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Chapter

The Infection Unit: An Overlooked Conceptual Unit for Arbuscular Mycorrhizal Function

Yoshihiro Kobae

Abstract

Most land plant species have their roots colonized by arbuscular mycorrhizal fungi (AMF). These symbiotic associations are often found in the roots of field crops. The biological basis and practical significance of this symbiosis have been extensively studied, and the molecular mechanisms underlying the initial colonization process and the nutrient exchange between the host plant and the AMF have been elucidated. However, developmental processes and turnover of elements of the mycorrhiza, and the resulting changes in mycorrhizal function, are not well understood. The enigmatic nature of the development-function relationship is probably due to the short life span of the infection unit, which has largely been overlooked in studies investigating mycorrhizal function at the macroscopic level. This paper outlines the concept of the infection unit and functional expression patterns in terms of the transient aspects of the micro-symbiont during its life cycle in this symbiosis.

Keywords: arbuscular mycorrhiza, functional molecular markers, infection unit, life cycle, live imaging

1. Introduction

The roots of approximately 95% of vascular land plant species, with the exception of some families (e.g., the Brassicaceae, Amaranthaceae, and Polygonaceae), are colonized by symbiotic fungi which form a mutualistic relationship (mycorrhiza) with the host plant roots [1, 2]. Approximately 10% of vascular plant species, mostly woody species, are colonized by ectomycorrhizal fungi, which belong to the *Basidiomycota*, *Ascomycota*, and (less commonly) *Zygomycota*, and the fungal hyphae grow extracellularly, forming a mantle of mycelium around the roots [3, 4]. Most of the remaining mycorrhizal fungi, with the exception of family-specific mycorrhiza, such as the ericoid or orchid-specific mycorrhizal fungi, colonize nonwoody plant species and belong to the subphylum *Glomeromycotina* [3, 5]. This fungal group is generally known as the arbuscular mycorrhizal fungi (AMF), because these fungi form highly branched hyphal structure, known as arbuscules, in root cortical cells, and spread intercellularly (*Arum*-type) or intracellularly (*Paris*-type) [6]. The formation of arbuscules has been regarded as the unique morphological feature of this symbiosis responsible for the nutrient (particularly phosphorus) exchange between the host plant and the AMF [7]. Arbuscule formation occurs in parallel with the

expression of a specific cellular system to allow the accommodation of AMF within the root tissue and to achieve nutrient (e.g., phosphorus and nitrogen) uptake via AMF mycelia [8, 9]. Genetic disruption of genes in the symbiotic system of model plants (e.g., *Medicago truncatula*, *Lotus japonicus*, *Oryza sativa*) has revealed the nutritionally beneficial relationships between plants and AMF [10–12].

AMF can also colonize thalli of the early nonvascular land plants, namely, the liverworts and hornworts [13, 14]. Phylogenetic analyses indicated that symbiotic genes are present in the genomes of these early land plants, with the functions of the encoded proteins being conserved, suggesting that this symbiosis is phylogenetically widespread in plants [15]. AM symbiosis is beneficial for plants in relation not only to nutrition but also to the mitigation of biotic and abiotic stresses (e.g., resistance to pathogens, tolerance of drought and toxic element stress, increased biomass production, and secondary metabolite accumulation) [16–19]. Hence, the functionality of AM symbiosis can influence the productivity and quality of crops.

The effects on the host plant of AM symbiosis are commonly investigated by inoculation of the roots of the host plant with a (usually) single-species AMF in pot culture. However, field-grown inoculated roots can harbor AMF species other than the test AMF species, the functionality of which would not have been tested using an inoculation test because not all AMF in the roots can sporulate [20]. In addition, not all AMF which colonize roots are active and functional [21, 22], and the colonization process and the stability of the colonization by diverse AMF species in the same field-grown roots are unclear [23]. Overall, under field conditions, the functionality of AM symbiosis is probably based on unknown but highly dynamic associations between plants and a diverse range of AMF species. To better understand this complex association, in the current article, the colonization dynamics of AMF species in roots will be discussed. To understand the functional unit of the plant-AMF symbiosis, it is important to outline the concept of the colonization unit.

