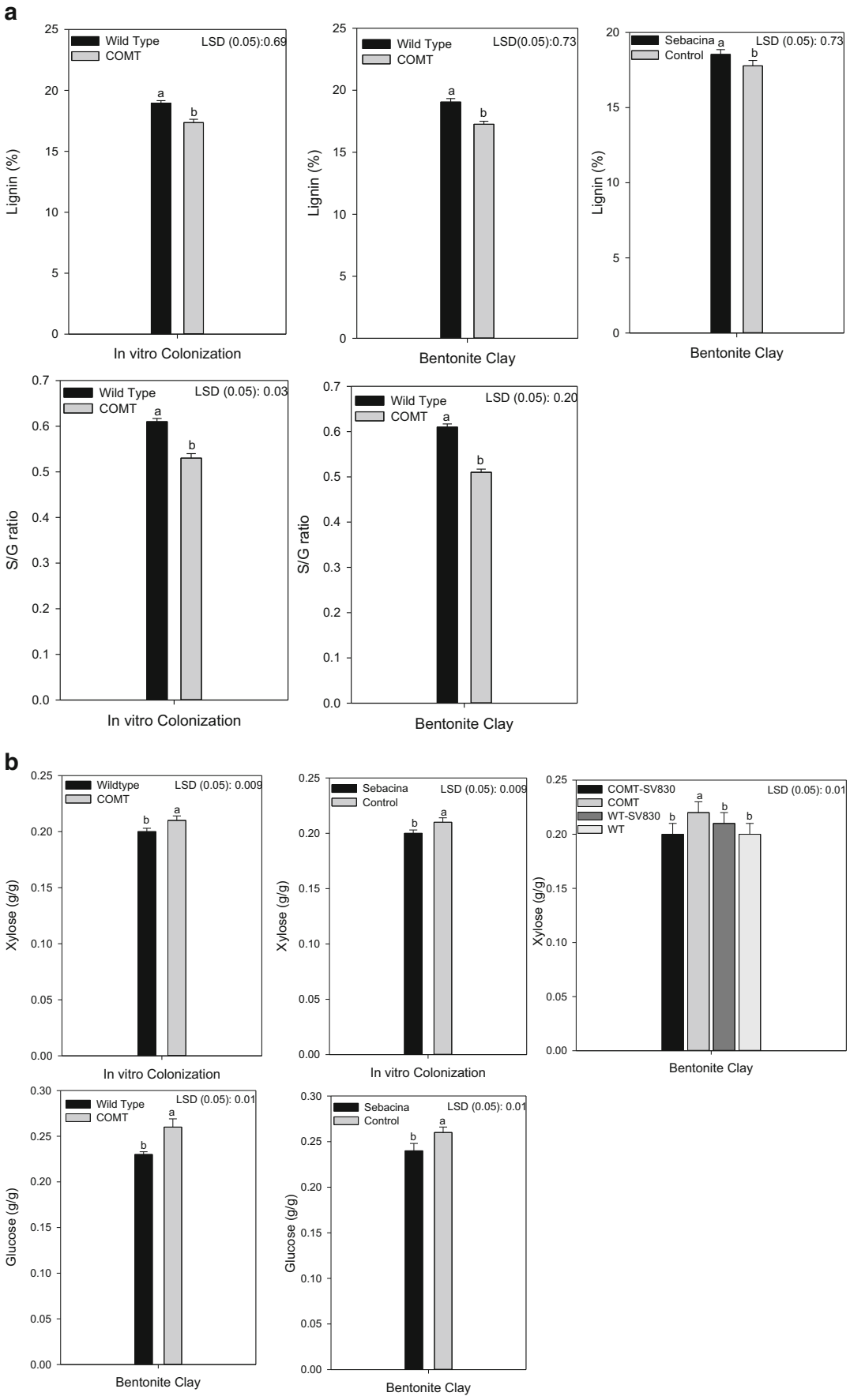
Cell Wall Composition Analysis

The shoot biomass was oven dried at ~40 °C for 72 h and milled to 1 mm particle size (mesh size 20) using a Model 4 Thomas Wiley® Mill (Thomas Scientific, Swedesboro, NJ). The milled samples were analyzed for lignin content and sugar release characteristics at the National Renewable Energy Laboratory (NREL) (Fig. 5a, b).

Cell wall chemistry analysis was performed on ~4 mg of plant material. Mass spectra data analysis comprises (1) multivariate statistical methods to select cell wall chemistry phenotypes that significantly differ from cell wall chemistry of controls and (2) estimating changes in concentration or structure of cell wall components from peak intensities. Lignin content and S/G ratio were analyzed by pyrolysis molecular beam mass spectrometry (py-MBMS) [20].

