

## Mini-Review

# Do symbiotic microbes have a role in plant evolution, performance and response to stress?

Jerry R. Barrow,<sup>1,\*</sup> Mary E. Lucero,<sup>1</sup> Isaac Reyes-Vera<sup>2</sup> and Kris M. Havstad<sup>1</sup>

<sup>1</sup>USDA-ARS Jornada Experimental Range; <sup>2</sup>Department of Agronomy and Horticulture; New Mexico State University; Las Cruces, New Mexico USA

**Abbreviations:** AM, arbuscular mycorrhiza; EM, ectomycorrhiza; DSE, dark septate endophytes

**Key words:** arid, ecosystem, genetic modification, germplasm, photosynthesis, plant breeding, plant microbe interactions

Vascular plants have been considered as autonomous organisms especially when their performance has been interpreted at the genome and cellular level. In reality, vascular plants provide a unique ecological niche for diverse communities of cryptic symbiotic microbes which often contribute multiple benefits, such as enhanced photosynthetic efficiency, nutrient and water use and tolerance to abiotic and biotic stress. These benefits are similar to improvements sought by plant scientists working to develop ecologically sustainable crops for food, fiber and biofuels.

Native desert plants include a community of indigenous endosymbiotic fungi that are structural components with cells, tissues, cell cultures and regenerated plants. These fungi regulate plant growth and development and contribute genes and natural products that enable plants to adapt to changing environments. A method developed for transferring these endophytes from cell cultures to non-host plants promises to be a revolutionary approach for the development of novel plant germplasm and has application in the field of plant biotechnology.

## Introduction

Population growth with corresponding decreases of arable land, available water and nutrients, under biotic and abiotic stress presents major challenges for the production of sufficient food, fiber and biofuels in the coming century.<sup>1</sup> Food production has kept abreast of population growth through developing new cultivars that respond to increased inputs such as improved cultural practices, fertilizers and chemical pesticides. These advances have been based on the assumption that plants function as autonomous organisms regulated by their genetic code and cellular physiology. This concept was developed from early evolutionary theories that plants and animals evolved by accumulating gene changes within species.<sup>2</sup>

Yet in reality it is well recognized that symbiosis is a common and fundamental condition of plants.<sup>3</sup> Current research suggests

that all plants in native ecosystems are symbiotic with fungi and other microbes (bacteria, yeast) on their leaf and root surfaces, rhizosphere and internal tissues that influence their performance.<sup>4-8</sup> It was suggested in the late 1800's and now confirmed by DNA based detection technology that plastids and mitochondria of the eukaryotic cell were derived from a consortium of primitive microbes.<sup>3,9,10</sup> Cytoplasmic organelles, each with their own DNA, are replicated and passed from generation to generation through the egg. Similar origins have been proposed for other cytoplasmically inherited organelles such as cilia and centrioles.<sup>2</sup> The continuity of microbial associations with plants from their origin suggests that plants have not functioned as autonomous individuals, but their internal tissues provide a unique ecological environment for diverse communities of symbiotic microbes, which have had a major influence on plant adaptation and evolution.<sup>3,11-17</sup>

Plants with endosymbiotic microbes have similarities to lichens.<sup>18-20</sup> Lichens are the simplest model of symbiosis between fungi and photosynthetic organisms. Fungi are structurally integrated with either photosynthetic algal or cyanobacterial cells to form a single thallus that dramatically differs from the component organisms and shows structural convergences with vascular plants. The fungus obtains carbon from the photosynthetic microbe, while providing a protective cortex composed of a dense layer of hyphae embedded within a polysaccharide matrix (biofilm) that accesses and retains water. Pigmentation within the cortex may protect photosynthetic components from exposure to intense sunlight. Fungi also secrete organic acids and enzymes to acquire mineral nutrients from recalcitrant sources such as rock surfaces, soil and organic substrates. This unique symbiosis results in a superorganism capable of coping with extreme stress in ecosystems where neither component could survive by its self.<sup>18</sup> The similarity of symbiotic microbes associated with desert plants to lichens and their potential role in coping with severe drought stress in arid environments has stimulated our scientific investigations and this review.