2. Latent colonization dynamics in arbuscular mycorrhizas

In 1905, an illustration by Gallaud showed that root cortical cells often contain "clumps" of arbuscules (**Figure 1**) [24]. Subsequent morphological examination of mycorrhizal roots at the cellular level suggested that this intracellular colonization may be ephemeral [25–27]. Following this, morphometric studies, coupled

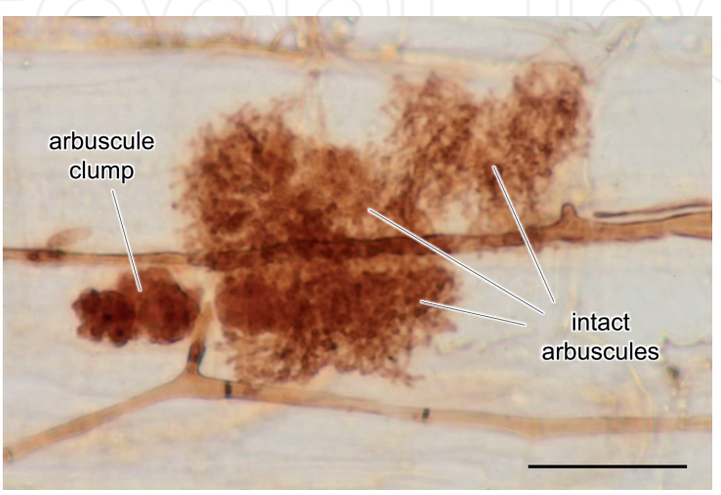


Figure 1.
Arbuscule has a short life span. Image of sunflower (Helianthus annuus) mycorrhizal roots grown in field soil. 3,3'-diaminobenzidine (DAB) staining with horseradish peroxidase (HRP)—Wheat germ agglutinin (WGA) [65]. Bar = 50 μm.

with electron microscopy, calculated that the life span of an intact arbuscule in several plant species was a minimum of 2.5 days [28, 29]. Such a limited life span of the units of intracellular colonization by AMF can be generalized because these arbuscule clumps have been observed in many plant species, including the relatively primitive nonvascular plant, the liverwort [13]. Why does this mutually beneficial association exhibit such short-lived units of intracellular colonization? Unfortunately, our understanding of this phenomenon has not increased much since the first observation of arbuscule clumps by Gallaud more than 110 years ago.

Advances in the forward and reverse genetic approaches available to decipher the molecular mechanism for the AMF colonization process have shown that several signaling mutants of model plants exhibited compromised AMF epidermal penetration and altered chemical and cellular crosstalk in the initial stages between plants and AMF [10, 30]. Recently, several mutant lines exhibited suppressed intraradical colonization, forming prematurely senescent or stunted arbuscules [11, 31]. Some plant genes have been implicated in the cellular process of arbuscule degeneration [32–35]. The degeneration process of arbuscules has also been shown to be related to colonization level, illustrating the importance of arbuscule life cycle to the development of mycorrhiza. In addition, live imaging of the green fluorescent protein (GFP)—symbiotic phosphate transporter (PT11) fusion protein in the roots of mycorrhized rice seedlings—also revealed the limited life span of the units of intracellular colonization, in which the rapid collapse of arbuscules was observed [36]. The mechanism of mycorrhization, coupled with such a short life span of the individual colonization unit, has been addressed by continuous (long-term) live imaging of the symbiotic marker secretory carrier membrane protein (SCAMP) [37], where successive de novo colonizations underlie AM development (SubSection 2.2). These findings emphasize the importance of the life cycle of colonization when we consider the dynamics of AM functionality.

2.1 Concept of the infection unit

In soils, there are many different AMF species, and no strict host-AMF specificity has been observed [38, 39]. Accordingly, under field conditions, co-colonization of the same root by multiple AMF species can occur [2]. It is likely that the functionality of these diverse AMFs is not the same, and colonization by each AMF may last only a short time. Therefore, to correctly characterize the functionality of field mycorrhizas, the colonization process, the dynamics, and the functionality of the diverse AMFs in the roots need to be understood.

