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Perspective

Chemical Ecology of Endophytic Fungi: Origins of Secondary Metabolites

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Endophytes constitute a remarkably multifarious group of microorganisms ubiquitous in plants and maintain an imperceptible association with their hosts for at least a part of their life cycle. Their enormous biological diversity coupled with their capability to biosynthesize bioactive secondary metabolites has provided the impetus for a number of investigations on endophytes. Here, we highlight the possible current and future strategies of understanding the chemical communication of endophytic fungi with other endophytes (fungi and bacteria) and with their host plants, which might not only allow the discovery and sustainable production of desirable natural products but also other mostly overlooked bioactive secondary metabolites.

INTRODUCTION

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease (Bacon and White, 2000). "Endophytism" is, thus, a unique cost-benefit plant-microbe association defined by "location" (not "function") that is transiently symptomless, unobtrusive, and established entirely inside the living host plant tissues (Kusari and Spiteller, 2012b). During this association, none of the interacting partners is discernibly harmed, and the individual benefits depend on both the interacting partners. The subtleties of such a complex interaction can be represented between extremely dedicated mutualism and ardent parasitism or saprophytism or exploitation, which might bear the potential to shift variably or progressively toward a more specialized interaction (Millet et al., 2010; Zuccaro et al., 2011). Evidence of plant-associated microorganisms found in the fossilized tissues of stems and leaves has revealed that endophyte-plant associations may have evolved from the time higher plants first appeared on the earth (Redecker et al., 2000). The existence of fungi inside the organs of asymptomatic plants has been known since the end of the 19th century (Guerin, 1898), and the term "endophyte" was first proposed in 1866 (de Bary, 1866). Since endophytes were first described in the Darnel (*Lolium temulentum*) (Freeman, 1904), they have been isolated from various organs of different plant species, aboveground tissues of liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns, and spermatophytes from the tropics to the arctic, and from the wild to agricultural ecosystems (Arnold, 2007), and to date, all plant species studied have been found to harbor at least one endophyte. A milestone in the history of endophyte research was the discovery of the endophytic fungus *Neotyphodium coenophialum* as the causative organism of "fescue toxicosis," a syndrome suffered by cattle fed in pastures of the grass *Festuca arundinacea* (Bacon et al., 1977). It was later found that these infected plants contained several toxic alkaloids and that *Neotyphodium* species could be beneficial to their plant hosts, increasing their tolerance of biotic and abiotic stress factors (Schardl et al., 2004). The most frequently encountered endophytes are fungi (Staniek et al., 2008), and currently, to our knowledge, all reported endophytes are fungi or bacteria (including actinomycetes), but it is possible that future discoveries might also reveal the endophytic nature of other non-endophytic microorganisms (Strobel et al., 2004). Endophytic fungi are a very diverse polyphyletic group of microorganisms; they can thrive asymptomatically in the tissues of plants aboveground as well as belowground, including stems, leaves, and/or roots.

Many endophytes have the potential to synthesize various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases (Strobel et al., 2004; Staniek et al., 2008; Aly et al., 2010; Kharwar et al., 2011; Kusari and Spiteller, 2012b). Occasionally, endophytes that produce host plant secondary metabolites with therapeutic value or potential have been discovered; some examples include paclitaxel (also known as Taxol) (Stierle et al., 1993), podophyllotoxin (Eyberger et al., 2006; Puri et al., 2006), deoxypodophyllotoxin (Kusari et al., 2009a), camptothecin and structural analogs (Puri et al., 2005; Kusari et al., 2009c, 2011b; Shweta et al., 2010), hypericin and emodin (Kusari et al., 2008, 2009b), and azadirachtin (Kusari et al., 2012). The production of bioactive compounds by endophytes, especially those exclusive to their host plants, is not only important from an ecological perspective but also from a biochemical and molecular standpoint. Exciting possibilities exist for exploiting endophytic fungi for the production of a plethora of known and novel biologically active secondary metabolites. For example, using controlled fermentation conditions by altering the accessible culture and process parameters (such as media type and composition, aeration, pO_2, pCO_2, pH, temperature, agitation, sampling, and harvest points), the compounds produced by fungal endophytes might be optimized. This could lead to a cost-effective,

