

1 The Ectomycorrhizal Fungus *Sebacina vermifera*, Enhances Biomass Production of
2 Switchgrass (*Panicum virgatum* L.) Under Drought Conditions

3

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7

8 **Abstract**

9 Experiments were conducted to examine the effects of cocultivating the important
10 bioenergy crop switchgrass with the ectomycorrhizal fungus, *Sebacina vermifera* under
11 severe drought conditions. Plants cocultivated with the fungus produced significantly
12 higher biomass and had a higher macronutrient content than uninoculated control plants
13 under both well watered and drought conditions.

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15 **Running title:**

16 Symbiosis offsets drought related biomass losses

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19 Drought is a predominant factor limiting plant growth and yield in both dry land
20 and irrigated agriculture (6, 27). Lack of soil water has a wide range of effects on
21 morphological and biochemical processes in plants, including nutrient uptake from the
22 soil, negatively impacting crop productivity (2). Unfortunately, the influence of drought
23 on agriculture is expected to worsen in the future due to climate change (8) and
24 increasing demands for water for municipal and residential consumptions (9). Thus,
25 various strategies are being developed to maximize water use efficiency and minimize the
26 effects of drought on agriculture (3, 12, 14, 18, 23, 26, 29). However, the utilization of
27 naturally occurring symbiotic microbes to enhance drought tolerance of agricultural crops
28 has remained largely unexplored.

29 Most plant species in natural ecosystems are symbiotic with mycorrhizal and /or
30 endophytic fungi (21). Members of the newly defined basidiomycete order Sebaciales
31 naturally form a wide spectrum of mycorrhizal types (31) with the roots of various mono-
32 and dicotyledonous plants (4, 11, 15, 28, 30). Two species in particular, *Sebacina*
33 *vermifera* [= *Serendipita vermifera* (Oberw.) P. Roberts, comb. nov]. and its close relative
34 *Piriformospora indica*, have stimulated considerable attention over the past several years
35 because they form endophytic and mycorrhiza-like associations with most plant species
36 studied to date (30, 32). This is of great interest because both species are axenically
37 cultivable, possess plant growth-promoting characteristics and contribute several other
38 benefits to their host plants (4, 11, 15, 28, 30). Two previous studies have shown that
39 colonization of roots by *P. indica* confers drought tolerance in *Arabidopsis thaliana* and
40 Chinese cabbage (24, 25). However, no similar studies have been performed to evaluate
41 the potential of *S. vermifera* to impart drought tolerance to host plants. Our objective here

42 was to investigate the effect of *S. vermifera* in mitigating biomass losses in switchgrass
43 due to drought, with the ultimate goal of maximizing the utility of this important
44 bioenergy crop and the range of lands upon which it can be grown.

45 *In-vitro* study was performed in 175 ml plant containers (dia. 65 mm x ht. 65 mm)
46 with lids, filled with 25 ml of modified PNM culture medium (24), and overlaid with a
47 nylon disk (mesh size 50 μm). Two strains of *S. vermifera*, MAFF-305828 and MAFF-
48 305830 were used in the study. One 5 mm plug of fungal hyphae from an actively
49 growing colony on malt extract agar (MEA) was placed at the center of the nylon disk
50 and allowed to grow for two weeks, and a plug of similar size from an uninoculated MEA
51 plate was used as a control. Subsequently, four germinated Alamo seeds with no visible
52 contamination of fungi and bacteria were placed on each nylon disk at an equidistance of
53 15 mm from the plug. Containers were incubated at 24°C with a 16/8 h light/dark cycle
54 and light illumination of 165 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for six weeks. The lids were then replaced
55 with air-pore filters to allow evaporative and transpiration water loss at ambient
56 temperature. On the 7th day of drought exposure leaf color, plant stature and total fresh
57 weight were recorded. The fibrous roots were stained (16) and examined under a
58 microscope for fungal colonization. Each treatment consisted of 10 to 13 experimental
59 units (containers) and the experiment was performed twice.