When the fungal spore germ tubes approach the root surface, chemical crosstalk occurs between the roots and the AMF hyphae, triggering the molecular and cellular remodeling process necessary for hyphal entry into the roots [11]. Coinciding with this pre-symbiotic crosstalk, AMF hyphae around the roots are often highly branched, giving rise to a characteristic cascade-like mycelium, composed of lateral branches [2]. Several plant mutants exhibiting disruption of the early signaling process fail to allow hyphal penetration of the epidermal layer [10, 11], suggesting the importance of this initial mutual recognition process.

After hyphopodia are formed, hyphae penetrate the rhizodermal layer and grow longitudinally in the root cortex. Short branches from the longitudinally extending hyphae penetrate the cortical cell walls and branch dichotomously in the cell lumen to give rise to arbuscules. Importantly, the maximum elongation in the cortex of hyphal structures derived from a single or a few hyphal penetrations of the epidermis are reported to be up to 20 mm [2]. In rice seedlings, however, the maximum elongation in the cortex of hyphal structures derived from the entry point is only 0.5 mm [37]. In general, the area occupied by each colonization unit derived from a single epidermal entry is difficult to recognize, because intraradical hyphae derived from different

entry points immediately overlap one another to form a larger colonization area within the cortex. The rates of growth of intraradical colonization are reported to range from 0.13 to 1.22 mm/day [2]. In the live imaging of mycorrhizal rice roots, the rate varied from 0.42 to 1.68 mm/day [36]. Although the maximum length of intraradical colonization derived from one or a few penetrations varied greatly among studies, these independent colonies of mycelia are called the "infection unit" (**Figure 2**) [25].

2.2 Development and functionality of mycorrhizas: an infection unit-based view

The development of the mycorrhiza and the dynamics of the infection unit are tightly linked processes during mycorrhization. It is also likely that the functionality of mycorrhiza in the field is also variable, depending on the functionality of the infection unit that is derived from different AMF species. Live in situ imaging revealed that multiple infection units overlap to form a larger infection ("colonized region")