Sugar release estimates were generated using a high-throughput screening method that involved hot-water pretreatment and enzymatic hydrolysis [21, 22]. Approximately 200 mg of each biomass sample was rolled up in a teabag wrapped with stainless steel wire. The wrapped samples were treated with glucoamylase (Spirizyme Ultra—0.25 %) and alpha-amylase (Liquozyme SC DS—1.5 %) in 0.1 M sodium acetate (24 h, 55 °C, pH 5.0) to remove starch (16 ml enzyme solution per 1 g biomass) [23]. After extensive rinsing with water, the biomass samples, still in the teabags, were cycled for 24 h in a Soxhlet extractor using 190 proof ethanol to remove extractives. After drying overnight, the samples were transferred to the hoppers of a Symyx Powdernium MTM solids dispensing robot (Freeslate, Sunnyvale, CA). Triplicate sample aliquots of 5 mg (±0.5 mg) were weighed into


a
Sebacina
Control
LSD (0.05):6.20
Shoot Dry Wt. (g)
In vitro Colonization
LSD (0.05): 2.42
Root Dry Wt. (g)
LSD (0.05):4.01
Bentonite Clay
LSD (0.05): 2.19
b
Wild Type
COMT
LSD (0.05): 1.55
Tiller Number
LSD (0.05):1.55
COMT-SV830
WT-SV830
WT
LSD (0.05): 2.47
ab
bc
c
LSD (0.05):14.3
Tiller Height (cm)
LSD (0.05): 12.52


a
Wild Type
COMT
LSD (0.05):0.69
Lignin (%)
In vitro Colonization
LSD(0.05):0.73
Bentonite Clay
Sebacina
Control
LSD (0.05): 0.73
S/G ratio
LSD (0.05): 0.03
LSD (0.05): 0.20
b
Wildtype
LSD (0.05): 0.009
Xylose (g/g)
COMT-SV830
WT-SV830
WT
LSD (0.05): 0.01
Glucose (g/g)

◀ **Fig. 5** **a** Cell wall composition of low lignin (COMT down-regulated) and wild-type switchgrass either colonized in vitro or using bentonite clay as carrier 90 days after infection. *Bars* with *different letter* indicate significant difference between treatments at $p \leq 0.05$. **b** Cell wall composition of low lignin (COMT down-regulated) and wild-type switchgrass either colonized in vitro or using bentonite clay as carrier 90 days after infection. *Bars* with *different letter* indicate significant difference between treatments at $p \leq 0.05$

the wells of a custom-built 96-well Hastelloy reactor plates. Water was added (250 μl) and the plates were sealed with silicone adhesive Teflon tape. Stacked plates were reacted at 180 °C for 17.5 min in a Parr steam reactor and rapidly quenched with tap water to eliminate differential cooling rates through the stack. Once cooled, the plates were centrifuged at 1600×*g* for 10 min, the sealing tape was removed, and 40 μl of 8 % CTec2 (Novozymes) in 1.0 M sodium citrate buffer was added to each sample. The plates were then resealed with tape and magnetic plates, mixed thoroughly by inversion, and left to statically incubate at 50 °C for 70 h. After 70 h, an aliquot of the saccharified hydrolysate was diluted and tested using GOPOD (glucose oxidase/peroxidase) and XDH (xylose dehydrogenase) assays (both from Megazyme, Intl., Bray, Ireland). Results were calculated using mixed glucose/xylose standard solutions. The average of the three replicates for each sample was used for subsequent analyses.

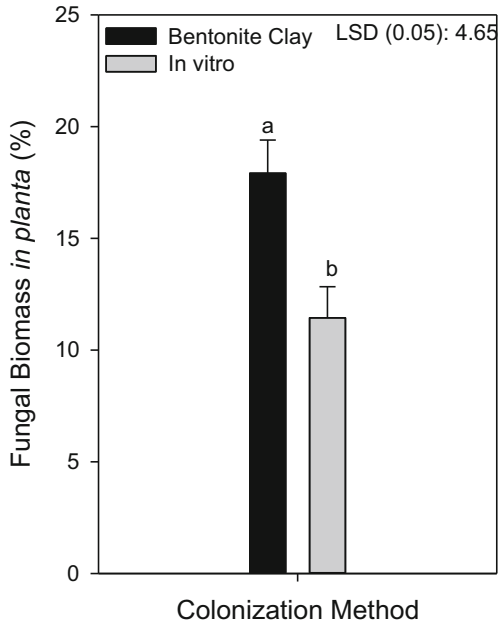


Fig. 6 *S. vermifera* root colonization in terms of fungal biomass in planta (%) in low lignin (COMT down-regulated) and wild-type switchgrass either colonized in vitro or using bentonite clay as carrier 90 days after infection. Bars with different letter indicate significant difference between treatments at $p \leq 0.05$