60 In a subsequent greenhouse experiment, six week-old rooted explants of
61 switchgrass genotype VS16 were grown in 3.8 L pots filled with 4:1 (v/v) mixture of
62 sterile Metromix-350 (Scotts-Sierra Horticultural Products, Marysville, Ohio) and *S.*
63 *vermifera* (strain MAFF-305828) colonized sorghum grains (Fig. 1). Control seedlings
64 were grown in a mixture prepared with sterile sorghum grains. Plants were maintained at

65 24°C/18°C, 16/8 h light/dark cycle with light illumination of $165 \mu \text{ mol m}^{-2} \text{ s}^{-1}$ for five
66 weeks. Individual seedlings from both treatments were subsequently planted in 1L pots
67 filled with a 3:1 (v/v) Metromix-350 and sand mixture, and grown in the greenhouse with
68 an average temperature and average relative humidity of 20.2 °C and 39.3%, respectively.

69 All plants were watered to saturation at planting. Thereafter, half of the plants from
70 cocultivation and control treatments were subjected to drought whereas the other half was
71 watered normally. Experimental pots were checked individually for soil volumetric water
72 content (VWC) on alternate days using the Field Scout® TDR 100 (Spectrum
73 Technologies, Plainfield, Illinois). Plants receiving drought treatments were allowed to
74 grow until the VWC dropped to $\leq 1\%$, at which point a subset of droughted and non-
75 droughted plants were harvested. Plants with normal watering were maintained at a
76 constant 10-20% VWC, but were harvested at the same time points as droughted plants.
77 These water levels were derived empirically in a previous experiment where switchgrass
78 seedlings at 10-20% VWC grew normally, seedlings at 5% VWC showed initial wilt
79 symptoms and plants wilted permanently when grown for 2 days at $\leq 1\%$ VWC.

80 Experimental plants were arranged in a factorial randomized block design. Each
81 treatment had 48 individually potted plants. One third of the experimental plants were
82 harvested after the first drought stress (i.e. when the average VWC in the corresponding
83 drought treatment pots reached 1%). The remaining plants were subjected to a second
84 drought stress and a half of these were subjected to a subsequent third drought stress. All
85 experimental plants were re-watered to saturation prior to subsequent drought stresses.
86 Data on tiller number, shoot length, root length, shoot dry matter (DM) and root DM
87 were recorded in each harvest. Leaf chlorophyll content was measured using the

88 chlorophyll meter SPAD-502 Plus (Konica Minolta Sensing Americas Inc., Ramsey,
89 New Jersey). The shoot and root tissues were analyzed for nitrogen (N), phosphorous
90 (P), potassium (K) calcium (Ca), magnesium (Mg) and sulfur (S) content at Ward
91 Laboratories, Inc. (Kearney, Nebraska). Data on biomass and biomass related parameters
92 were analyzed using PROC GLM of SAS statistical software package version 9.1.3. (22)
93 and LSD tests were performed to compare treatments at $\geq 95\%$ confidence levels.

94 Roots of cocultivated plants from *in-vitro* study were effectively colonized by *S.*
95 *vermifera* (Fig. 2). After a week-long exposure to ambient temperature, the appearance of
96 cocultivated plants was barely affected whereas control seedlings were pale green and
97 withered (Fig. 3). Plants cocultivated with MAFF-305828 and MAFF-305830 produced
98 71% and 53% higher fresh biomass, respectively, than control plants (Table 1; $P < 0.01$).
99 The absence of fungal mediated water and nutrient uptake might be the reason for poor
100 performance of control seedlings. Similar effects on drought tolerance of *Arabidopsis*
101 *thaliana* seedlings inoculated with *P. indica* were observed previously, with up to a 300%
102 increase in fresh biomass production (24).

103 In the greenhouse study, treatments differed significantly for shoot length, shoot
104 DM, root DM and the shoot to root DM ratio (Table 2). Shoot length increased by 109%,
105 59% and 95%, and shoot biomass increased by 337%, 215% and 267%, respectively at
106 first, second and third harvests in response to inoculation under well-watered conditions.
107 Intriguingly, cocultivated plants exposed to drought produced significantly taller plants
108 with higher shoot DM than well-watered control plants. Under well-watered conditions,
109 cocultivated plants consistently produced higher root biomass (290%, 270% and 166%
110 respectively, at first, second and third harvest) than the controls. Further, when subjected

