

# *Sebacina vermifera*: a unique root symbiont with vast agronomic potential

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**Abstract** The Sebaciniales belong to a taxonomically, ecologically, and physiologically diverse group of fungi in the Basidiomycota. While historically recognized as orchid mycorrhizae, recent DNA studies have brought to light both their pandemic distribution and the broad spectrum of mycorrhizal types they form. Indeed, ecological studies using molecular-based methods of detection have found Sebaciniales 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*, the model plant traditionally viewed as non-mycorrhizal. The orchid mycorrhizal fungus *Sebacina vermifera* (MAFF 305830) was first isolated from the Australian orchid *Cyrtostylis reniformis*. Research performed with this strain clearly indicates its plant growth promoting abilities in a variety of plants, while demonstrating a lack of specificity that rivals or even surpasses that of arbuscular mycorrhizae. Indeed, these traits thus far appear to characterize a majority of strains belonging to the so-called “clade B” within the Sebaciniales (recently reclassified as the *Serendipitaceae*), raising numerous basic research questions regarding plant–microbe signaling and the evolution of mycorrhizal symbioses. Further, given their proven beneficial impact on plant growth and their apparent but cryptic ubiquity, sebacinoid fungi should be considered as a previously hidden, but amenable and

effective microbial tool for enhancing plant productivity and stress tolerance.

**Keywords** *Sebacina vermifera* · Serendipitaceae · Growth promotion

## Introduction

Mycorrhizas are highly evolved mutualistic associations between soil fungi and plant roots. The partners in these associations are members of most fungal classes (Basidiomycetes, Ascomycetes and Zygomycetes) and the large majority of vascular plant (Brundrett 1991; Harley and Smith 1983). In the mycorrhizal literature, the term mutualism is often used to describe this symbiotic relationship where the host plant receives mineral nutrients while the fungus obtains photosynthetically derived carbon compounds. More than 80 % of plant families are associated with arbuscular mycorrhizae (AM) in comparison to only 3 % that are associated with ectomycorrhizae (Smith 1997). However, in contrast to AM fungi, a large number of fungal species form ectomycorrhizal associations, mainly belonging to the basidiomycetes and ascomycetes.

The Sebacinaceae (Class Basidiomycota; Order Sebaciniales) encompass a taxonomically, ecologically and physiologically diverse group of fungi with a broad geographical distribution. On the basis of culturable isolates and morphological taxonomy, they were mostly categorized as orchid mycorrhizae. However, more recent culture-independent efforts utilizing sequence-based classification have demonstrated both their pandemic distribution and the broad spectrum of mycorrhizal types they form (Oberwinkler et al. 2013). Such endeavors have indeed led to article titles such as “*Sebaciniales everywhere*” that document their presence

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in field specimens of bryophytes (moss), pteridophytes (fern) and all families of herbaceous angiosperms (flowering plants) from diverse agro-climatic regions. These natural host plants include, among others, liverworts, wheat, maize and *Arabidopsis thaliana*, a genetic model plant traditionally viewed as non-mycorrhizal (DeMars and Boerner 1996), (Selosse et al. 2002a), (Weiss et al. 2011).

The two most studied species of the order Sebaciniales are (a) *Sebacina vermifera* (= *Serendipita vermifera*), isolated from the Australian orchid *Cyrtostylis reniformis* (Warcup 1988) and (b) *Piriformospora indica*, isolated from the rhizosphere of *Prosopis juliflora* and *Zizyphus nummularia* Rajasthan, India (Verma et al. 1998). Both of these species are reported to induce beneficial effects on plant performance including growth promotion, increased nutrient uptake, enhanced seed production and increased resistance against different biotic and abiotic stresses (Barazani et al. 2007; Dolatabadi et al. 2011; Ghahfarokhi and Goltapeh 2010; Ghimire and Craven 2011; Ray et al. 2015b; Waller et al. 2008). The plant growth-promoting aspects of *P. indica* have already been reviewed thoroughly (Franken 2012; Varma et al. 2012, 2013). Further, the taxonomy, detailed ultrastructure and biodiversity of this enigmatic fungus has been well reviewed (Oberwinkler et al. 2013). The current review aims at evaluating the vast unexplored agronomic potential of *Sebacina vermifera* and its close relatives.

## Taxonomy

Oberwinkler (1964) described *Sebacina vermifera* as “re-spinate basidiomycetous fungi with longitudinally septate basidia and clampless hyphae, including very scanty basidiocarps with scattered hyphae and separated basidia or small clusters of them, but not forming a hymenium”. Because of its vermiform (L, *vermes* worms; *formes* shaped), nematode-like basidiospores, the species was named *S. vermifera*. The family Sebacinaceae has been proposed (Wells and Oberwinkler 1982) to cover species of the genera *Sebacina*, *Tremelloscypha* and *Tremellodendron*. Of these, *Sebacina* belonging to the order Sebaciniales are characterized by longitudinally septate basidia, imperforate parentheses (the membranous structure present in the cytoplasm on either side of the dolipore septum is continuous or imperforate) and lack of clamp connections (Selosse et al. 2007). More recently, a growing number of fungal DNA sequences derived directly from plant roots can be assigned to the Sebacinaceae. Using this approach, it has become evident that members of this family fairly ubiquitously distributed and are involved in a wide spectrum of mycorrhizal types: ectomycorrhizas (Glen et al. 2002; Selosse et al. 2002a; Urban et al. 2003), orchid mycorrhizas (McKendrick et al. 2002; Selosse et al.

