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The Mycorrhizal Fungus, *Sebacina vermifera*, Enhances Seed Germination and Biomass Production in Switchgrass (*Panicum virgatum* L)

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Abstract Seed dormancy and slow seedling establishment are two major concerns in switchgrass (*Panicum virgatum* L.) production, often resulting in a poor stand with reduced productivity. Studies were conducted to investigate the stability of artificial associations between switchgrass and the ectomycorrhizal fungus, *Sebacina vermifera*, and to evaluate the potential benefits of this novel association in seed germination and biomass production. All six strains of *S. vermifera* tested had a high frequency of colonization on switchgrass roots of a synthetic cultivar NF/GA-993. The positive effects of the associations were reflected in plant height, root length, and biomass production. Inoculated plants produced as much as 75%, 113%, and 18% more shoot biomass than un-inoculated control plants in the first, second, and third harvest, respectively, with no consequent reduction in root biomass. Further, culture filtrates from some strains of *S. vermifera* increased seed germination in the switchgrass cultivar Kanlow by 52% over the control ($p<0.05$). This study illustrates the great potential of microbial associations to increase biomass production and productivity of switchgrass.

Keywords Bioenergy crop · Ectomycorrhizae · Symbiosis

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Abbreviations

AMF	Arbuscular mycorrhizal fungi
FGP	Final germination percentage
ha	Hectare, 10,000 m^2
Mg	Megagram, 1,000 kg
MYP	Malt extract, yeast extract, peptone
PBS	Phosphate buffer saline

Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial C_4 grass native to North America, primarily in the tall-grass prairie region of the Great Plains [20]. The US Department of Energy (DOE) has identified switchgrass as a promising bio-energy crop for cellulosic ethanol production because of its abundant biomass, excellent nutrient-use efficiency, and broad adaptability [5, 6, 36, 37]. The ambitious goal set by the DOE to replace 30% of current US petroleum consumption with bio-ethanol by 2030 will require billions of tons of biomass annually, with the contribution of perennial crops predicted to account for approximately 342 million tons [5, 25]. If switchgrass is to be a central component of this strategy, perhaps contributing up to 70% of this amount, approximately 15 million hectares of arable land would need to be dedicated to switchgrass at a productivity level of 16 Mg ha^{-1}. The acreage would increase dramatically if cultivated on marginal lands, where estimates of biomass yield drop between 5.2 and 11.1 Mg ha^{-1} [38]. Therefore, increasing switchgrass productivity and further enhancement of its already prodigious nutrient-use efficiency is paramount to maximizing the potential of this species as a sustainable cellulosic feedstock and ecological restoration agent.

The profitability of switchgrass as a forage or biomass crop can be enhanced if yield can be increased with a concomitant decrease in chemical inputs, particularly N and P fertilizers [7]. One means of accomplishing this is through utilization of symbiotic relationships wherein a microbial partner provides or enhances nutrient acquisition for its plant host. Arbuscular mycorrhizal fungi (AMF) are ubiquitous in terrestrial plant communities, forming symbiotic associations with the roots of the large majority of plant species [15, 32]. In these relationships, AMF often provide enhanced uptake of nutrients, abiotic stress tolerances, and even reduced pathogenic infection in exchange for photosynthetically derived carbon from its host plant [1, 10, 16, 22]. In fact, AMF have also been shown to increase dry matter production and uptake of nutrients in switchgrass grown in acidic soils and P-limited environments, reflecting the common observation that these associations are most beneficial for the host under stressful environmental conditions [8, 9]. Despite these obvious benefits, the application of AMF in switchgrass at the commercial level is limited as these fungi can be grown only in the presence of their hosts [12, 30].