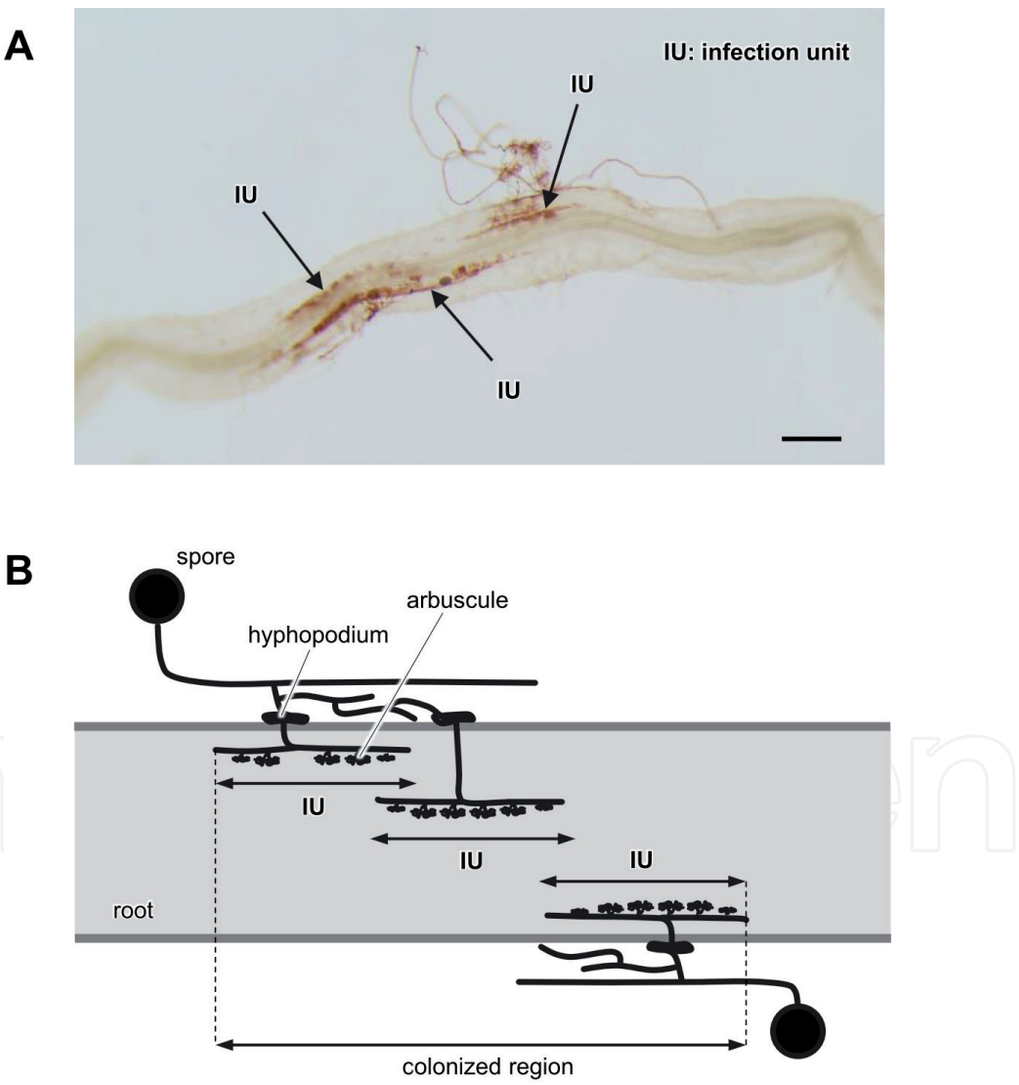


Figure 2.
The relationship between infection unit and mycorrhizal development. (A) Image of Lotus japonicus seedlings grown in field soil taken at 14 days post plantation. 3,3'-diaminobenzidine (DAB) staining with horseradish peroxidase (HRP)—Wheat germ agglutinin (WGA) [65]. Bar = 200 μm. (B) Model diagram of mycorrhization process. AMF spores germinate in the soil, and germ tubes approach the root surface and form a hyphopodium. The young infection unit, comprising an internal mycelium arising from one hyphal entry, grows and develops new arbuscules at the infection fronts. In many cases, new infection units develop immediately adjacent to established infection units. Colonized regions that were bound by two infection fronts, comprising intercellular and intracellular hyphae, develop through the successive formation of infection units. Arbuscules collapse from near the hyphopodia as a result of their short life span.

within a few days [36, 37, 40], making the delimitation of each infection unit experimentally difficult. Coupled with the difficulty of differentiating between different AMF species in the roots, using morphological or molecular approaches [41, 42], the dynamic process of mycorrhization has probably hampered progress in characterizing the infection unit-based functionality of field mycorrhizas. Live imaging of rice mycorrhizal roots revealed that the expansion of the colonized region occurs in concert with successive de novo formation of multiple infection units (**Figure 2**) [37].

However, a better understanding of the dynamics of the mycorrhization process depends substantially on the quality and the ease of imaging of the hyphae within the roots. Traditionally, cytological studies of the colonization process have been performed with chemical staining of fixed (i.e., dead) AMF structures or in situ visualization of their enzymatic activities. In recent studies, however, imaging of non-fixed root samples by means of fluorescent molecular markers of the symbiotic process has been used to improve our understanding of colonization dynamics at the cellular level. As many parts of the molecular mechanism implicated in AM symbiosis are common to nodule symbiosis, several molecular markers are available in the model legume plants, *M. truncatula* and *L. japonicus* [30]. However, live imaging of mycorrhizas in model legumes by means of fluorescent markers is difficult due to the presence of highly autofluorescent materials in the root tissue and the presence of thick (multiple) cortical cell layers that decrease transparency [43]. Such poorly transparent root tissues are not particularly useful for macroscopic imaging of the dynamics of the infection unit.