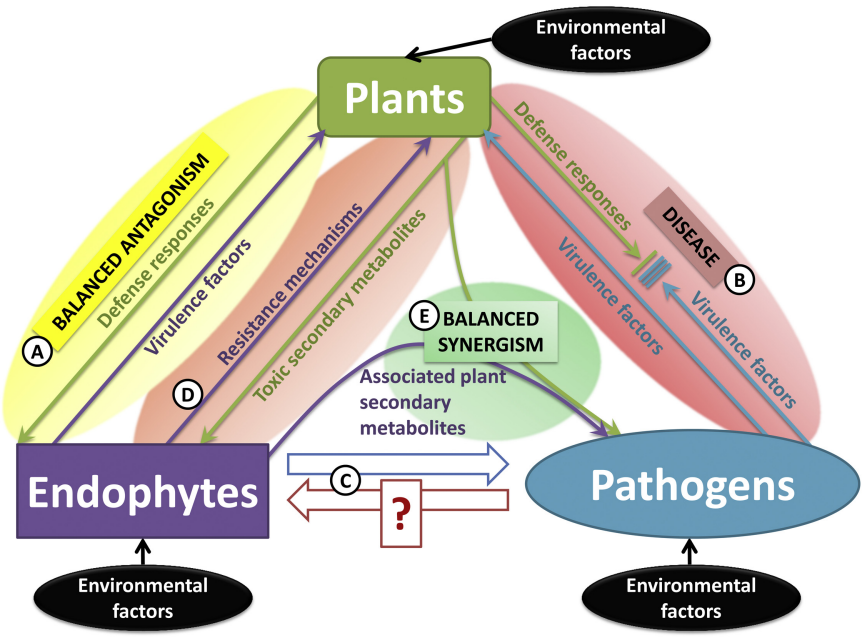

Figure 1. Chemical-Ecological Schematic Interpretation of Plant-Fungus Cost-Benefit Interactions with Emphasis on Endophytic Fungi

(A) Balanced antagonism hypothesis is shown.
(B) Plant disease caused by pathogenic fungi is presented.
(C) Endophyte-pathogen reciprocity is demonstrated. The question mark (?) indicates that this phenomenon might not be universal, and further research is necessary for verification.
(D) Endophyte survival strategy is illustrated.
(E) Balanced synergism is shown.

environmentally friendly, continuous, and reproducible yield compliant to commercial scale-up. In the case of endophytes capable of producing host plant compounds, such production (under optimized fermentation conditions) would then be independent of the variable quantities produced by plants influenced by environmental conditions (vide infra Future Considerations: Resolving The Present Challenges). However, the practicality of commercial production of compounds by endophytic fungi still remains unproven. The reduction of secondary metabolite production on repeated subculturing under axenic monoculture conditions is one of the key challenges that needs to be addressed in order to establish, restore, and sustain the in vitro biosynthetic potential of endophytes (Kusari and Spiteller, 2011). This problem is intensified by the fact that almost all efforts to obtain natural products from endophytes have so far been made by the "classical" approach, under axenic monoculture conditions (Winter et al., 2011). This has occasionally led to the rediscovery of known secondary metabolites, mostly overlooking the repertoire of "cryptic" natural products that are not produced under standard in vitro conditions (Scherlach and Hertweck, 2009; Walsh and Fischbach, 2010). To overcome the aforementioned challenges, in this *Perspective* we highlight the basic principles of chemical communication strategies of endophytic fungi with their host plants and with other endophytes (both fungi and bacteria) with emphasis on the future directions and the virtually inexhaustible possibilities for discovery and sustainable production of target and nontarget secondary metabolites utilizing endophytes.

Plant-Endophyte Interactions

Any plant-fungal interaction is preceded by a physical encounter between a plant and a fungus, followed by several physical and chemical barriers that must be overcome to successfully establish an association. The "balanced antagonism" hypothesis (Schulz et al., 1999; Schulz and Boyle, 2005) was initially proposed to address how an endophyte avoids activating the host defenses, ensures self-resistance before being incapacitated by the toxic metabolites of the host, and manages to grow within its host without causing visible manifestations of infection or disease (Arnold, 2005, 2007, 2008; Schulz and Boyle, 2006) (Figure 1A). This hypothesis proposed that asymptomatic colonization is a balance of antagonisms between the host and the endophyte. Endophytes and pathogens both possess many virulence factors that are countered by plant defense mechanisms. If fungal virulence and plant defense are balanced, the association remains apparently asymptomatic and avirulent. This phase is only a transitory period where environmental factors play a major role to destabilize the delicate balance of antagonisms. If the plant defense mechanisms completely counteract the fungal virulence factors, the fungus will perish. Conversely, if the plant succumbs to the virulence of the fungus, a plant-pathogen relationship would lead to plant disease (Figure 1B). Because many endophytes could possibly be latent pathogens, they might be influenced by certain intrinsic or environmental factors to express factors that lead to pathogenicity (Arnold, 2008) (Figure 1C). For example, expression of the stress- and mitogen-activated protein kinase gene (*sakA*) of endophytic *Epichloë festucae* is shown to be vital for maintaining its mutualistic association with host *Lolium perenne* (perennial ryegrass) and preventing this association to become pathogenic (Eaton et al., 2010, 2011).