Members of the family *Sebacinaceae* (class: *Basidiomycete*; order: *Sebacinales*) have a broad geographical distribution and are known to form various types of mycorrhizal associations with a wide range of plant species [28, 31, 41]. Several recent studies have shown that one of the *Sebacinales* in particular, *Piriformospora indica*, is capable of colonizing the roots of numerous mono- and dicotyledonous plants [26, 35]. Further, these associations are apparently stable, and in most instances, an enhancement of growth and protection against various biotic and abiotic stresses have been documented [11, 29, 34, 39]. As plant enhancement applications involving *P. indica* have been patented [33], its application in commercial production of switchgrass may not be feasible or economical. However, isolates of a closely related genus to *P. indica*, *Sebacina vermifera*, form orchidaceous mycorrhizal associations with several Australian terrestrial orchids of the genera *Cladenia*, *Glossodia*, and *Macrotis*. Intriguingly, this fungus has also been used to form artificial ectomycorrhizal associations with the roots of non-orchid plants [40], and several strains provide comparable fitness enhancements to *P. indica* when inoculated onto barley (*Hordeum vulgare* L.) roots, including both biomass gains and systemic resistance to powdery mildew [11]. Further, *S. vermifera* colonization stimulated plant growth, seed germination, and production in *Nicotiana attenuata* [3]. Of critical importance, and in contrast to AMF, *S. vermifera* can be easily cultured in the laboratory.

This study explores the possibility of establishing a novel association between switchgrass and the ectomycorrhizal fungus, *S. vermifera*. The specific objectives of this study were: (1) to assess compatibility between *S. vermifera* and promising switchgrass cultivars, (2) to evaluate the effect of *S. vermifera* infection on biomass production of switchgrass, and (3) to examine the effect of culture filtrate from *S. vermifera* on seed germination of two switchgrass cultivars. We explore the potential application of this novel association in the commercial production of switchgrass grown as a bioenergy crop.

Methods

Plant and Fungal Materials

A synthetic switchgrass cultivar NF/GA-993 (representative of lowland ecotypes) was used for compatibility assessments and biomass production studies. Switchgrass seeds were received from the Forage Improvement Division of the Samuel Roberts Noble Foundation, Ardmore, OK. Six *S. vermifera* strains used in this study (MAFF-305828, MAFF-305830, MAFF-305835, MAFF-305837, MAFF-305838, and MAFF-305842) were obtained from the National Institute of Agro-biological Sciences, Tsukuba, Japan.

Seedlings and Fungal Inoculum Production

Switchgrass seeds were acid scarified by submerging the seeds in 8 M sulfuric acid for 5 min followed by rinsing under running water for 5 min [42]. Seeds were surface sterilized using a previously described method [23] except that the sterilization period was extended to 30 min. Seeds were rinsed several times with sterile distilled water for 20 min. Seeds were dried overnight and plated on 2.5% water agar in 90 mm Petri dishes. Plates were incubated at 24°C in the dark for 4 days to induce germination then transferred to a light chamber.

All *S. vermifera* isolates were grown in MYP broth (7 g L^{-1} malt extract, 1 g L^{-1} peptone, and 0.5 g L^{-1} yeast extract) at room temperature. The fungal mycelia were harvested from 3-week-old cultures, rinsed three times with sterile water, and homogenized in an aqueous solution of 0.05% Tween-20 [11].

Plant Inoculation and Maintenance

Ten-day-old switchgrass seedlings were inoculated with *S. vermifera* strains by immersing roots in the homogenized fungal mycelial suspension for 3 h. Seedlings for the control treatment were immersed into aqueous solution of 0.05% Tween-20 for the same length of time. Three-day-old seedlings of barley cultivar Golden Promise were inoculated with *S. vermifera* strains to examine the effectiveness of the inoculation technique. Each test strain was inoculated onto 288 seedlings, with an equal number for water-treated controls. Inoculated seedlings were transplanted in a 2:1 mixture of turface (Profile Products, Buffalo Grove, IL,

USA) and bentonite clay conditioner (BWI, Texarkana, TX, USA) in 96-cell plastic trays for 2 months. Two-month-old seedlings were subjected to a subsequent inoculation with their respective strain. Inoculated seedlings were transplanted into 1.5 L plastic pots containing five seedlings per pot.

Experimental trays and pots were arranged in a randomized block design and maintained in the glasshouse. Plants in trays were watered regularly with tap water and fertilized with a solution containing 12:4:6 (N/P/K) at 2-week intervals. Plants in pots were watered with tap water and fertilized with Peters' professional 20:10:20 (N/P/K) general purpose water soluble fertilizer (The Scott Company, Marysville, OH, USA) at 4 week intervals.