Rice (*O. sativa*) is commonly grown in paddy fields, where it is rarely colonized with AMF. However, rice roots are colonized with AMF under semidry conditions. The root systems of leguminous species have cambium, and the diameters of primary and lateral roots are generally uniformly large. On the other hand, grass roots do not have cambium, and lateral roots are smaller in diameter than the primary (crown) root. In rice seedlings, lateral roots with a few cortical layers are the main site of AMF colonization, and the average diameter of roots is less than 200 μm [36, 44]. In addition, the concentrations of autofluorescent root materials are quite low, and some symbiotic molecular markers are available for live imaging [36, 37, 45].

Transcriptome analysis of mycorrhized rice roots revealed that an AM-specific marker gene of rice, *AM42*, which encodes a SCAMP, is specifically expressed in mycorrhized roots [46, 47]. A GFP-tagged SCAMP protein was localized in the endomembrane systems of colonized cells and even in cells with collapsed arbuscules, allowing live imaging, coupled with GFP-SCAMP, to evaluate the colonization and recolonization sequences. Live imaging of GFP-SCAMP revealed that the average lifetime of intact arbuscules was 1–2 days. Cortical cells with collapsed arbuscules were rarely recolonized, whereas new colonizations occurred in close proximity to cells containing collapsed arbuscules, contributing to the expansion of the colonized region. Collapsed arbuscule-containing cells are intact [2]; however, colonization spread readily into an uncolonized region of roots but sparsely into a previously colonized region, suggesting that successive formation of new infection units is required for continuous mycorrhization [37, 48]. It is unlikely that the collapse and the presumed digestion of arbuscules play a significant role in nutrient transfer from fungus to plant [2].

The concept that mycorrhization is linked to the successive formation of infection units is supported by the observation that decreased hyphopodium formation leads to decreased mycorrhization. Under low-phosphate conditions, roots secrete strigolactones (SLs), which are carotenoid-derived phytohormones. A chemical analog of SLs, GR-24, activated mitochondrial respiratory activity and facilitated hyphal branching of *Gigaspora margarita* or *G. rosea* under in vitro conditions [49–51]. In SL biosynthesis-defective rice mutants, the hyphal branching of a model AMF, *Rhizophagus irregularis*, around the roots (rhizospheric hyphal branching) was