Results

Assessment of Inoculum Viability

Air-dried *S. vermifera*-coated clay particles were able to produce fresh fungal colonies when plated in MMN agar media and further were not prone to contamination under standard laboratory conditions (Fig. 1b). Purity of the fungal colonies was verified by PCR followed by sequencing using specific primers and direct visualization using a light microscope. No abnormality with respect to morphology or growth pattern was observed. Further, direct imaging of the *S. vermifera*-coated clay particles with the Hitachi TM3000 tabletop scanning electron microscope showed homogenous coating on the surface of the clay particles (Fig. 1f).

Bioleaching of Macronutrients by *S. vermifera*

The data were analyzed by ANOVA and the treatment means were compared through LSD value by DMRT at $p \leq 0.05$ (Fig. 2). Water collected from clay particles coated with *S. vermifera* was found to have significantly lower concentrations (mg/l) of ammonium, phosphate, and potassium with respect to the control and absolute control. In contrast, concentrations (mg/l) of calcium, magnesium, and sulfate were significantly higher in water with *S. vermifera*-coated clay particles. These results clearly demonstrate that the presence of *S. vermifera* alters nutrient availability and may be able to mobilize nutrients trapped inside the clay particles.

Assessment of the Colonization Efficiency of the Inoculum

Colonization efficiency of the inoculum was confirmed by PCR using root-derived DNA and *Sebacina* specific primers. Further, to confirm that *Sebacina* was present within the plant roots and not just on the surface, confocal microscopy was used. We visualized normal, healthy fungal hyphae packed inside in the cortical roots cells of switchgrass (*P. virgatum* L) (Fig. 3b).

Estimation of Fungal Biomass in Planta

S. vermifera biomass (%) in planta was determined from the ratio of fungus to plant DNA, as measured by real-time quantitative PCR. *S. vermifera* colonization enhanced switchgrass biomass in either of the colonization method (Fig. 4a). Both low lignin (COMT down-regulated) and wild-type switchgrass inoculated with the clay particle-based method showed significantly ($0.001 < p \leq 0.01$, **) more fungal biomass in planta with respect to in vitro colonized plants (Table S4, Fig. 6). We found that COMT down-regulation had no preference ($p > 0.05$, ns) over specific colonization methods (Table S4).

Estimation of Plant Biomass and Cell Wall Composition

Significant ($p \leq 0.05$) main effects, and interactions of *S. vermifera* colonization and COMT down-regulation on plant biomass and cell wall composition are graphically presented in Figs. 4a, b and 5a, b.

Shoot and Root Dry Weight

S. vermifera colonization significantly increased shoot and root dry weight in switchgrass with respect to un-inoculated controls (Fig. 4a, Table S1).

Shoot dry weight increased by 22.06 and 12.39 % when inoculated with *S. vermifera*-coated bentonite clay as the carrier and 19.09 and 33.10 % when inoculated in vitro in low lignin (COMT down-regulated) and wild-type plants, respectively. With similar colonization methods, root dry weight increased by 44.32 and 72.87 %, and 19.09 and 33.10 % in low lignin (COMT down-regulated) and wild-type plants, respectively (Table S1).

COMT down-regulation had no effect in altering shoot or root dry weight (Table S1).

Tiller Number and Height

S. vermifera colonization and COMT down-regulation affected tillering when colonized in vitro and plant height when colonized using bentonite clay as carrier (Fig. 4b). While *S. vermifera* colonization improved positively, COMT down-regulated plants reported reduced tiller number and tiller height (Fig. 4b) in either of the colonization methods.

We observed an interaction (COMT × *Sebacina*) on tillering when plants were colonized using bentonite clay as carrier. *S. vermifera* increased the tiller number in low lignin (COMT down-regulated) and wild-type switchgrass with respect to un-inoculated control (Fig. 4a, Table S1). Tiller number increased by 17.91 and 48.86 % in low lignin (COMT down-regulated) and wild-type plants, respectively. Therefore, we conclude, tiller number in wild-type plants was significantly enhanced by *S. vermifera*, strikingly so in plants inoculated with bentonite clay particles, whereas tillering in low lignin plants was more modestly but positively affected.