Recently, it was revealed that the plant-endophyte interaction might not be just equilibrium between virulence and defense, but a much more complex and precisely controlled interaction (Figure 1D). For instance the plant *Camptotheca acuminata* (happy tree) produces the anticancer compound camptothecin that inhibits topoisomerase I by binding and stabilizing the covalent complex of topoisomerase I-DNA (Kusari and Spiteller, 2012a). A camptothecin-producing endophyte (*Fusarium solani*) isolated from the inner bark tissues of *C. acuminata* ensures protection from its own and plant camptothecin by specific amino acid residue alterations in the camptothecin-binding and catalytic domains of its topoisomerase I (Kusari et al., 2011a). Similarly, the topoisomerase I encoded by another endophyte isolated from the same tissue but that does not produce camptothecin also contains the same changes to make it resistant to the action of camptothecin. On the one hand this points toward

similar evolutionary preadaptation of endophytes infecting the same plant, regardless of their biosynthetic capability. It is known that plants utilize camptothecin as a mode of chemical defense against insect and pathogen attack (Sirikantaramas et al., 2009). Any fungus trying to infect a camptothecin-producing plant will immediately come in contact with the plant camptothecin. The invading fungus will, therefore, be killed by camptothecin that will target its topoisomerase I-DNA complex, unless it intrinsically possesses the ability to resist the attack of the host camptothecin after its infection. In this case the infecting endophytic fungus, *F. solani*, had to be pre-equipped to resist the camptothecin toxicity conferred by the host *C. acuminata* plant, before evolving toward biosynthesizing camptothecin itself as dictated by the in planta selection pressures. Some plants have also demonstrated resistance to camptothecin vested by specific amino acid residues in the camptothecin-binding and catalytic domains of their topoisomerase I enzymes. For example *Ophiorrhiza japonica* exhibits partial resistance to camptothecin in vivo, although it does not produce this compound itself (Sirikantaramas et al., 2009). This suggests the contribution of yet unknown specific amino acid residues, which are responsible for topoisomerase I preadaptation in *O. japonica*. On the other hand the concept of time-dependent target-based resistance features (coevolutionary adaptation) in various species when differentiating the resistance-mediating topoisomerase I alterations in camptothecin-producing plants and human camptothecin-resistant cancer cells (CEM/C2) has been well elaborated by Sirikantaramas et al. (2009). It is conceivable that some specific mutations are only found in plants (Sirikantaramas et al., 2008) because of the much longer evolutionary period of exposure to camptothecin in plants than in endophytic fungi. Furthermore, because endophytic *F. solani* is capable of producing camptothecin, it might develop additional target-based camptothecin-resistance features in driving the course of evolution. In either case it would seem that these types of endophyte-plant interactions should, therefore, be very specific and strongly selected toward steady coexistence.

According to the plant-endophyte coevolution hypothesis (Ji et al., 2009), it might be possible for endophytes to assist the plant in chemical defense in planta by producing bioactive secondary metabolites. Two parallel intriguing propositions have been made. According to the ''mosaic effect'' theory, endophytes might protect host plants by creating a heterogeneous chemical composition within and among plant organs that are otherwise genetically uniform (Carroll, 1991). Consequently, these organs would vary arbitrarily in lusciousness or worth for herbivores, and in terms of infectivity for pathogens. The other theory holds that endophytes might assist their corresponding host plants as ''acquired immune systems'' (Arnold et al., 2003). The recently proposed ''xenohormesis'' hypothesis by Howitz and Sinclair (2008) states that signaling and stress-induced molecules from plants can be sensed by heterotrophs (animals and microbes), which have developed such ability under evolutionary selective pressures. The heterotrophs might have retained the capacity to sense chemical cues in plants to start producing similar secondary metabolites again, though they have gradually lost the capacity to biosynthesize these compounds. Hence, it is possible that certain gene clusters have remained homologous over evolutionary time across plants, microbes, and animals, and these might be activated by suitable plant-endophyte and/or endophyte-endophyte associations. Recently, for example, it was revealed that mammals can also synthesize morphine, which was originally considered exclusive only to *Papaver somniferum* (poppy plant) (Grobe et al., 2010). Thus, it is compelling that compounds formerly believed to be synthesized only by plants can also be produced by endophytes.