2002b), ericoid and cavendishioid mycorrhizas (Allen et al. 2003), and even jungermannioid mycorrhizas (Kottke et al. 2003). Additionally, many sequences have been obtained from surface-sterilized plant tissues, and have thus been designated as endophytic (Allen et al. 2003; Tao et al. 2008). Such was the diversity and abundance of these fungi, Weiss et al. (Weiss et al. 2004) constructed a new fungal order, the Sebaciniales to encompass them all. He further divided the order Sebaciniales into two clades; clade A whose species form ectomycorrhizae (and at the same time, mycorrhizae on heterotrophic orchids) and clade B, whose species are either endomycorrhizal on autotrophic orchids and Ericaceae, or jungermannioid mycorrhiza to liverworts. Most recently, Oberwinkler et al. (2014) cited the tendency of the latter to be culturable and their distinct rDNA sequences that clearly segregate them away from clade A members. Therefore, he has proposed to refer to clade B collectively as Serendipitaceae and its type member *Sebacina vermifera* as *Serendipita vermifera*. Clade A members are retained under the family Sebacinaceae.

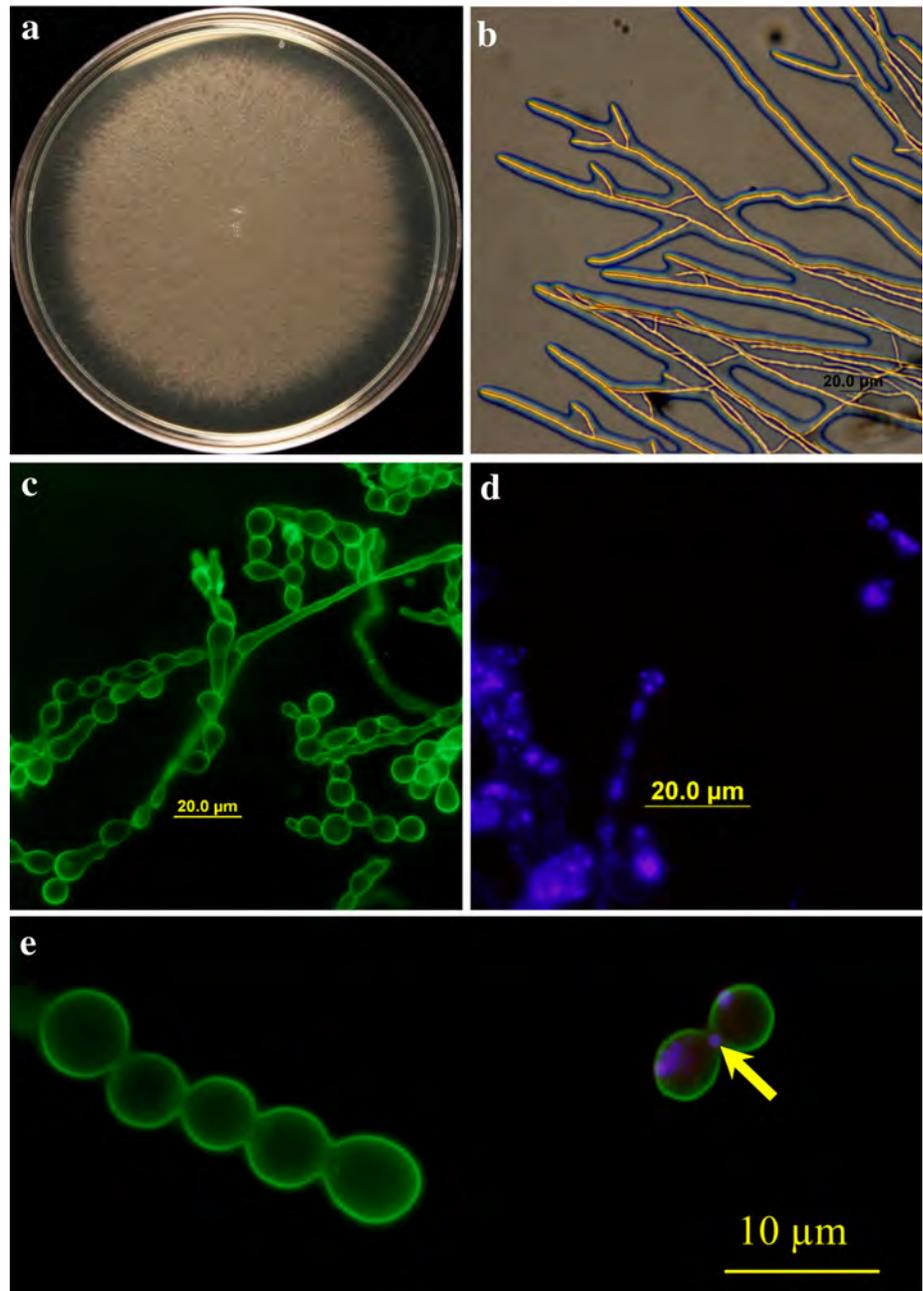
Interestingly, the orchid mycorrhizal species of Sebaciniales are distributed in both Serendipitaceae and Sebacinaceae. In the latter they colonize achlorophyllous species, such as *Neottia nidus-avis* and *Hexalectris spicata* (Selosse et al. 2002b; Taylor et al. 2003), and simultaneously form ectomycorrhizas with diverse surrounding trees (Selosse et al. 2002a, 2004) suggesting a tripartite association, where the orchid derives carbohydrate resources from the tree via the sebacinoid mycobiont.