S. vermifera colonization increased the tiller height with respect to un-inoculated control when colonized in vitro (Fig. 4b, Table S1). Height of low lignin (COMT down-regulated) and wild-type plants increased by 12.61 and 10.46 % respectively (Table S1).

In summary, a clear role of *S. vermifera* can be depicted in improving plant fitness parameters in either of the colonization methods, except in tillering when colonized using bentonite clay as carrier, where we observed significant interaction between colonization and COMT down-regulation.

Cell Wall Composition Analysis

Lignin and S/G Ratio

S. vermifera showed significant effect in modifying lignin content, only when colonized using bentonite clay as a carrier (Table S2).

As expected, lignin content (% of dry matter) in low lignin (COMT down-regulated) plants was consistently less compared to the wild-type control plants in either of the colonization method (Fig. 5a). *S. vermifera* colonization reported marginally higher lignin content when colonized using bentonite clay as carrier.

Production of fuels from biomass crops is limited by the recalcitrance of lignocellulose to hydrolysis into its component pentose and hexose sugars. A lower S/G ratio implies more potential for covalent crosslinking and expected to result in less hydrolysis which has a strong influence on the efficiency of the overall process [24]. A decrease in S/G ratio was observed in low lignin (COMT down-regulated) switchgrass, compared to the wild-type control (Fig. 5a). *S. vermifera* colonization had no impact on modifying S/G ratio (Table S2).

Sugar Release

S. vermifera and COMT down-regulation showed a significant effect in glucose and xylose release when colonized using bentonite clay as carrier and in vitro, respectively (Table S2). While sugar released was higher in COMT down-regulated plants, *S. vermifera* colonized plants reported less sugar release with respect to un-inoculated control (Fig. 5b).

We observed an interaction (COMT × *Sebacina*) on xylose release when plants were colonized using bentonite clay as carrier. Un-inoculated low lignin (COMT down-regulated) plants reported higher sugar release compared to the rest of the treatments.

In summary, we claim that *S. vermifera* colonization had little or no effect in altering lignin content or S/G ratio. Further, *S. vermifera* colonized plants reported less sugar release with respect to un-inoculated control, except xylose when colonized using bentonite clay as carrier.

Discussion

We have previously demonstrated that the orchid mycorrhiza, *S. vermifera* (MAFF-305830), dramatically increases the biomass of switchgrass, particularly under abiotic stress such as drought conditions [6, 7]. Here, we have developed a method for large-scale dissemination of inoculum containing this growth-promoting fungus for switchgrass field trials. Further, we demonstrate the effectiveness of this carrier-based method for colonization of switchgrass in greenhouse trials and also

show that similar biomass gains are achieved in low lignin (COMT down-regulated) plants, as we have previously shown in wild-type switchgrass.

During the course of this study, we evaluated several methods (details not reported in this paper) of producing inoculum for large-scale dissemination, including using wheat straw as a substrate for the fungus, creation of *Agrobacterium rhizogenes* hairy root co-cultures, in vitro colonization, and the bentonite clay method described herein. Of these, we believe the most promising method for large-scale delivery is the latter. Any carrier material used for inoculation substantially affects the success of a mycorrhizal inoculation program. The inoculant must remain viable during storage and transport, maintaining its infectivity for several months after its production. Furthermore, the formulated inoculant must be easy to apply and must also be free of contamination by plant pathogens and any free-living microorganisms that could affect inoculant viability [25, 26]. The clay particles can be inoculated en masse, are not prone to contamination, and can be stored under standard laboratory conditions for extended periods of time. Another important concern is the potential for inhibition of the mycorrhizal fungus by the carrier material itself. Bentonite clay did not retard either the growth of the plants or the fungus. Further, many plant-growth-promoting microorganisms, such as *S. vermifera*, play a significant role in regulating the availability of macro- and micronutrients such as N, P, and K. Our results clearly showed the ability of *S. vermifera* to mobilize several essential micronutrients from the clay particles, demonstrating not only the chemical compatibility of the carrier and the fungus but also the potential for the fungus to contribute to the dynamics of nutrient management.