The temperature in the glasshouse varied from 22.5°C to 29.7°C during the experimental period, and relative humidity ranged from 39% to 74%. Plants were grown in a 16:8 h light/dark cycle.

Microscopic Examination

Two-month-old inoculated seedlings were uprooted, rinsed several times with tap water, and blot dried. Roots were cut into small pieces and stained with the chitin specific green fluorescent dye WGA-AF 488 (Invitrogen, Carlsbad, CA, USA). Root segments were incubated at room temperature for 20 min in phosphate buffer saline (PBS) containing dye at 10 μg mL^{-1} and then rinsed with 1× PBS (pH 7.4). For the microscopic examination, root segments were washed with PBS and placed on a glass slide in PBS and mounted using a cover glass. Fluorescence microscopy was performed using a BioRad MRC 1024 ES Confocal Laser Scanning Microscope (BioRad, Hercules, CA, USA). Optical sections were acquired by scanning multiple sections, and the z-series projections were generated with the software provided with the BioRad MRC 1024 ES CLSM.

Observation, Data Collection, and Statistical Analysis

Microscopic observations were carried out on 2-month-old seedlings (48 from each interaction and the water inoculated control) to estimate the association frequency. Barley roots were also examined to determine the effectiveness of the inoculation technique. Data on biomass (shoot and root) and biomass-related parameters (plant height, number of tillers, and root length) were collected three times (2, 3.5, and 7 months). Root biomass data were collected at first and third harvests, and plant nutrient uptake (nitrogen and phosphorus) was determined at second and third harvests. Data for biomass and biomass-related parameters at 2 months were collected from at least 24 individual plants per interaction, and subsequent data on biomass and biomass-related parameters were collected from 90 plants. Data were analyzed within the framework of a general linear model using PROC GLM of the SAS statistical software package version 9.1 [27]. Least significant difference (LSD) tests were performed to compare the treatment effect on test parameters.

Seed Germination Study

Acid scarified and surface-sterilized seeds of two switchgrass cultivars, Alamo and Kanlow, both representing lowland ecotypes, were used in this study. Culture filtrates from six strains of *S. vermifera* were collected from 3-week-old cultures in MYP broth and filter sterilized using 0.22 μM Steriflip Millipore filters (Millipore Corporation, Billerica, Massachusetts, USA). Two control treatments, MYP broth alone and sterile water, were included in the study. Water agar was amended with filter-sterilized culture filtrate, MYP, or water at 50°C in a ratio of 4:1 (*v*/*v*) and a final water agar concentration of 2.5% and poured into 90 mm Petri plates. Each treatment had 600 seeds (six plates, 100 seeds per plate). Seeds were incubated at 24°C in the dark throughout the study period. A total of 4,800 seeds of each genotype were used in the study.

Seed germination was counted at 4 days after plating, and subsequent counts were made at weekly intervals for a 3-week period. Seeds producing both a radicle and a plumule were considered germinated. Germinated seeds were removed from the plate after each recording. Final germination percentage (FGP) was calculated for each replication by summing total seeds germinated. Data on seed germination were analyzed within the framework of a general linear model using PROC GLM of the SAS statistical software package version 9.1 [27]. Least significant difference tests were performed to compare the treatment effect on seed germination.

Results

Association of *S. vermifera* on Switchgrass Roots

All six strains of *S. vermifera* had a high frequency of association with roots of the synthetic cultivar NF/GA-993 when examined 2 months after inoculation. The association frequency of each strain ranged from 94% to 100%. Confocal microscopy images depicting fungal colonization of roots of switchgrass and barley (positive control) are presented in Fig. 1.