111 to one, two or three drought cycles, cocultivated plants produced 303%, 127% and 112%
112 higher root biomass, respectively, than control. Further, as was evident for shoot tissues,
113 cocultivated plants exposed to one, two or three drought stresses produced respectively,
114 353%, 131% and 148% higher root biomass than corresponding well-watered controls
115 (Table 2; $P \leq 0.01$). Thus, plants colonized by *S. vermifera* exhibited a simultaneous
116 increase in both shoot and root biomass, indicating that above ground biomass gains are
117 not simply a consequence of reallocated carbohydrate. Indeed, cocultivated plants
118 consistently produced higher root biomass than control plants suggesting a greater
119 potential to sequester carbon and hold soils, both highly desired properties in a crop
120 grown under a low-input regime.

121 Except for the second harvest cocultivated plants had significantly higher shoot-to-
122 root DM ratio than corresponding control plants (Table 2). In all treatments, the shoot-to-
123 root DM ratio was highest at the first harvest and declined in the subsequent harvests
124 falling between 66 to 77% from the first to the third harvest. These results may suggest
125 that nutrient availability determines how plants allocate their resources to root or shoot
126 tissues. As experimental plants were grown in pots without supplemental nutrition, plants
127 at the first harvest may have had access to relatively higher nutrient conditions than those
128 of second and third harvests. Accordingly, development prior to subsequent harvests may
129 have shifted in favor of root growth (thereby reducing shoot-to-root DM) to maximize
130 nutrient acquisition potential. These observations are consistent with other studies that
131 reported an increased biomass allocation to roots under low nutrient conditions (7, 17).

132 Cocultivated plants had lower concentrations of several macronutrients in shoot and
133 root tissues compared to control plants (Tables S1 and S2; $P \leq 0.05$). However, the total

134 acquisition of all macronutrients (except Ca in root) was significantly higher in
135 cocultivated plants compared to controls, likely reflective of their larger stature. As the
136 plants were grown on the same amount of soil substrate, larger plants would have
137 depleted this resource faster than smaller plants. Moreover, most plant species, especially
138 perennials, effectively allocate resources to transport, growth, defense and reproduction
139 (10), with the remainder being committed to storage. The lower concentrations of these
140 nutrients in *Sebacina*-infected plants observed in this study may reflect a depletion of
141 cellular stores to fuel the demands for growth and maintenance in these substantially
142 larger plants (19). Some of these macronutrients (e.g. N and Mg) are critical constituents
143 of chlorophyll and their lower concentrations in cocultivated plants might have affected
144 the observed decline in leaf chlorophyll content (13).

145 This study confirms that cocultivation imparts extraordinary biomass gains to
146 switchgrass, so much so that the yield of such plants grown under our defined drought
147 stress conditions significantly exceeds that of control plants grown under normal or
148 restricted water levels. The cocultivated plants consistently produced higher root biomass
149 than control plants suggesting a greater potential to sequester carbon and hold soils, both
150 highly desired properties in a crop grown under a low-input regime. As with many warm-
151 season perennial grasses, switchgrass can be difficult or slow to establish (1, 20) and this
152 is a major impediment to its implementation as a primary bioenergy crop (5). Therefore,
153 both the shoot and root growth promoting effects of *S. vermifera*, particularly early on in
154 plant development, are likely to improve competitiveness of switchgrass seedlings
155 especially during the establishment process.

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272 **FIG. 1.** Sorghum grains (a) colonized by *Sebacina vermifera* and, (b) uninoculated
273 control.

274

275 **FIG. 2.** Switchgrass root (a) uninoculated control (b) roots of cocultivated switchgrass
276 seedling with Aniline blue stained mycelium on the root surface (black arrows) and, (c)
277 *Sebacina vermifera* chlamydo spores in the epidermal cells (red arrows).

278

279 **FIG. 3.** Switchgrass seedlings after exposure to the mild drought stress. Cocultivated
280 seedlings with *Sebacina vermifera* strain MAFF 305830 (left), with *S. vermifera* strain
281 MAFF 305828 (middle) and controls (right).

282

283 **List of Tables**

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285 **TABLE 1.** Effect of *Sebacina vermifera* and switchgrass cocultivation on switchgrass
286 biomass production under drought stress in *in-vitro* conditions.