## Morphology and growth characteristics

The young mycelia of axenic *S. vermifera* are mostly hyaline, but with cream to light brown coloration, lying flat and submerged into the substratum. The hyphae are thin walled with diameter approximately 2 µm. Hyphae elongate by splitting into Y-shaped ramifications, and will often produce hyphal coils. In older cultures, the hyphal tip will bulge out and produce septate, swollen moniloid or bulbous structure with a diameter of approximately 8 µm. These bulbous structures are arranged in chains and are usually multinucleate (Fig. 1).

To determine the physiologically optimal pH and temperature, as well as the range within which *Serendipita vermifera* (MAFF305830) could grow, the fungus was grown on Modified Melin Norkan's (MMN) (Marx 1969) agar at seven different temperatures ranging from 4 to 30 °C with four different pH (5, 6, 8 & 10) for each temperature point. No buffering agent was used as it may affect the growth of the fungus (Giltrap 1981). The optimum pH for the growth of *Serendipita vermifera* (MAFF305830) ranges from pH 6 ~ 8 in MMN agar media at temperatures ranging from 20 to 30 °C (Figs. 2, 3).

**Fig. 1** Colony morphology of *Serendipita vermifera* (MAFF305830) (a) 14 days old fungal colony in MMN agar (b) fungal hyphae (c) WGA-AF 488 stained moniloid hyphae (d) DAPI stained hyphae (e) left: WGA-AF 488 stained moniloid hyphae featuring septal pore; right: DAPI stained nucleus migrating through septal pore (arrow head)



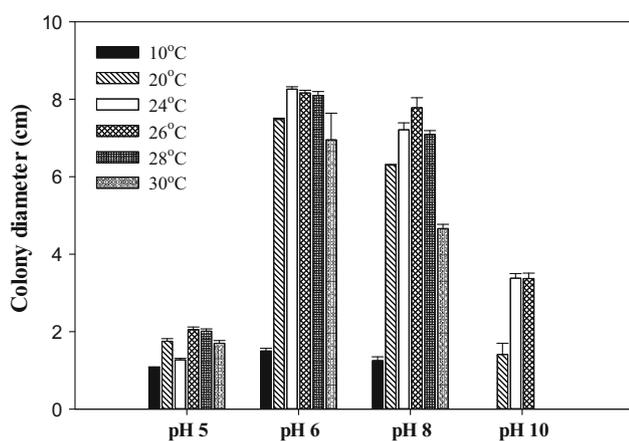
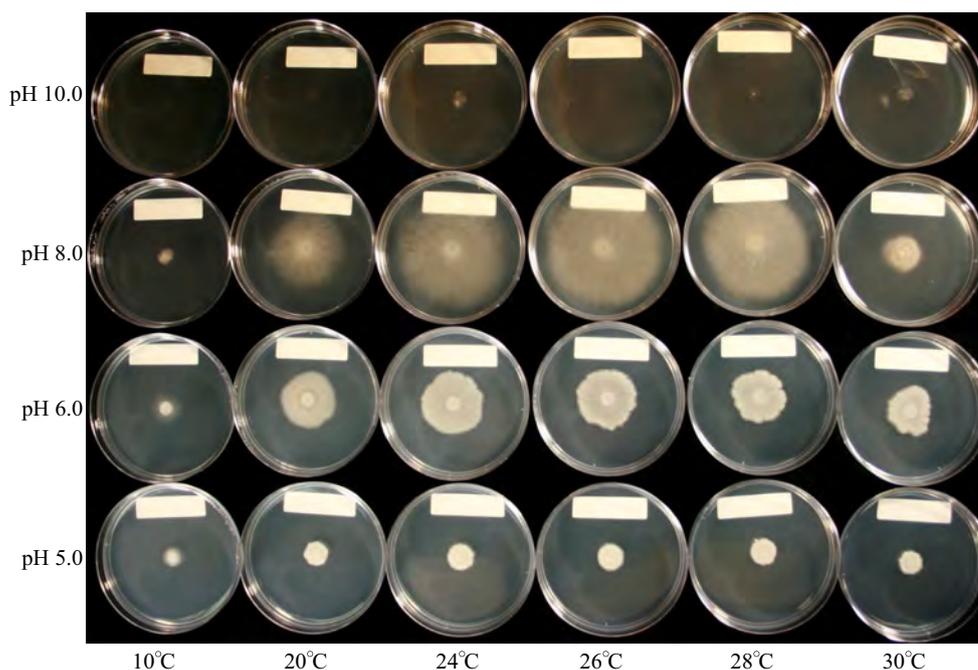
### Fungal morphology in planta