Shoot Length, Root Length, and Biomass Production at First Harvest

Treatment effects were observed in root and shoot lengths, and treatments differed significantly ($p<0.05$) for both parameters (Table 1). The mean shoot length ranged from 30 to 40 cm. The longest shoots were produced by the plants inoculated with strain MAFF-305828, and the shortest shoots

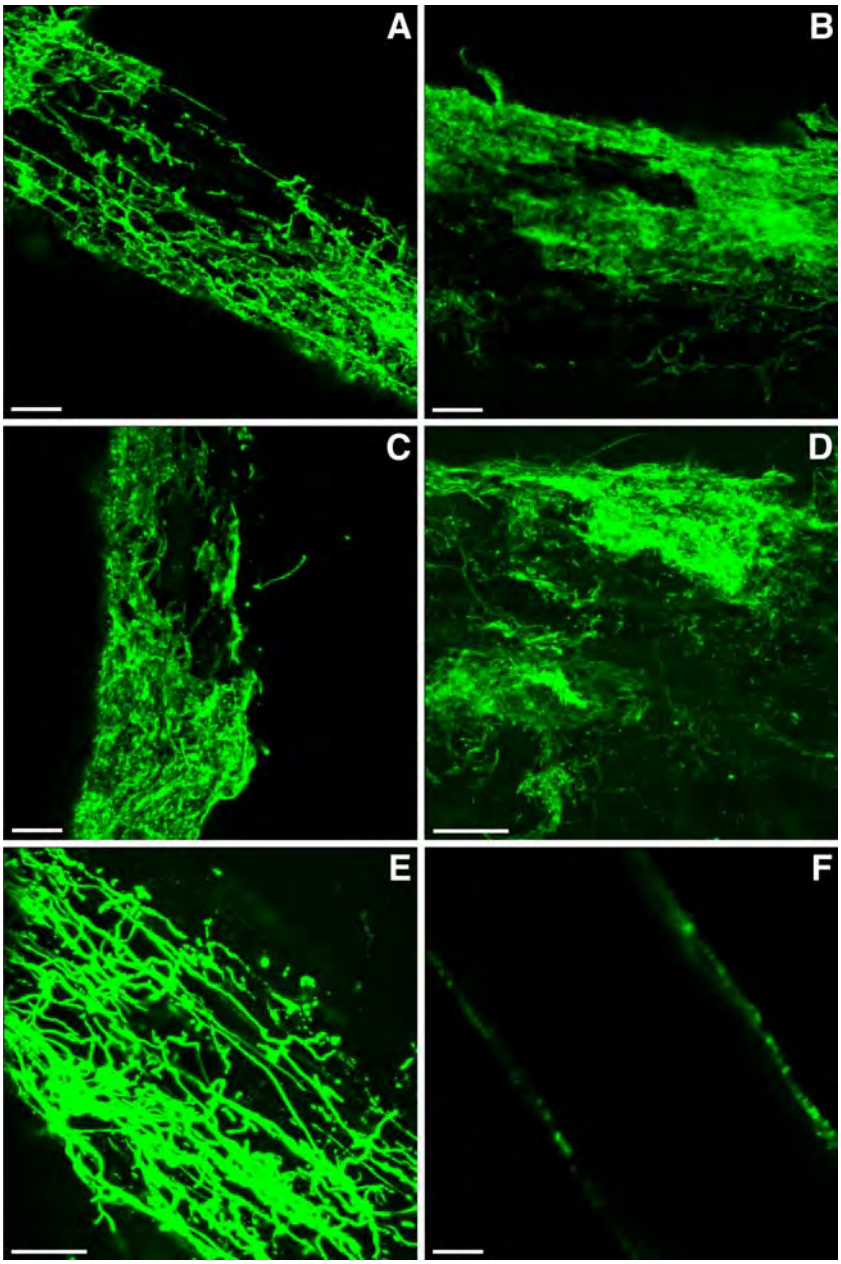

Fig. 1 Colonization of switchgrass and barley roots by *S. vermifera*. Switchgrass root colonization by strains MAFF-305828, MAFF-305830, MAFF-305835, and MAFF-305838 (**a**–**d**, respectively); MAFF-305830 colonized barley root (**e**) and uninoculated control switchgrass root (**f**). Root staining was performed using WGA-AF 488, and scale bar is 50 μm

Table 1 Effect of *S. vermifera* colonization on shoot length, shoot biomass, root length, and root biomass in switchgrass synthetic cultivar NF/GA-993 at first harvest