287

288 **TABLE 2.** Effect of *Sebacina vermifera* on switchgrass biomass production under well-
289 watered and drought conditions in greenhouse.

290

291 **TABLE S1.** Macronutrients concentration and total content in shoot tissues of
292 switchgrass seedlings grown under different treatments in greenhouse conditions.

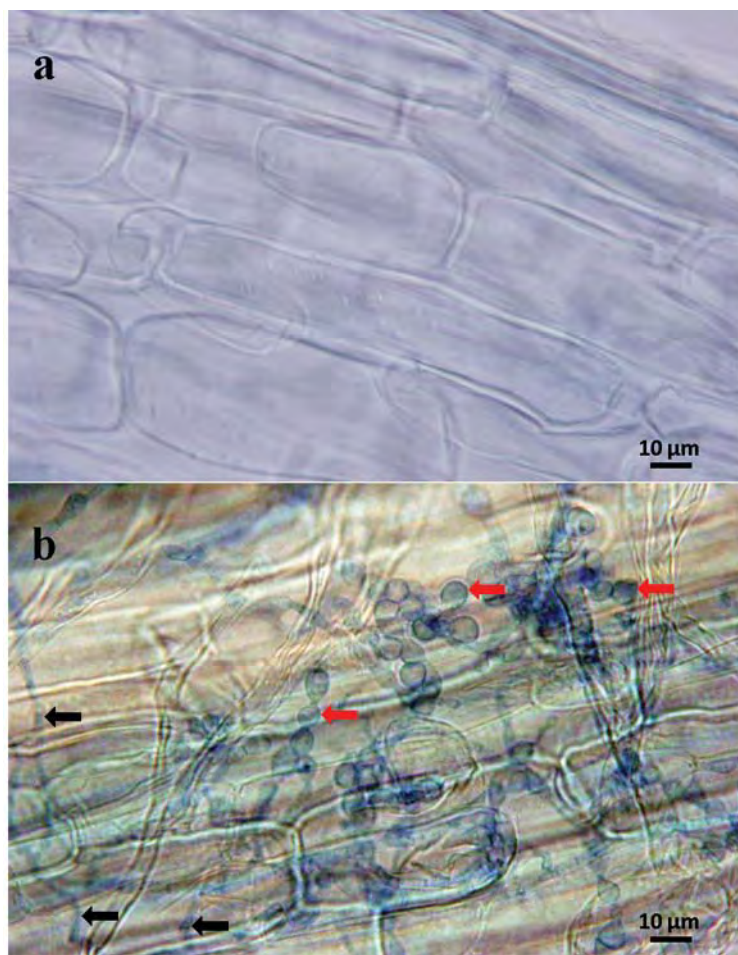
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294 **TABLE S2.** Macronutrients concentration and total content in root tissues of switchgrass
295 seedlings grown under different treatments in greenhouse conditions.

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1 **TABLE 1.** Effect of *Sebacina vermifera* and switchgrass cocultivation on switchgrass biomass
 2 production under drought stress in *in-vitro* conditions (means \pm LSD)
 3

Treatments	Seedlings per container at		Fresh weight (mg/container)
	Planting	Drought exposure	
Experiment 1			
Cocultivation			
Strain MAFF 305828	4	3.46 \pm 0.26 ^a	148.62 \pm 13.45 ^a
Strain MAFF 305830	4	3.69 \pm 0.26 ^a	143.08 \pm 13.45 ^a
Control	4	3.54 \pm 0.26 ^a	97.46 \pm 13.45 ^b
Significance tests			
Treatment	-	n.s.	**
Experiment 2			
Cocultivation			
Strain MAFF 305828	4	3.80 \pm 0.20 ^a	122.59 \pm 12.20 ^a
Strain MAFF 305830	4	3.60 \pm 0.20 ^a	96.48 \pm 12.20 ^b
Control	4	3.10 \pm 0.20 ^b	58.11 \pm 12.20 ^c
Significance tests			
Treatment	-	**	**
Experiment 1 and 2			
Cocultivation			
Strain MAFF 305828	4	3.61 \pm 0.17 ^a	137.30 \pm 10.65 ^a
Strain MAFF 305830	4	3.65 \pm 0.17 ^a	122.82 \pm 10.65 ^a
Control	4	3.35 \pm 0.17 ^a	80.35 \pm 10.65 ^b
Significance tests			
Treatment	-	ns	**