The production of natural products by endophytic fungi, once considered exclusive to plants, also raises intriguing questions regarding the original source organism. Actually, it is possible that various so-called ''plant metabolites'' could in fact be the biosynthetic products of their endophytes. An important example is production of the very potent antitumor maytansinoid ansamitocin, originally isolated from higher plants, by the Actinomycete *Actinosynnema pretiosum* ssp. *auranticum* (Yu et al., 2002). This study substantiated the possibility that the true biosynthetic source of the maytansinoid backbone could be a bacterial endophyte. Although horizontal gene transfer may explain the production of maytansinoids by plants, a more likely scenario is the production of maytansinoids by symbionts (Cassady et al., 2004).

Plant-Endophyte Interspecies Crosstalk

Considering the fact that endophytes reside within plants and are continuously interacting with their hosts, it is conceivable that plants would have a substantial influence on the in planta metabolic processes of the endophytes. For example plant homoserine and asparagine act as host signals to activate expression of a lethal gene in virulent strains of *Nectria haematococca* that is only expressed in planta (Yang et al., 2005). Furthermore, expression of the gene cluster for lolitrem biogenesis in endophytic *Neotyphodium lolii* resident in perennial ryegrass is high in planta, but low to undetectable in fungal cultures grown in vitro, lending support to the notion that plant signaling is required to induce expression (Young et al., 2006). Another convincing example is that of the symbiotic association between dicotyledonous plants (Convolvulaceae) and clavicipitaceous fungi leading to synthesis of ergoline alkaloids by the fungus, and question the origin of these compounds in plants (Kucht et al., 2004; Steiner et al., 2006; Leistner and Steiner, 2009). Recently, it was found that a camptothecin-producing endophyte, *F. solani* isolated from *C. acuminata* (Kusari et al., 2009c), could indigenously produce the precursors of camptothecin. However, a host plant enzyme absent in the fungus, strictosidine synthase, was employed in planta for the key step in producing camptothecin (Kusari et al., 2011b). This was the main reason for substantial reduction of camptothecin production on subculturing under axenic conditions. Such plant-fungus interactions compel reconsidering whether horizontal gene transfer (plant to endophyte genome or vice versa) is the only mechanism by virtue of which endophytes produce associated plant compounds (Kusari and Spiteller, 2011).

Endophyte-Endophyte Interspecies Crosstalk

It is rather uncommon that a plant is colonized by only a single type of endophyte. In fact usually the presence of diverse microorganisms is observed in plant tissues, and it is obvious that a given endophyte directly or indirectly interacts with other

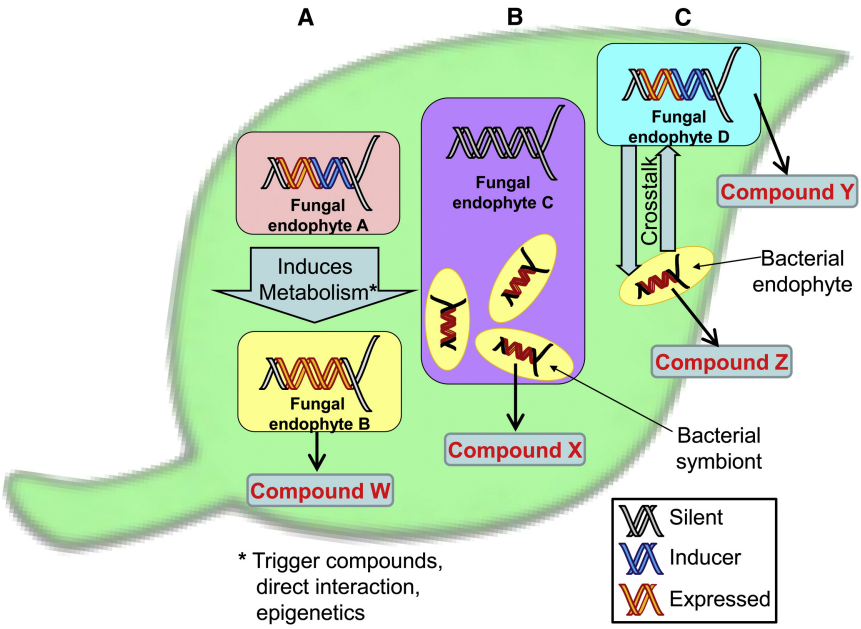

Figure 2. Schematic Representation of Endophyte-Endophyte Interspecies Crosstalk
(A) Fungus-fungus crosstalk is illustrated.
(B) Fungus-bacterial endosymbiont crosstalk is demonstrated.
(C) Fungus-bacteria crosstalk is presented.