We were able to successfully colonize a variety of agronomically-important crops, including switchgrass, tall fescue and wheat. We visualized normal, healthy looking hyphal branches, aggregates of swollen moniloid or moniliform fungal cells in the cortical plant cells and root hairs completely packed with fungal hyphae (Fig. 4). These structures resemble hyphal protrusions bearing terminally swollen parts formed by some ericoid mycorrhiza on the surface of colonized roots. We assume that hyphae

proliferates primarily by intracellular growth, resulting in a coiled appearance, typical of ericoid and orchid mycorrhizal colonization. Colonization appears to progress to adjoining cells by migration of hyphae through intercellular spaces and is limited to the outer cortex, never advancing into the endodermis or vascular bundle.

It is currently unclear whether there is a plant membrane surrounding the fungal hyphae, although the fluorescence of plant nuclei suggests they are still intact. No defining structures of ectomycorrhiza (i.e. a hyphal mantle around the root and/or a Hartig's net between cortical cells) were

**Fig. 2** Growth and colony morphology of *S. vermifera* (MAFF305830) with response to different temperature and pH in MMN agar 14 *dpi*



**Fig. 3** Growth of *S. vermifera* (MAFF305830) in terms of colony diameter (cm) in MMN agar media in response to different temperature and pH 14 *dai*. Each data point is mean of three replicates with standard error of mean

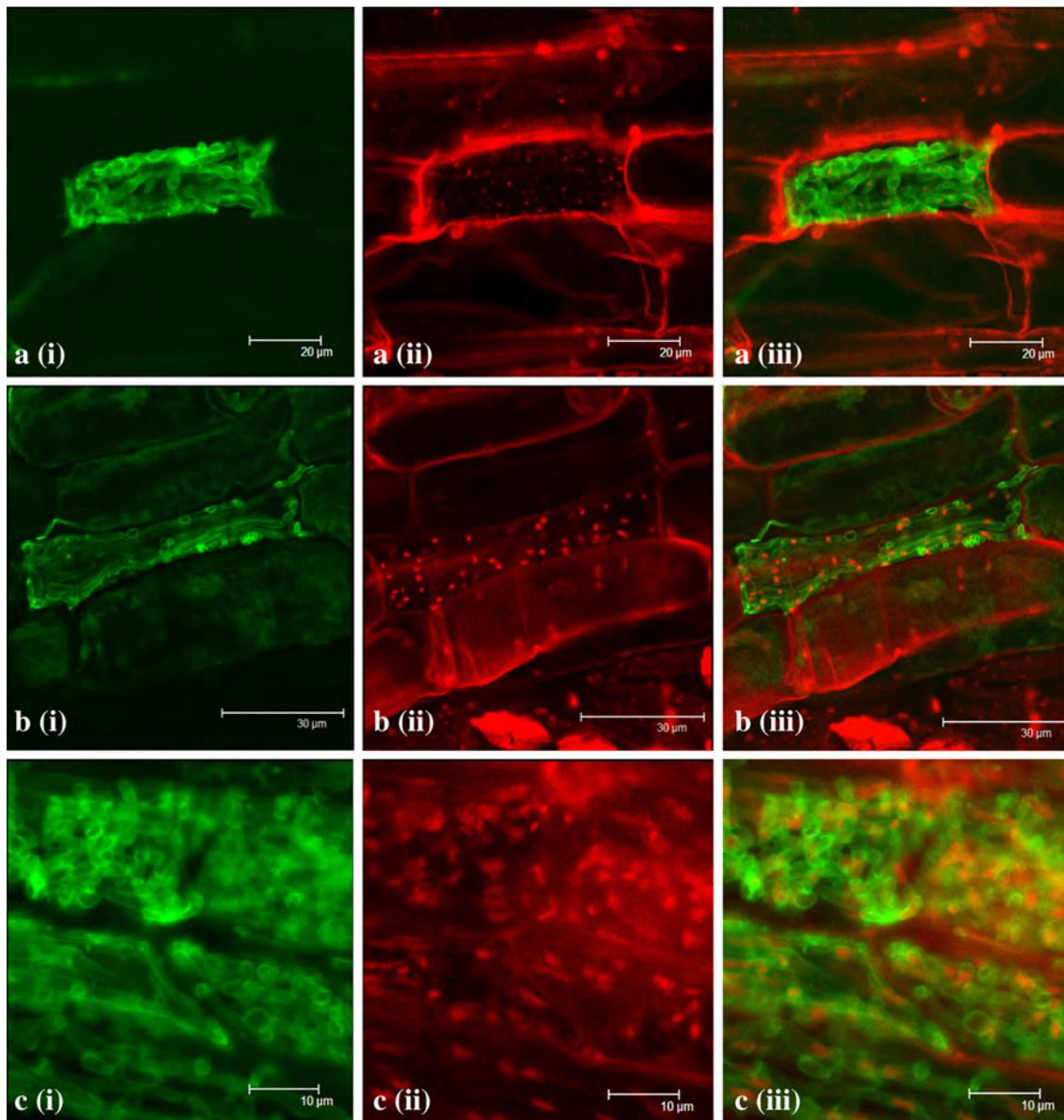
observed. On the basis of this observation, we believe that the colonization pattern of this orchid mycorrhiza *Serendipita vermifera* in all non-orchid hosts thus far examined resembles the ericoid mycorrhizal type.