Treatments	Shoot length (cm)	Shoot dry wt. (mg plant^{-1})	Root length (cm)	Root dry wt. (mg plant^{-1})
MAFF-305828	40.27 a	312 a	38.44 ab	138 a
MAFF-305830	36.78 b	208 bc	40.74 a	112 ab
MAFF-305835	31.83 c	156 e	34.47 bc	59 c
MAFF-305837	38.30 ab	186 cd	35.51 bc	86 bc
MAFF-305838	35.82 b	231 b	36.24 bc	81 bc
MAFF-305842	35.71 b	228 b	32.99 c	110 ab
Control	30.21 c	178 de	35.68 bc	87 bc
LSD $_{(0.05)}$	2.60	28	4.40	34

Values with different letters are statistically significant with 95% confidence

were from the control plants. Similarly, the root length ranged from 33 to 41 cm, where the longest roots were from the plants inoculated with strain MAFF-305830 and the shortest from the plants inoculated with MAFF-305842.

Differences in shoot dry weight were observed among the treatments ($p<0.05$) and ranged from 156 to 312 mg plant^{-1}. The highest shoot dry biomass was recorded from the plants inoculated with MAFF-305828, which was 75% greater than control plants. Increases in the dry shoot biomass were evident in four of the six inoculated treatments (Table 1). Similarly, a significant treatment effect was observed in root biomass production. The highest dry root biomass was obtained from the plants inoculated with MAFF-305828, a 59% increase over the control treatment (Table 1).

Shoot Length and Biomass Production at Second Harvest

Treatments differed significantly for shoot length. The tallest plants were recorded from MAFF-305838 (61 cm) and the shortest from the control (44 cm). All inoculated plants performed significantly better than the control ($p<0.05$), and the difference among strains for shoot length was less than 4 cm (Table 2). There was a 29% to 38% increase in shoot length over control at second harvest.

Similarly, treatments differed significantly for biomass production ($p<0.05$). Biomass production ranged from 1.75 to 3.74 g pot^{-1}. Strain MAFF-305838 produced the highest shoot biomass and was followed by strains MAFF-305837 and MAFF-305842 and the least by the control (Table 2). Inoculation with *S. vermifera* strains increased biomass production by 77% to 113% over the control at second harvest.

Tiller numbers, Plant Height, Root Length, and Biomass Production at Third Harvest

Treatment effects were observed on tiller production and shoot and root biomass ($p<0.05$; Table 3). Mean tiller production was the highest from the plants inoculated with strain MAFF-305835 (6.72) and the least from the control plants (5.19). Shoot biomass production ranged from 10.04 to 11.86 g pot^{-1}. Strains MAFF-305838, MAFF-305835, and MAFF-305837 produced significantly higher shoot biomass than control ($p<0.05$). These three strains were also the highest root biomass producers (Table 3). Root biomass production ranged from 13.45 to 16.63 g pot^{-1}, and the lowest biomass production was from the control. Roots had formed a thick mat at the base of pot, making accurate root length measurements difficult at this harvest.

Nutrient Uptake

Colonization by *S. vermifera* strains increased N and P content in shoots by 39% to 64% and 44% to 98%, respectively, at second harvest, and the differences among treatments were significant ($p<0.05$; Table 2). Colonized plants had 2% to 31% higher P content at third harvest. However, N content was not significantly different among the treatments in the third harvest (Table 3).

Seed Germination

Culture filtrates from *S. vermifera* had variable effects on seed germination of two switchgrass cultivars. Culture filtrates from three strains (MAFF-305828, MAFF-305830, and MAFF-305842) significantly increased FGP over water control in cultivar Kanlow. The best-performing treatment increased FGP by 41% to 52% compared to MYP broth control and water control, respectively (Fig. 2). The FGP among treatments ranged from 63% to 70% in Alamo, but the treatment effect was not significant.

Discussion

For switchgrass to play a major role in the production of biomass for cellulosic ethanol production, a major allocation

Table 2 Effect of *S. vermifera* colonization on plant height, shoot biomass and nutrients uptake in switchgrass synthetic cultivar NF/GA-993 at second harvest