## Mycorrhizal Fungi

Mycorrhizal like associations assisted the early colonization of terrestrial systems by vascular plants.<sup>12,21-23</sup> Early observations on mycorrhizal fungi in the late 1880's and hypothesis on their role on evolution, ecology and physiology flew in the face of conventional

\*Correspondence to: Jerry R. Barrow; USDA-ARS Jornada Experimental Range; P. O. Box 30003; MSC, 3JER, NMSU 88003; Las Cruces, New Mexico 88003 USA; Tel.: 575.646.7015; Email: jbarrow@nmsu.edu

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wisdom at that time. Those observations are now supported by tens of thousands of scientific papers, yet the full nature of symbiotic fungal organisms is still in the process of examination by the scientific community.<sup>16</sup> Most extensively studied fungal symbionts are the arbuscular mycorrhizae (AM), which are associated with approximately 90% of all land plants and contribute multiple benefits to their host plants. Similar to the fungal component of lichens, mycorrhizal fungi mineralize and transfer phosphorous and other essential mineral nutrients to the plant. Other benefits include improved water acquisition and use, drought tolerance, increased tolerance to pathogens, heavy metals, herbivory and enhanced soil stability.<sup>23-25</sup> Similar to lichens, the fungal component contributes these benefits to the photosynthetic partner for organic carbon. Mycorrhizal fungi are perceived as essential for survival and well being of individual plants and they also influence community and ecosystem structure.<sup>16,23,25-27</sup> Attempts to incorporate these valuable symbionts into mainstream agricultural production practices have not been successful.<sup>25</sup>

Ectomycorrhizal fungi (EM) are the major root symbionts of important woody trees and shrubs of the northern boreal and temperate forests. EM fungi are generally free living in soil, exist on soil organic matter and are readily cultured on a wide range of simple to complex artificial media. A similarity between mycorrhizal roots and lichens is noted by the dense hyphal mantle that is formed on the surface of mycorrhizal roots and is similar to the fungal cortex in lichens. This interface between the root and soil suggests protection against pathogens and fluctuating soil moisture and is thought to regulate bidirectional flow of photosynthetic carbon and mineral nutrients between plants and fungi. EM fungi are transferred horizontally from colonized roots or soil to new roots.<sup>23</sup>

## Endosymbiotic Fungi

Numerous examples of other non-mycorrhizal fungal endophytes associated with plants are accumulating. These fungal symbionts also had early origins with land plants. Thin petrographic sections of a 400-year-old Rhynie chert plant *Nothia aphylla* revealed three different non-mycorrhizal fungal endophytes that modified root tissues.<sup>28</sup> Currently the most extensively studied are the clavicipitaceae (Ascomycota) endophytes of the cool season C<sub>3</sub> forage grasses. These fungi grow within the intercellular spaces of above ground leaves and into developing embryos and are vertically transmitted by seed. They produce toxic alkaloids that protect host plants against insect and grazing herbivores and present a serious economic problem for the grazing livestock industry. They influence community structure, host metabolism and physiology which, similar to lichens, enable both fungus and photosynthetic host to exploit novel or extreme hot, dry habitats that are typically inhospitable to perennial C<sub>3</sub> grasses.<sup>14</sup>

As new examples of endosymbiotic fungi are discovered, questions arise as to their role and function on the genetics, physiology, ecology and evolution of plants. The ability of these fungi to survive in extreme environments, harvest nutrients and transport them through filamentous hyphae over extended distances make them valuable symbionts of vascular plants.<sup>29</sup> Our basic understanding of microbial morphology, taxonomy and molecular profiles has been derived from those that can be cultured on artificial media. Yet, 90 to 99% of microbes cannot be cultured using standard techniques.<sup>30</sup> Currently

fungal presence in plants has been determined by symptoms, by isolation and culture or by microscopic detection of specifically stained plant tissues that selectively highlight fungal wall components (chitin) with minimal background staining of plant tissue.<sup>31-33</sup>

Many plant endophytes are cryptic since they induce no symptoms and escape detection using current histochemical, microscopic, isolation and cultural methods.<sup>34,35</sup> More innovative molecular and microscopy methods are required for more detailed studies. Even biochemical and molecular detection methods remain insufficient for appreciating synergistic roles endophytes play.<sup>36</sup> In addition fungal colonization is often localized and minute fungal structures are hard to interpret. Active fungal structures may be overlooked when they differ from typical recognized fungal morphology, particularly when they are intimately integrated with cell walls and membranes.<sup>37,38</sup> Successful chemical detection, immunological methods or direct amplification of fungal DNA from colonized plant tissues can occur, but proving that the detected products are of fungal origin still requires separating the fungus from plant tissues. Recognition of uncultured fungi is only successful if the molecular profiles of the uncultured species resemble profiles of known, cultured fungi. The unique plant environment that harbors diverse populations of endosymbiotic fungi suggests novel unexplored fungi that differ from existing fungal species described from other habitats, making molecular identification difficult. It has been estimated that these may represent a million or more new species.<sup>4,5,32,39</sup> Their presence suggests enormous carbon expenditures by the host, which might be expected to place undue stress on the host metabolism.<sup>39</sup> Numerous examples in the literature suggest that these novel endophytes have significantly contributed to the ability of host plants to adjust to multiple stresses induced by changing environments, which would justify their presence.<sup>39-41</sup> Little is known of their function, but endophytes that enhance nutrition, photosynthesis, productivity, resistance to stress and regulate ecosystem processes are more likely a benefit than a burden.<sup>32</sup>