### Agronomic potential

*Serendipita vermifera* (MAFF 305830) has the remarkable ability to establish beneficial interactions with many different experimental hosts, including the model plant *Arabidopsis*. Colonization of the roots by this strain results in enhanced biomass production as well as increased

resistance against numerous biotic and abiotic stresses (Barazani et al. 2007; Dolatabadi et al. 2011; Ghahfarokhi and Goltapeh 2010; Ghimire et al. 2009; Ghimire and Craven 2011; Pirdashti et al. 2012; Ray et al. 2015b). *S. vermifera* colonization in coyote tobacco (*N. attenuate*) stimulated seed germination, increased growth and stalk elongation, and resulted in early flowering and a greater number of mature seed capsules compared to the non-inoculated controls (Barazani et al. 2007). However, *S. vermifera* colonization appeared to compromise plant defense against *Manduca sexta* larvae by reducing the expression of trypsin proteinase inhibitors (TPI), known defensive proteins (Barazani et al. 2005). While this is unclear how *S. vermifera* compromises with the plant defense response, the role of mycorrhizal fungi in regulating plant defense responses is not unusual. For example, it's been shown that arbuscular mycorrhizal colonization involves a temporal and spatial activation of different defense mechanisms, further contributing to the mutualistic nature of the interaction (García-Garrido and Ocampo 2002).

*Mentha piperita* and *Thymus vulgaris* are aromatic plants of economic importance in the family *Lamiaceae*. Dolatabadi et al. (2011) reported not only increased biomass in *M. piperita* and *T. vulgaris* when colonized with *S. vermifera*, but an increased level of thymol in the oil of thyme as an effect of colonization. Similar investigations with Fennel (*Foeniculum vulgare*) demonstrated increased biomass and essential oil content, as well as an increased level of anethole content in the oil due to *S. vermifera* colonization (Dolatabadi et al. 2011).



**Fig. 4** Visualization (2-D maximum projection image) of *S. vermifera* colonization of root cells by confocal microscopy. **a** tall fescue, **b** switchgrass and **c** wheat. **i** FITC (493–538 nm), **ii** TRITC (548–700 nm), **iii** overlay

Similarly, podophyllotoxin (PPT), and its synthetic derivatives display a wide selection in medical applications such as purgative, vesicant, antirheumatic, antiviral, and antitumor agents. Cell cultures of *Linum album* are well for their ability to produce PPT (Seidel et al. 2002) and it has been shown (Baldi et al. 2008) that co-cultures of *L. album* cells with *Serendipita vermifera* enhanced podophyllotoxin and 6-methoxypodophyllotoxin production along with an increase in biomass compared to the control cultures.

While the exact mechanism(s) behind this unique ability of *S. vermifera* to alter the plant metabolomics profile has not yet been elucidated, preliminary data generated by our