Treatments	Plant height (cm)	Shoot dry wt. (g pot^{-1})	N uptake (mg pot^{-1})	P uptake (mg pot^{-1})
MAFF-305828	56.99 b	3.314 cd	24.11 ab	7.29 a
MAFF-305830	56.82 b	3.355 bcd	22.81 ab	5.70 b
MAFF-305835	58.49 ab	3.111 d	21.90 b	5.29 b
MAFF-305837	58.26 ab	3.615 ab	24.29 ab	5.78 b
MAFF-305838	60.52 a	3.741 a	25.74 a	6.17 ab
MAFF-305842	59.85 ab	3.550 abc	25.56 a	5.86 b
Control	43.90 c	1.754 e	15.72 c	3.77 c
LSD $_{(0.05)}$	2.94	0.248	3.21	1.30

Values with different letters are statistically significant with 95% confidence

Table 3 Effect of *S. vermifera* colonization on plant height, shoot biomass and nutrients uptake in switchgrass synthetic cultivar NF/GA-993 at third harvest

Treatments	Plant height (cm)	No. of tillers	Shoot dry wt. (g pot^{-1})	Root dry wt. (g pot^{-1})	N uptake (mg pot^{-1})	P uptake (mg pot^{-1})
MAFF-305828	84.54 a	5.30 c	10.28 bc	15.28 abc	58.12 a	9.25 b
MAFF-305830	82.58 a	5.58 bc	10.76 abc	14.88 abc	64.27 a	10.04 ab
MAFF-305835	84.44 a	6.72 a	11.78 ab	15.76 ab	61.57 a	10.60 ab
MAFF-305837	83.97 a	6.21 ab	11.45 ab	16.63 a	65.34 a	11.07 a
MAFF-305838	85.22 a	6.16 ab	11.86 a	15.85 ab	63.89 a	11.46 a
MAFF-305842	82.50 a	5.66 bc	10.71 abc	14.06 bc	58.83 a	10.71 ab
Control	86.28 a	5.19 c	10.04 c	13.45 c	61.04 a	9.04 b
LSD $_{(0.05)}$	5.39	0.69	1.34	2.11	15.26	1.81

Values with different letters are statistically significant with 95% confidence

of land must be dedicated to its cultivation. Studies have shown that the smallest viable ethanol plant would require about 630,000 Mg switchgrass per year, accounting for 70,000 ha with an average yield of 9 Mg ha^{-1} [13]. Much of the great appeal of this native grass is derived from the fact that it can be grown in marginal soils, thus minimizing the use of arable cropland and helping to stabilize and replenish these depleted areas. Importantly, it is precisely in these deficient soils where the utilization of beneficial microbes can potentially have the greatest impact. Our approach was to determine whether we could enhance further the biomass and inherent nutrient-use efficiency of switchgrass and simultaneously address some current limitations by incorporating a group of fungi known to have beneficial effects on their hosts. To our knowledge, this study is the first to document such effects from a novel association created between switchgrass and the ectomycorrhizal fungus *S. vermifera*.

Plants colonized by *S. vermifera* exhibited an increase in shoot and root length and enhanced shoot biomass production up to 113% over uninoculated controls (Table 2). These results are particularly significant as the increase in shoot biomass came at no observable loss in root biomass, suggesting the aboveground gains are not simply a consequence of reallocated carbohydrate nutrition. From an ecological perspective, this translates into retention of the soil-binding capacity of the switchgrass root system, a priority for efforts to prevent soil erosion. These growth-promoting effects were obvious despite fairly limited colonization of switchgrass roots by *S. vermifera*, and this differed significantly than growth on barley (Fig. 1). On switchgrass, the fungus typically forms a thick mat on the