4
 5 ** $P \leq 0.01$; n.s. = not significant; values within the column with different letters for a given experiment are
 6 significantly different at $\geq 99\%$ confidence level.
 7
 8

1 **TABLE 2.** Effect of *Sebacina vermifera* on switchgrass biomass production under well-watered
 2 and drought conditions in greenhouse (means \pm SE)

Treatments	Shoot length (cm)	Root length (cm)	Shoot DM (mg/plant)	Root DM (mg/plant)	Shoot to root ratio	No. of tillers	Chlorophyll content (%)
First harvest (one drought cycle)							
Cocultivation							
Watered	70.5 \pm 1.1	24.1 \pm 0.4	752 \pm 29	316 \pm 16	2.48 \pm 0.06	2.38 \pm 0.06	31.0 \pm 0.3
Dry	56.9 \pm 1.0	30.5 \pm 0.9	500 \pm 23	367 \pm 16	1.40 \pm 0.03	2.06 \pm 0.03	30.9 \pm 0.3
Control							
Watered	33.8 \pm 0.6	20.7 \pm 0.5	172 \pm 9	81 \pm 4	2.16 \pm 0.07	2.38 \pm 0.06	29.7 \pm 0.3
Dry	28.5 \pm 0.7	20.3 \pm 0.4	105 \pm 6	91 \pm 5	1.23 \pm 0.07	1.88 \pm 0.10	31.7 \pm 0.3
Significance tests							
Cocultivation	**	**	**	**	*	n.s.	n.s.
Drought	**	*	**	n.s.	**	**	n.s.
Interaction	*	**	*	n.s.	n.s.	n.s.	n.s.
Second harvest (two drought cycles)							
Cocultivation							
Watered	73.6 \pm 1.0	36.2 \pm 0.6	1600 \pm 53	1236 \pm 80	1.46 \pm 0.05	2.56 \pm 0.06	28.6 \pm 0.3
Dry	61.8 \pm 1.0	36.7 \pm 0.8	900 \pm 20	771 \pm 22	1.20 \pm 0.03	2.69 \pm 0.06	32.2 \pm 0.3
Control							
Watered	46.2 \pm 0.8	37.9 \pm 0.7	508 \pm 17	334 \pm 11	1.56 \pm 0.05	2.63 \pm 0.06	31.1 \pm 0.3
Dry	36.5 \pm 0.7	41.8 \pm 0.6	388 \pm 14	339 \pm 11	1.18 \pm 0.04	2.50 \pm 0.07	34.2 \pm 0.5
Significance tests							
Cocultivation	**	*	**	**	n.s.	n.s.	**
Drought	**	n.s.	**	**	**	n.s.	**
Interaction	n.s.	n.s.	**	**	n.s.	n.s.	n.s.
Third harvest (three drought cycles)							
Cocultivation							
Watered	72.9 \pm 0.7	43.3 \pm 0.9	1598 \pm 35	2342 \pm 41	0.68 \pm 0.01	2.69 \pm 0.08	16.7 \pm 0.9
Dry	61.8 \pm 1.0	41.1 \pm 0.8	1047 \pm 25	2189 \pm 40	0.48 \pm 0.01	2.38 \pm 0.08	16.5 \pm 1.2
Control							
Watered	37.4 \pm 1.1	43.8 \pm 0.9	435 \pm 20	882 \pm 36	0.49 \pm 0.01	2.25 \pm 0.07	24.7 \pm 0.3
Dry	34.0 \pm 0.5	47.6 \pm 1.5	406 \pm 16	1032 \pm 43	0.41 \pm 0.01	2.31 \pm 0.06	28.4 \pm 0.4
Significance tests							
Cocultivation	**	n.s.	**	**	**	n.s.	**
Drought	**	n.s.	**	n.s.	**	n.s.	n.s.
Interaction	*	n.s.	**	n.s.	**	n.s.	n.s.

3 * $P \leq 0.05$; ** $P \leq 0.01$; n.s. = not significant