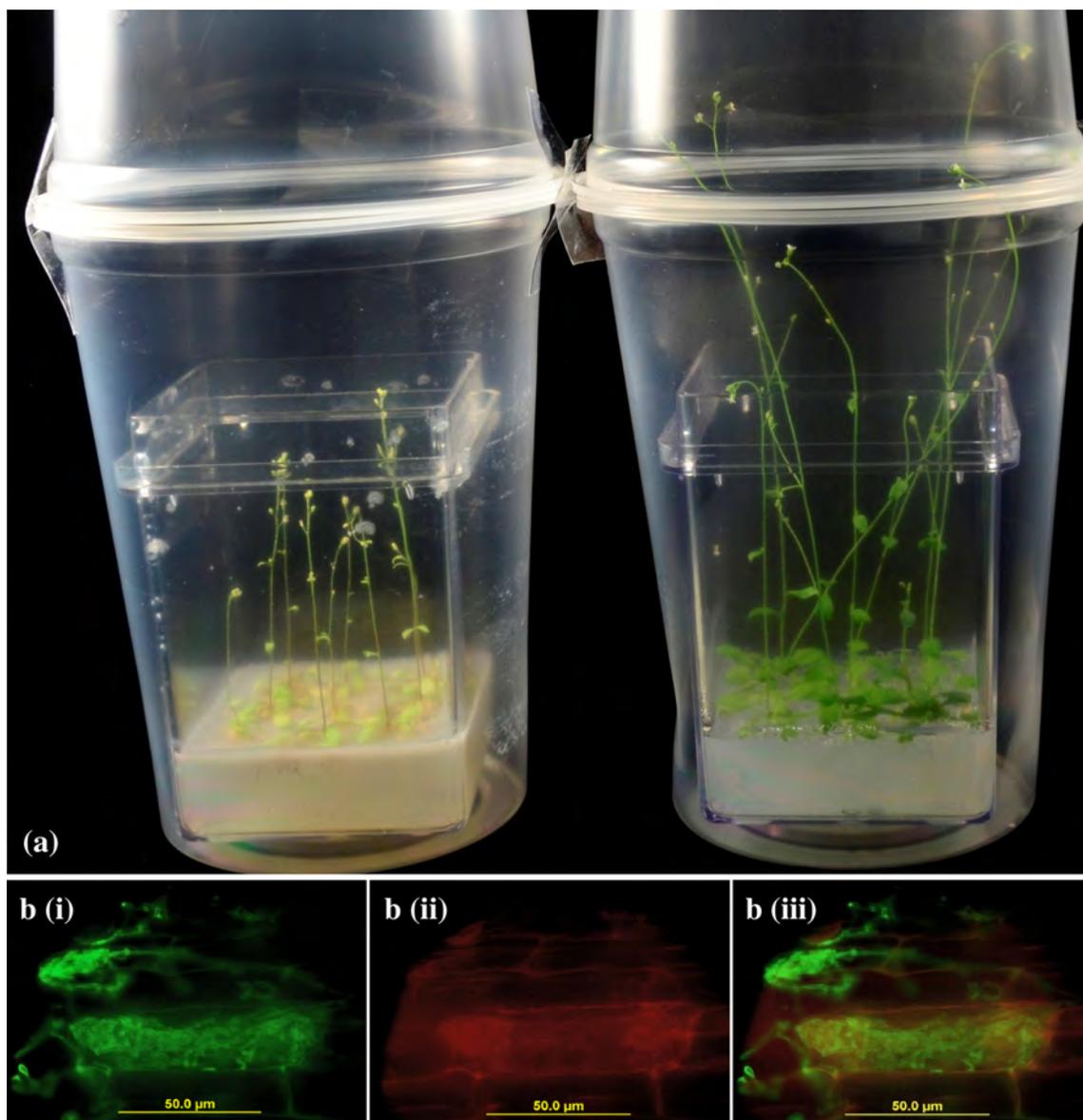
lab indicates that *Serendipita vermifera* produces mevalonic acid in axenic culture that could potentially be used in the plant terpenoid pathway to produce either growth promoters, such as gibberellins, or inhibitors, such as ABA, that improve plant stress response. It is possible that *S. vermifera* imparts a greater metabolic plasticity to the plant that can result in improved defense, water stress tolerance, and productivity (Tim Tschaplinski, ORNL, personal communication).

While *Serendipita vermifera*-mediated growth promotion studies with agronomically important crops belonging to the family Brassicaceae has not yet been done, we find

that *S. vermifera* can indeed colonize the model plant *A. thaliana*, even though this species and related Brassicas are traditionally recognized as non-mycorrhizal. This is clearly not the case, as *S. vermifera* has been reported to colonize *A. thaliana* under natural conditions (Weiss et al. 2011), as well as in vitro. Although we didn't observe any improvement in plant fitness in *Arabidopsis* colonized with *S. vermifera* (MAFF305830) (Fig. 5), the colonization ability per se opens up the possibility for inoculation studies involving agronomically important crops belonging to the family Brassicaceae.