Traditional breeding techniques have increased yield performance of switchgrass by 20–30 % from existing parent types [8]. In the present study, we found that *S. vermifera* colonization mediated by bentonite clay as a carrier significantly increased the shoot biomass of low lignin (COMT down-regulated) switchgrass by 22 % (dry-weight basis). The percentage increase in biomass was not only comparable to the increased biomass of in vitro colonized plants (19 %), which we considered as a bench mark for this study, but is obviously comparable to other endeavors engaged in improving switchgrass biomass. Annual biomass yields of established switchgrass fields average from 5.2 to 11.1 Mg/ha [11]. Assuming an estimated conversion rate of 0.38 l ethanol/kg harvested biomass [10], a 22 % increase in biomass would translate into increased production of 433–927 l ethanol/ha. It is expected that further improvements in both genetics (hybrid cultivars, molecular markers) and agronomics (production system management practices and inputs) will be achieved for dedicated energy crops such as switchgrass, which will further improve biomass yields, conversion efficiency, and net energy values (NEV) [27].

Switchgrass is not only a model bioenergy energy crop; its deep root system holds great potential for improving soil quality by reducing nutrient loss [28, 29], preventing erosion, and sequestering carbon (C) in the soil-switchgrass agro-ecosystem [30]. Similarly to shoot biomass, *S. vermifera* colonization using bentonite clay as a carrier or in vitro significantly increased the root biomass of low lignin (COMT down-regulated) switchgrass up to 44 and 40 % (dry-weight basis), respectively. Such increases in root biomass would likely translate into multi-fold benefits for the target plant and the surrounding community including increased capacity to utilize water and nutrients from deeper soils, enhanced protection from soil erosion, increased enrichment of soil carbon from root turnover, increased activity of microbial communities, and increased capacity of switchgrass to store and mobilize energy and nutrients needed to regrow following cutting or grazing [8].

In conclusion, we were able to successfully coat bentonite clay with the beneficial fungus, *S. vermifera* (MAFF-305830), and demonstrate subsequent high-efficiency colonization of the root cortex of switchgrass (*P. virgatum* L). Colonization by the fungus with this method resulted in significant growth promotion of transgenic low lignin (COMT) and wild-type switchgrass in greenhouse conditions. In fact, bentonite clay coated with *S. vermifera* as a carrier more effectively colonized plants than our in vitro colonization method. This clay-based inoculum is not prone to contamination and can be stored at room temperature. These features together with the mineral composition of the clay illustrate the potential for packaging the fungus with a nutrient source en masse for large-scale delivery to the field. We are currently taking this next step, initiating field trials of transgenic and natural varieties of switchgrass for *S. vermifera*-mediated crop improvement studies.

Acknowledgments *S. vermifera* (MAFF-305830) used in this study was obtained from the National Institute of Agro-biological Sciences, Tsukuba, Ibaraki, Japan. The COMT lines used in this study were provided by Chunxiang Fu and Zeng-Yu Wang, Forage Improvement Division, The Samuel Roberts Noble Foundation. We thank Crissa Doeppke, Melissa Glenn, Kimberly Mazza, Logan Schuster, and Kevin Cowley in NREL for their efforts in preparing samples for the HTP recalcitrance pipeline; Erica Gjersing Robert Sykes and Mark Davis in NREL for cell wall composition analysis; David Huhman for ion chromatography; Stacy Allen for qRT-PCR; Jin Nakashima for assistance with SEM and confocal microscopy; Stephen L. Webb for assistance with statistical analysis; and Myoung-Hwan Chi, Blue Stewart, Colleen Elles, and Amanda Hammon for greenhouse assistance. This work was supported by the Bioenergy Science Center, a US Department of Energy Bioenergy Research Center, through the Office of Biological and Environmental Research in the DOE Office of Science.

Conflict of Interest All the authors in this manuscript declare no conflict of interests inherent to this submission.