## Fungal Associations with Native Desert Plants

We extensively studied fungal endophytes associated with grasses and shrubs native to the Northern Chihuahuan Desert where organisms are exposed to chronic light and temperature extremes, extended drought periods and recalcitrant mineral availability. Initial interpretations were that roots were most extensively colonized by dark septate fungi (DSE).<sup>42</sup> These fungi are characterized as melanized, dark pigmented hyphae and microsclerotia and are reported in some 600 plant species in stressed environments.<sup>43-46</sup> They coexist with mycorrhizal fungi and are thought to benefit host plants in nutrient acquisition similar to mycorrhizal fungi.<sup>47-50</sup>

Analysis of desert plants over years, seasons and variable climatic conditions revealed that the characteristic melanized structures were most prevalent in roots of dormant plants. Long periods of dormancy are interrupted by intense precipitation events during the summer monsoons which initiate growth and high levels of physiological activity. Dual staining methods that targeted fungal wall material and internal fungal lipid bodies revealed that fungal morphology in physiologically active plants was variable and fungal presence was more extensive than when only stained or melanized structures were considered. Fungal wall structure in physiologically active plants varied from melanized, stained to hyaline. Often fungi

functioned without walls as membrane bound protoplasts and escaped detection by conventional staining methods.<sup>37,38</sup> Fungi were found on both leaf and root surfaces and were observed to be associated with all cells of roots and leaves. We concluded that native desert plants are colonized by diverse groups of symbiotic fungi that differ from mycorrhizal, DSE and other recognized fungi. Plants were regenerated from cell cultures initiated from aseptically prepared embryonic tissues of germinating seedlings.<sup>51</sup> Consistent with other reports,<sup>52-54</sup> we unexpectedly found that cell cultures and regenerated plants were not microbe free, but housed several different endosymbiotic fungi.<sup>55</sup> We concluded that these cryptic endophytic fungi were generally noncultivable and were vertically transmitted from generation to generation.

An astonishing example of a conserved lichen-like fungal association was obtained by microscopic analysis of the epidermis of native C<sub>4</sub> desert grasses that revealed an integrated chimera of plant and fungal cells. A precisely organized fungal network was associated with all epidermal plant cells. Bicellular cells attached to the fungal network, previously described as plant trichomes, were similar to teliospores produced by Uredomycetes. Their attachment to the fungal network, staining specifically for fungal tissue and initiation of fungal hyphae verified that these structures were fungal and not plant cells.<sup>56</sup> This integrated pattern of plant and fungal tissue also developed on leaves of regenerated *Bouteloua eriopoda* plants confirming the conserved plant-fungus association by vertical transmission from cell to cell in the regeneration process. This symbiotic association with fungi results in a superorganism where the fungal component similar to lichens, is essential for warm season C<sub>4</sub> grasses to survive under arid conditions where they may retain water and regulate light.

## Endophyte Transfer

Callus cultures initiated from aseptic tissues of native grasses and shrubs examined by scanning electron microscopy further revealed that they were completely encapsulated with a fungal enmeshed biofilm. Similar biofilms were reported encapsulating pine callus cultures suggesting a fungal role in pine bud development.<sup>52</sup> We also observed fungal biofilms on root and leaf surfaces of native plants suggesting that they protect cells from desiccation and possibly pathogen invasion. Endophytes did not grow from cell cultures on to the enriched carbon-nutrient media, nor could endophyte free plants be regenerated from axenic cell cultures of native plants. Aseptically cleansed germinating seedlings of tomato, chile and several native grasses were co-cultured with callus tissues of native plants in an attempt to transfer indigenous native endophytes to non-host plants.<sup>57,58</sup> Fungal hyphae were microscopically observed growing from the callus to the seedlings. Phenomenal responses in root initiation, branching and biomass were observed in some combinations of co-cultured non-host plants. Transfers from calluses generated from some native plants to non-host native grasses not only enhanced root and shoot biomass, but substantially induced earlier flowering, greater seed production and seedling vigor. Not all transfer combinations were positive, some were neutral and others were negative indicating differences in fungus-host interactions. These differential responses suggested that indigenous endophytic microbes function to regulate photosynthesis, physiology and ecology of native plants in a complex fashion.