associated endophytes within plants (fungus-fungus, fungus-bacteria, and/or bacteria-bacteria). Many recent studies provide compelling evidence that microbial interactions can play a major role in the onset of metabolite production in bacteria and fungi (Figure 2). These encounters may involve small, diffusible signaling molecules, such as quorum-sensing signals or other elicitors, which may trigger otherwise silent biosynthetic pathways (Keller and Surette, 2006; Hughes and Sperandio, 2008; Scherlach and Hertweck, 2009). However, intimate physical interactions between fungi (*Aspergillus nidulans*) and bacteria (*Streptomyces rapamycinicus*) have also been observed (Schroeckh et al., 2009), which result in an epigenetic regulation involving Saga/Ada-mediated histone acetylation of fungal secondary metabolism (Nützmann et al., 2011). This unexpected interaction led to the production of orsellinic acid-derived polyphenols such as cathepsin K inhibitors and lecanoric acid. The observation of the latter is intriguing because it is an archetype lichen metabolite (Schroeckh et al., 2009).

In light of these recent observations, it is remarkable that almost all efforts to obtain natural products from endophytes have so far been made only under axenic monoculture conditions. Thus, it would be intriguing to evaluate the endophyte-endophyte interactions and to study in more detail the secondary metabolite function in complex environments as found for endophytes. In these microbial communities, potentially every natural product could have an impact on the metabolic profiles of the microorganisms sharing the same habitat. Very likely, the interplay between endophytes within the plant results in a significantly higher natural product diversity than what is observed in individual, axenic cultures under laboratory conditions. From the point of view of the host, one should also consider synergistic effects of the "antibiotics" released, which could play a role in plant protection.

Apart from the potential cooperative role of microorganisms resulting in metabolite production, there is potentially another level of complexity, which has been neglected until recently. A recent study revealed that rhizoxin, the causative agent of rice seedling blight, is not biosynthesized by the pathogenic fungus *Rhizopus microsporus* as previously thought but by an endosymbiotic bacterium of the genus *Burkholderia* residing within the fungal cytosol (Partida-Martinez and Hertweck, 2005). Interestingly, the endosymbiont not only produces the phytotoxin but also evades fungal-resistance mechanisms (Leone et al., 2010), and controls the differentiation and sporulation of the fungal host (Partida-Martinez et al., 2007; Lackner et al., 2011). Similar scenarios are also conceivable for endophytic fungi (Hoffman and Arnold, 2010) (Figure 2B), and indeed, related symbioses involving arbuscular mycorrhizal (AM) fungi have been reported (Bianciotto and Bonfante, 2002; Tarkka et al., 2009).

Current Challenges of Exploiting Endophytic Fungi

Bioprospecting endophytes capable of producing desired bioactive secondary metabolites traditionally involves screening of a plethora of different endophytes isolated from a single host plant for identifying the "competent" endophyte with the desired trait (Scherlach and Hertweck, 2009). When employing the classical approach, often, only a few or even none of the endophytes is capable of possessing the desired potential (Kusari and Spiteller, 2011). The rest so-called "incompetent" endophytes are discarded without further investigation leading to the loss of the entire suite of natural products that they might produce under suitable conditions mimicking their natural habitat. However, recent whole-genome sequencing strategies have revealed that the number of genes encoding the biosynthetic enzymes in various fungi and bacteria undoubtedly is greater than the known secondary metabolites of these microorganisms (Scherlach and Hertweck, 2009; Winter et al., 2011). Therefore, it is compelling that the discarded endophytes might actually express only a subset of their biosynthetic genes under in vitro standard laboratory conditions such that only a minor portion of their actual biosynthetic potential is harnessed. The large reservoir of "cryptic" natural metabolites is, thus, yet to be exploited. It is even possible that they produce the desired target compounds in quantities below the limit of detection, sometimes coupled with a large "metabolic background" and discrete culture conditions. Hence, it is necessary to understand and unravel the chemical ecological interaction of endophytes to fully exploit their inexhaustible potential of natural product biosynthesis.