References

1. Oberwinkler F, Riess K, Bauer R, Selosse MA, Weiss M, Garnica S, Zuccaro A (2013) Enigmatic Sebacinales. Mycol Prog 12(1):1–27. doi:10.1007/s11557-012-0880-4
2. DeMars B, Boerner R (1996) Vesicular arbuscular mycorrhizal development in the Brassicaceae in relation to plant life span. Flora 191
3. Selosse MA, Bauer R, Moyersoen B (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155(1):183–195. doi:10.1046/j.1469-8137.2002.00442.x
4. Weiss M, Sykorova Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS ONE 6(2):e16793. doi:10.1371/journal.pone.0016793
5. Warcup J (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. New Phytol 110(2):227–231
6. Ghimire SR, Charlton ND, Craven KD (2009) The mycorrhizal fungus, *Sebacina vermifera*, enhances seed germination and biomass production in switchgrass (*Panicum virgatum* L). Bioenergy Res 2(1–2):51–58
7. Ghimire SR, Craven KD (2011) Enhancement of switchgrass (*Panicum virgatum* L.) biomass production under drought conditions by the ectomycorrhizal fungus *Sebacina vermifera*. Appl Environ Microbiol 77(19):7063–7067. doi:10.1128/AEM.05225-11
8. McLaughlin SB, Adams Kszos L (2005) Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. Biomass Bioenergy 28(6):515–535
9. Wang MQ (2001) Development and use of GREET 1.6 fuel-cycle model for transportation fuels and vehicle technologies. Argonne National Lab., IL (United States). Funding organisation: US Department of Energy (United States)
10. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 311(5760):506–508
11. Schmer MR, Vogel KP, Mitchell RB, Perrin RK (2008) Net energy of cellulosic ethanol from switchgrass. Proc Natl Acad Sci 105(2):464–469
12. Aden A, Foust T (2009) Technoeconomic analysis of the dilute sulfuric acid and enzymatic hydrolysis process for the conversion of corn stover to ethanol. Cellulose 16(4):535–545
13. Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction toward improved lignocellulosic production and processability. Plant Sci 178(2):61–72
14. Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M Jr, Chen F, Foston M, Ragauskas A, Bouton J, Dixon RA, Wang ZY (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci U S A 108(9):3803–3808. doi:10.1073/pnas.1100310108
15. Malusá E, Sas-Paszt L, Ciesielska J (2012) Technologies for beneficial microorganisms inocula used as biofertilizers. Sci World J 2012
16. Heijnen C, Hok-A-Hin C, Van Veen J (1992) Improvements to the use of bentonite clay as a protective agent, increasing survival levels of bacteria introduced into soil. Soil Biol Biochem 24(6):533–538
17. Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163
18. Charlton ND, Shoji J-Y, Ghimire SR, Nakashima J, Craven KD (2012) Deletion of the fungal gene soft disrupts mutualistic symbiosis between the grass endophyte *Epichloë festucae* and the host plant. Eukaryot Cell 11(12):1463–1471
19. Young C, Bryant M, Christensen M, Tapper B, Bryan G, Scott B (2005) Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. Mol Gen Genomics 274(1):13–29
20. Sykes R, Kodrzycki B, Tuskan G, Foutz K, Davis M (2008) Within tree variability of lignin composition in *Populus*. Wood Sci Technol 42(8):649–661
21. Selig MJ, Tucker MP, Sykes RW, Reichel KL, Brunecky R, Himmel ME, Davis MF, Decker SR (2010) Original research: Lignocellulose recalcitrance screening by integrated high-throughput hydrothermal pretreatment and enzymatic saccharification. Ind Biotechnol 6(2):104–111
22. Decker SR, Brunecky R, Tucker MP, Himmel ME, Selig MJ (2009) High-throughput screening techniques for biomass conversion. Bioenergy Res 2(4):179–192
23. Decker SR, Carlile M, Selig MJ, Doeppke C, Davis M, Sykes R, Turner G, Ziebell A (2012) Reducing the effect of variable starch levels in biomass recalcitrance screening. In: Biomass conversion. Springer, pp 181–195
24. Davison BH, Drescher SR, Tuskan GA, Davis MF, Nghiem NP (2006) Variation of S/G ratio and lignin content in a *Populus* family influences the release of xylose by dilute acid hydrolysis. Appl Biochem Biotechnol 130(1–3):427–435. doi:10.1385/Abab:130:1:427
25. Rossi MJ, Furigo A, Oliveira VL (2007) Inoculant production of ectomycorrhizal fungi by solid and submerged fermentations. Food Technol Biotechnol 45(3):277
26. Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9(5):501–515
27. Vogel KP, Jung H-JG (2001) Genetic modification of herbaceous plants for feed and fuel. Crit Rev Plant Sci 20(1):15–49
28. Huang Y, Rickerl D, Kephart K (1996) Recovery of deep-point injected soil nitrogen-15 by switchgrass, alfalfa, ineffective alfalfa, and corn. J Environ Qual 25(6):1394–1400
29. Schultz R, Collettil J, Isenhart T, Simpkins W, Mize C, Thompson M (1995) Design and placement of a multi-species riparian buffer strip system. Agrofor Syst 29(3):201–226
30. Ma Z, Wood C, Bransby D Management and soil influence on switchgrass carbon sequestration and biomass accumulation. In: Agronomy abstracts, ASA, Madison, WI, 1996. p 32