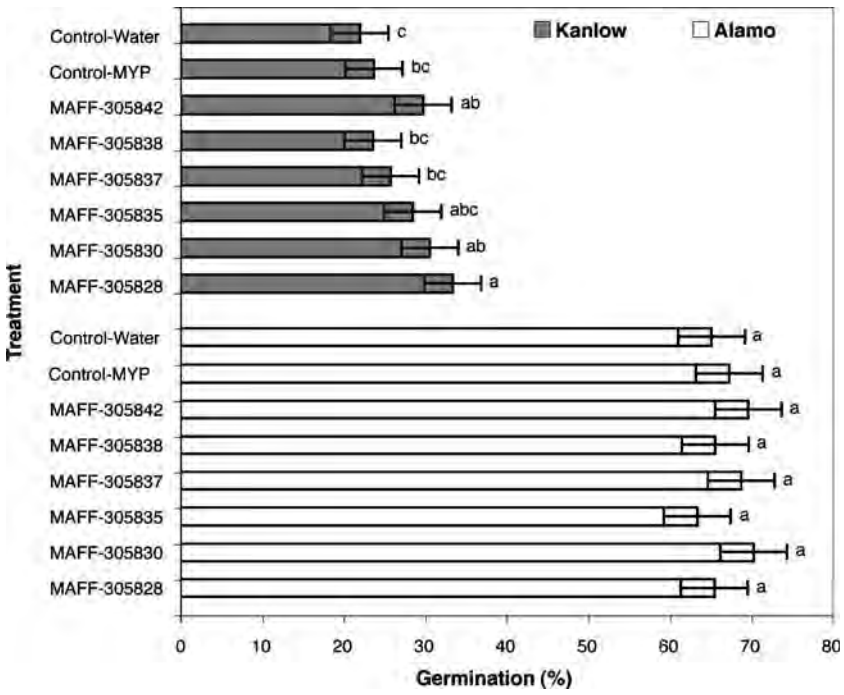

Fig. 2 Effect of culture filtrate from *S. vermifera* strains on FGP of switchgrass cultivars Kanlow and Alamo. *Error bars* are LSD, and the *bars labeled with different letters* are significant with 95% confidence

roots that is fairly localized to the site of inoculation, whereas growth on barley roots is sparser but more widespread (Fig. 1e). The root dip method of inoculation used here is labor intensive, and transplantation of switchgrass is usually not practiced in commercial production. Due to these limitations and because *S. vermifera* can easily be propagated on a large scale in axenic culture in the absence of a host plant (unlike AMF), we are currently investigating alternative inoculation techniques, including the feasibility of seed coating.

As with many warm-season perennial grasses, switchgrass can be difficult or slow to establish, particularly in unmanaged stands [2, 14, 17, 20]. There are several factors affecting the productivity of a stand in the field [20], and some of those are inherent to the species, such as seed dormancy [24]. Switchgrass seeds can be highly dormant [18], and the cultivars may vary in the degree of dormancy [21]. As a consequence, only a fraction of sown switchgrass seed will germinate at a given time, resulting in spotty stands prone to invasion by competing weedy species or other native plants [14, 19, 20]. This problem may be particularly significant in large-scale plantings on minimally or unmanaged lands, where removal of competing weeds is not an economically viable approach. Our results suggest that culture filtrate from the fungus stimulates greater seed germination in one of the two switchgrass genotypes tested, and we are currently using a proteomics approach to identify these putative compound(s).

Earlier work in other labs showed that inoculation of *N. attenuata* seeds with *P. indica* and *S. vermifera* stimulated seed germination and increased growth and stalk elongation. Plants inoculated with *S. vermifera* flowered earlier, produced more flowers, and had greater maturation of seed capsules than did uninoculated plants [3]. Although the underlying mechanisms or genes responsible for the beneficial effects of this relationship are largely unknown, some evidence suggests that *S. vermifera* promotes the growth and fitness of *N. attenuata* by inhibiting ethylene signaling [4]. Further, enhanced seed germination observed in the presence of the fungus could be mimicked by oxidizing ethylene with a $KMnO_4$ ethylene scrubber. Potential roles of phytohormone regulation and gene expression data for putative effectors in mediating this beneficial interaction are being explored.

Finally, we wish to note that, while switchgrass is native to North America, the strains of *S. vermifera* used in this study were originally isolated from the roots of Australian orchids. To the best of our knowledge, these two organisms do not have overlapping habitats or geographical ranges. Despite any known natural symbioses between these two organisms, the associations we created were mutually beneficial. While *S. vermifera* has not been reported as a pathogen on any plant species thus far, the possibility exists that the fungus could spread from infected switchgrass plants to colonize nearby plants, potentially resulting in enhanced growth and fitness of weeds and/or introduced species. Nonetheless, we fully acknowledge the regulatory concerns surrounding release of a non-native microbe, and given the successful results obtained herein, we have initiated a search for native species belonging to the *Sebacinales* and other mutualistic microbes in the native tall grass prairie of Oklahoma and surrounding regions.

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