## Symbiotic Microbes Offer Novel Tools for Biotechnology

Vascular plants do not function as autonomous individuals, but house diverse communities of symbiotic microbes. The role of these microbes can no longer be ignored. Like symbiotic lichens, microbial interactions are critical not only for host, but for fungal survival in stressed environments.<sup>59</sup> To date, improvements in plant quality, production, abiotic and biotic stress resistance, nutrient and water use have relied largely on manipulating plant genomes by breeding and genetic modification.<sup>60</sup> Increasing evidence indicates that the function of symbiotic microbes seem to parallel more than one of these characteristics. Past efforts to incorporate symbiotic microbe management into mainstream plant improvement and production practices have seen limited success for a number of reasons. These include difficulties associated with mass culture of microbes, performing inoculations and insuring persistence of beneficial symbionts in agricultural environments. The extreme and unquantified genetic diversity among beneficial microbes signifies complex potential interactions with host plants, making it difficult to predict uniform, successful outcomes across diverse plant cultivars.<sup>25</sup> Plant breeding has produced continuous supplies of improved cultivars and recent advances in genetic modification have successfully allowed transfer of desirable genes across kingdom barriers, yet these methods are expensive and time consuming and will be challenged by rapidly changing environments. It is becoming increasingly evident that cryptic symbiotic microbes may represent an enormous untapped genetic reservoir for plant improvement.<sup>25,39-41,61</sup> Transferring these endophytes from cell cultures of native plants to germinating seedlings of non-host plants promises a revolutionary biotechnology to rapidly improve plant germplasm.

A major objective of photosynthetic research is to increase its efficiency under biotic and abiotic stress.<sup>33,62,63</sup> These include altering photosynthetic pathways and plastid modification; both are attractive means of sustainable plant improvement. An example of enhanced photosynthetic efficiency induced by a symbiotic microbe was demonstrated by inoculating regenerated *Agave victoria-reginae* plants with a non-pathogenic strain of *Fusarium oxysporum*, indigenous to native plants. Inoculated plants had substantial increases in root length, branching, numbers of stomata, nocturnal acidity, malic acid, chlorophyll and sugar content compared to non-inoculated control plants.<sup>64</sup> Understanding physiological contributions of the fungal symbiont on enhancing photosynthetic efficiency should provide alternate approaches for plant improvement and management. Improved production of host plants by endosymbionts under stress suggests improvements in photosynthetic efficiency. *Neotyphodium* endophytes of cool season grasses also were shown to enhance photosynthetic rates under water and heat stress.<sup>65</sup> Increased biomass induced by transfer of endophytes of native grasses also suggests enhanced photosynthesis.<sup>57,58</sup>

A unique fungus was identified in association with *Dichanthelium lanuginosiae* growing in Yellowstone National Park in geothermic soils ranging from 20°C to 50°C. Like lichen associations, neither the grass nor the fungus could survive alone in the extreme temperatures. Heat tolerance was generated by a tripartite grass-fungus-virus association in *D. lanuginosiae*.<sup>40,66</sup> The genetic value of the endosymbiotic microbes was demonstrated when tomato and watermelon plants were able to survive temperatures of 50°C to 65°C after inoculation



with the endosymbiotic fungus *Curvularia* sp. The high level of heat tolerance conferred by the fungus-virus combination not only suggests its compatibility with a wide host range but also reveals high levels of heat tolerance which would be difficult to obtain by plant breeding or genetic modification.<sup>67</sup>

## Conclusion

A perceived future role of biotechnology is to introduce multiple choreographed genes into plants that would elicit multiple benefits to the plants such as resistance to stress, productivity and quality.<sup>68</sup> Microbial genomes that have co-evolved with native plant species may already be choreographed and compatible with a wide range of plant genomes and available in this vast unexplored genetic reservoir. Understanding microbial DNA and how it communicates with plant DNA for their mutual welfare and could lead to innovative methods of plant improvement.

It is our contention that native plants survive and flourish in stressed ecosystems because of endosymbiotic organisms that have co-evolved and were essential for their adaptation to changing environments. Some of these microbial components are non-cultivable and vertically transmitted from generation to generation. They represent a vast reservoir of heritable DNA that can enhance plant performance in changing environments and add genetic flexibility to adaptation of long-lived plants.<sup>40,69</sup> Our preliminary results suggest that uncultured endosymbiotic microbes may be vertically transferred in succeeding generations. If such endophytes can be identified that not only persist in progeny of novel hosts, but can confer benefits in mechanized, agricultural systems, they would be increasingly important in agricultural production and lead to a rapid and economical method of providing novel germplasms of native and crop plants. Many questions must be answered before systemic endophyte transfer to crop or native plants can become a standard practice. Better methods for identifying what is being transferred and for monitoring, how long these associations persist in field settings are required. The answer to these questions and others will require novel approaches of molecular technology.

The growing consensus is that microbial associations with higher plants are universal.<sup>4-8,33</sup> Plant growth and development cannot be adequately described without acknowledging microbial interactions. We need to determine the extent of microbial associations in the plant kingdom. This question will only be answered as technology is developed to detect their presence in plant tissues. What we have learned is that there is a need to understand how plant-microbes communicate in these endosymbiotic relationships, and how they regulate basic genetic and physiological functions.

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