We have shown that switchgrass (*Panicum virgatum* L.), an important bioenergy crop in the US, exhibited enhanced

plant biomass and macronutrient content when inoculated with *S. vermifera*, particularly under drought stress (Ghimire et al. 2009; Ghimire and Craven 2011). Given that the most cost prohibitive step to deployment of cellulosic crops for biofuels is the pretreatment of the biomass to yield something fermentable, we conducted a greenhouse trial to evaluate biomass enhancement of wild type and transgenic, low lignin (COMT down-regulated) switchgrass, an efficient transgenic switchgrass 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. We report *Serendipita vermifera* colonization increased the



**Fig. 5** a Effect on growth of *Arabidopsis thaliana* colonized in vitro with *S. vermifera* MAFF305830 (left box) with respect to control (right box), 30 days after colonization. b Visualization of colonized root cells. i FITC (493–538 nm), ii TRITC (548–700 nm), iii overlay

shoot biomass of low lignin (COMT down-regulated) switchgrass by 22 % (dry weight basis) and root biomass up to 44 % (dry weight basis), respectively (Ray et al. 2015b). Annual biomass yields of established switchgrass fields average from 5.2 to 11.1 Mg ha<sup>-1</sup> (Schmer et al. 2008). Assuming an estimated conversion rate of 0.38 l ethanol kg<sup>-1</sup> harvested biomass (Farrell et al. 2006), a 22 % increase in biomass would translate into increased production of 433–927 l ethanol ha<sup>-1</sup>. Further, an increase 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 (McLaughlin and Kszos 2005).

Wheat (*Triticum aestivum*) is a cereal grain, grown on more land area than any other commercial food. In terms of total production tonnage used for food, it is second only to rice as a primary human food crop. Wheat is the primary grain used in US grain products and is grown in 42 states in the United States.

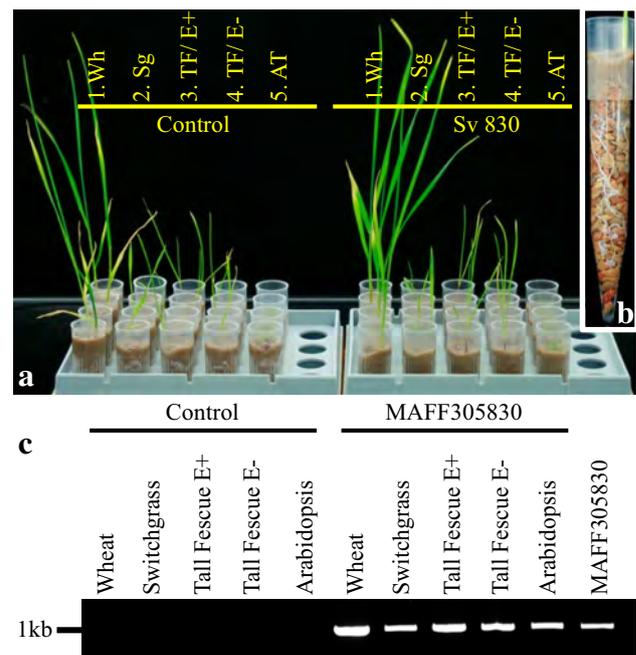
We tested the effect of *Serendipita vermifera* on yield and persistence of five different wheat cultivars in two different locations in southern Oklahoma differing in soil nutritional content and pH. The plants were inoculated using bentonite clay as a carrier coated with or without *S. vermifera* (Ray et al. 2015b). It was found that in one (NFEX25) of the five cultivars tested, *S. vermifera* colonization increased forage biomass and grain yield in both the field locations. Two cultivar (Gallagher & NFEX16) had no effect of fungal colonization, and in other two cultivars (Duster and NFEX28) *S. vermifera* colonization increased forage biomass and grain yield in the poorer, more marginal soil type only. This demonstrates how the fungus may differentially affect plant genotypes and even that the effect may predominate over differing soil conditions (Ray et al. 2015a).

### Large scale inoculum production

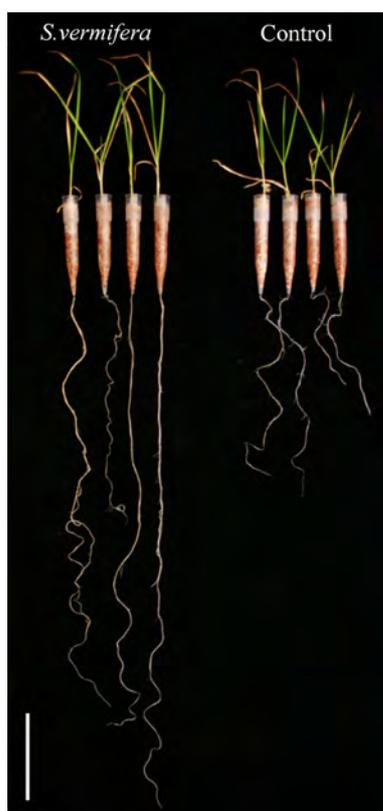
Considering the proven beneficial influence on a wide variety of plants and their apparent ubiquity (Weiss et al. 2011), Sebaciniales fungi should be considered as a previously hidden, but amenable and effective microbial tool for enhancing plant productivity and stress tolerance.