Future Considerations: Resolving the Present Challenges

Owing to the fact that the interaction between endophytic fungi with the host plant and other endophytes remains versatile, even slight variations in the in vitro cultivation conditions can impact the kind and range of secondary metabolites they produce. It is well established that the metabolic processes of microorganisms are critically dependent on the culture parameters (Scherlach and Hertweck, 2009). This is especially exemplified by endophytes because their range of interactions is so broad. For example the plant-associated *Paraphaeosphaeria quadriseptata* starts producing six new secondary metabolites when only the water used to make the media is changed from tap water to distilled water (Paranagama et al., 2007). Changing the medium from solid to liquid resulted in the production of radicicol instead of chaetochromin A by *Chaetomium chiversii* (Paranagama et al., 2007). Recently, the term "OSMAC" (*o*ne *s*train *ma*ny *c*ompounds) was suggested to describe the long-known effects of varying the fermentation parameters on the biosynthesis of secondary metabolites by any given microorganism, ranging from increasing the number of compounds produced to the accumulation of hitherto unknown natural products (Grond et al., 2002; Bode et al., 2000, 2002; Rateb et al., 2011). It was shown that varying the culture conditions like media composition, aeration, temperature, or shape of culturing flask led to discovery of novel natural products by various fungi and actinomycetes. Therefore, it is highly desirable to devise suitable coculture systems and challenge the complex endophyte interactions within the system by different accessible fermentation parameters, taking note of the secreted substances (such as inducers), the synergistic (or antagonistic) biotransformations, and the optimal growth and production conditions. Elucidating the optimal set of parameters will then enable the exploitation of the interspecies (or multispecies) biosynthetic pathway of endophytes in cocultures to achieve sustained production of a desired secondary metabolite (Bader et al., 2010).

The coculture systems can further be complemented by the emerging innovative biotechnological platforms encompassing evolutionary, comparative, and community genomics, proteomics, metabolomics, secretomics, transcriptomics, high-throughput and next-generation sequencing (NGS) technologies, and bioinformatics (Greenbaum et al., 2001). These will provide the comprehensive understanding of the endophytic molecular interactions and signal transduction, cross-species gene expression, and switch-on/off of the required gene cascades leading to the sustained production of a desired compound. The endophyte-endophyte differential gene expression can be enumerated using the conventional suppression subtractive hybridization (SSH) technique to generate subtracted cDNA or genomic DNA libraries (Diatchenko et al., 1996). Additionally, high-throughput tag-based methods such as serial analysis of gene expression (SAGE) (Velculescu et al., 1995), cap analysis of gene expression (CAGE) (Kodzius et al., 2006), and massive parallel signature sequencing (MPSS) (Brenner et al., 2000) that overcome the limitations of the conventional Sanger sequencing can be employed to quantify the precise digital gene expression levels of endophytes that ensue upon suitable association. Hybridization-based and inexpensive cDNA microarrays can also be used to monitor the endophyte gene expression patterns (Schena et al., 1995).

Recently, several NGS technologies have been developed that have many advantages over the aforementioned approaches (Metzker, 2010). For example the high-throughput mRNA deep sequencing (RNA-Seq) is a unique approach in mapping and quantifying transcriptomes (Wang et al., 2009). RNA-Seq overcomes the limitations of hybridization-based approaches in that it not only detects transcripts corresponding to existing (known) genomic sequences but also nonmodel organisms with undetermined genomic sequences. This makes it suitable for evaluating the endophyte-endophyte and endophyte-plant interactions and gene expressions, even when dealing with novel endophytes (genome not sequenced). Thus, the signaling of an endophyte with the plant and with other coexisting endophytes can be traced and quantified for a comprehensive characterization of their mutualistic association. Finally, it is even possible to sequence RNA isolated from just one endophytic hypha or its adjacent host plant cells by coupling such high-throughput methods to laser microdissection. For instance a TOM2 microarray coupled to laser microdissection systematically revealed the transcriptional changes triggered in *Solanum lycopersicum* (tomato plant) shoots and roots as a result of infection and colonization by the AM fungus, *Glomus mosseae* (Fiorilli et al., 2009). Thus, future studies to procure fundamental insights into endophyte-endophyte and plant-endophyte communication using the available and emerging tools would not only allow the discovery and sustainable production of desirable natural products but also other mostly overlooked secondary metabolites thereby unraveling the comprehensive potential of endophytes.

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