Towards this end, we have developed a bentonite clay particle delivery system for mass production and dissemination of *S. vermifera* for large-scale field trials. 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 (Heijnen et al. 1992; Malusá et al. 2012). 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. To evaluate these questions, we impregnated the fungus onto the bentonite clay particles by first soaking them in a nutrient broth. Mycelial plugs were then used to inoculate the clay which was subsequently allowed to sit at room temperature for 6 weeks. We were able to demonstrate that this technique is both efficient in colonization and transfer, and gives equivalent if not better fitness benefits than other, more time-intensive strategies. With this technology, we have demonstrated efficient colonization by *S. vermifera* in wheat, switchgrass, tall fescue and *A. thaliana* (Figs. 6, 7). When compared to an efficient *in vitro* colonization method, both methods enhanced plant biomass regardless of delivery method, but the percentage of fungal biomass *in planta* increased with the clay-based delivery system (Ray et al. 2015b). Further, we found that release of some clay minerals in solution was enhanced in the presence of *S. vermifera*, while others were seemingly reduced. This is the first report documenting the development of a bentonite clay particle based delivery system for



**Fig. 6** Evaluation of colonization efficiency of the inoculum. 1. Wh wheat, 2. Sg switchgrass, 3. TF/E+ tall fescue with endophyte, 4. TF/E- tall fescue without endophyte, 5. AT Arabidopsis (b) root development within 5 ml tip, (c) confirmation of root colonization by PCR using *Serendipita* specific primers. gDNA (extreme right lane) from *S. vermifera* (MAFF305830) was used as positive control



**Fig. 7** Root enhancement in winter wheat (NFEX28) due to *S. vermifera* colonization in vitro

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. We have since tested this clay-particle method in the field (on winter wheat) and found that the fungal inoculum survived the entire growing season of the crop and enhanced plant performance. This is the first report of a field trial utilizing this enigmatic root endophyte for any crop.

### Conclusions and future prospective

In this day and age, the application of inorganic fertilizers has proven to be both unsustainable and ecologically damaging. Current estimates suggest that minable, inorganic rock phosphorus will be depleted within 50–100 years, having reached peak production in approximately 2030 (Cordell et al. 2009). Unlike phosphorus, nitrogen isn't an inherent property of soils, and must instead be fixed from gaseous  $N_2$  by bacterial soil microbes or, to a lesser extent, by lightning strikes. With the world's natural resources being depleted daily, the race is on to find the technologies that satisfy the triple bottom line requirements of economic, social and environmental sustainability. Limitations in precious resources for agriculture

and crop sustainability can be ameliorated through the use of biotechnology which involves microbes that form symbiotic associations with plants. While the first Green Revolution was powered by a combination of new, high-yielding cereal grains, and synthetic fertilizers (among other things), our continued ability to provide the quantities and quality of food required to feed a burgeoning world population, a so-called *second* Green Revolution, may rely on our capacity to more effectively utilize such microbes in a sustainable manner with greater frequency and precision and without compromising with soil health.

A foundation for informing policy and research directives must be laid upon a comprehensive description of the microbes that are out there in natural ecosystems. Characterization of microbiomes associated with soil and plant is now possible with much greater resolution due to the rapid technological advancements underlying next generation sequencing technologies. Indeed, such studies have partly illuminated a great unseen world of “microbial dark matter”, although a tiny fraction of this inconspicuous majority have thus far been described. The Serendipitaceae fungi represent one of these groups that appear to be ubiquitously distributed worldwide and associate with a majority of plant families in an astoundingly diverse set of environments.

Several properties of these fungi lead us to believe they can be a very valuable tool in efforts to facilitate low-input agriculture. Firstly, they are culturable and can be manipulated fairly easily in the lab. As such, large amounts of inoculum can be readily generated relatively cheaply. We have used bentonite clay as a carrier, and find it to be stable over time and effective in transferring the fungus to the target host plant(s). We are currently pursuing additional technologies such as seed coating and spray application that could provide additional routes for inoculation. Secondly, an perhaps most importantly, we have found no evidence of host specificity to date. We have tried 16 fungal genotypes on a single plant species (switchgrass) and conversely, one fungal genotype on 8-10 plant species (Ghimire et al. 2009). All combinations have thus far been successful in our hands. Of particular note, we and others have successfully colonized *A. thaliana*, which has been touted as a non-mycorrhizal plant, along with most of its Brassicaceae family members. We are currently testing other such “non-hosts”, and if successful, this will provide a mycorrhiza for an important group of crops that have, until now, lacked usable fungal symbionts. While studies with sebacinoid fungi are relatively few, particularly regarding agricultural applications, the future seems bright for this previously hidden group of symbionts. Together with improving cultural practices such as precision pesticide and fertilizer application, continued improvements in breeding efforts led by high-throughput SNP genotyping,

and genetic modification that is deemed safe and effective, harnessing the potential of the plant microbiome can play an important role in promoting low-input agriculture and making the second Green Revolution a reality.

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#### Compliance with ethical standards

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

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