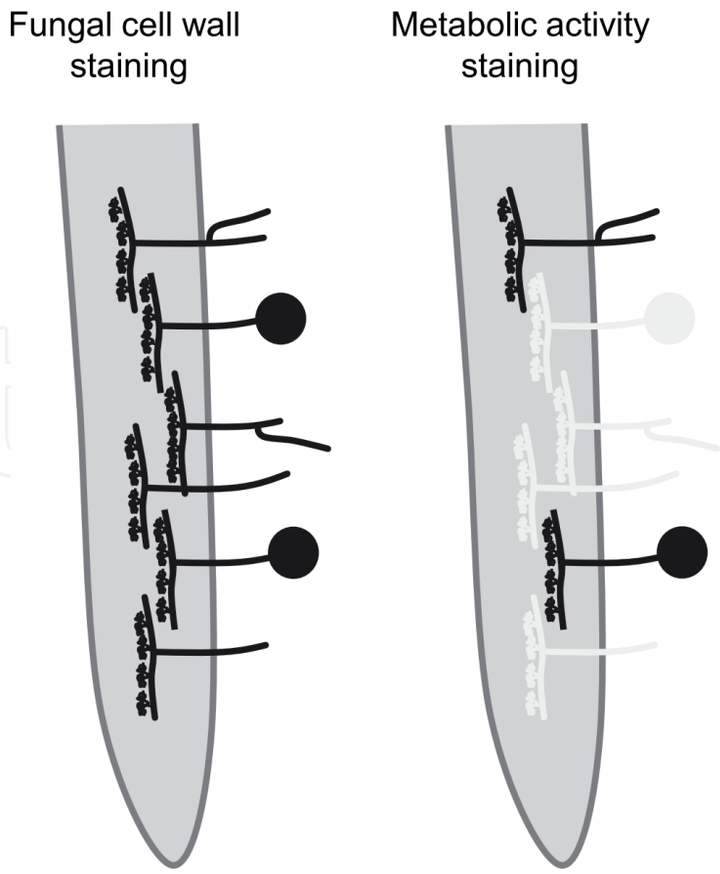


Figure 3.
Not all infection units revealed with fungal cell wall staining are metabolically active. Roots colonized with native AM fungi in field soils were subjected to cell wall (chitin) staining and vital staining to detect the presence of AMF and metabolically active AMF, respectively [60]. Vital staining, which histochemically visualizes the activity of succinate dehydrogenase (SDH), a tricarboxylic acid cycle enzyme in AM fungi, using the reduction of nitroblue tetrazolium (NBT) into insoluble formazan, detects metabolically active colonization [66]. The number of infection units detected by vital staining was lower than that determined by cell wall staining. In this analysis, rice (Oryza sativa L.) was used as the host plant because (i) the morphology of the development of infection units is well understood [36, 37]; (ii) active infection units rarely coalesce in roots [37], probably due to the small number of cortical cell layers [67, 68]; and (iii) the vital staining is convenient for detecting a single infection unit [36, 44].

normal [48]; however, in the SL biosynthesis-defective mutants of pea, tomato, and rice, the percentage root length colonization was significantly reduced [52–55]. In the rice SL biosynthesis mutants, the formation of the hyphopodium was delayed, compared with the wild type, but intraradical colonization was normal, indicating that the early formation of infection units, initiated by timely hyphal entry into epidermal cells, is necessary for the normal development of a colonized region [48].

In the field, multiple AMFs, from different species, can co-colonize roots, with multiple AMF species being detectable in only a 1-cm-long root fragment [56]. Thus, field roots can be regarded as a mosaic of the various functionalities of the different AMFs [57]; alternatively, only a portion of the AMF infection unit colonizing the roots may temporarily contribute to particular functions in response to specific environmental conditions [58]. However, the dynamics of the functionality of the respective AMFs in field roots have been little studied. Abiotic and biotic factors may influence the AMF composition, at least over a long time period [59], but the short-term effects of such environmental factors may also influence the various active (functional) AMFs as well as the functionality of the mycorrhiza as an entirety under field conditions. For example, not all infection units containing fine-branched arbuscules in roots grown in field soils are metabolically active (**Figure 3**) [60]. Further study will be needed to understand the functional dynamics of field mycorrhizas by considering infection unit-based colonization dynamics.

3. Conclusion

Our understanding of the molecular mechanism underlying the plant nutrient uptake system has been greatly advanced by the use of molecular studies and genetics. These studies have been based almost entirely on the plant alone. However, the plant nutrient uptake system is largely dependent on the largely undiscovered functionalities of diverse soil microorganisms. Roots of plants in the field are generally colonized with a range of different AMFs, but the functionality of these individual species is largely unknown. In addition, the genetic structure of AMF is quite enigmatic [41]. Recent studies into single-nucleus sequencing of some AMF culture lines demonstrated the presence of not only homokaryons but also dikaryons and heterokaryons [61, 62]. Furthermore, long-read whole-genome sequencing of *R. irregularis* DAOM197198 indicated that the genome contained 10 different rDNA sequences that were scattered (i.e., non-tandem repeats) around the chromosome [63]. These findings mitigate against the use of rDNA sequences to identify individual AM species in field AMF infection units, as a one-to-one relationship may not be applicable to the rDNA sequences and the genetic identities of the individual component species.

As mentioned before, AM infection units have a short life span and collapse within a few days, at least in the live imaging of rice seedlings [36, 37]. The development of mycorrhizal roots is associated with the turnover and de novo colonization by new AMFs, providing the opportunity to allow different AMF species to colonize the roots, depending on the context (environmental factors, plant growth stage, nutrition, etc.). Accordingly, field mycorrhizal roots can comprise multiple functionalities with different AMF species.

Plant breeding programs are not able to select for the genomic properties of plants adapted to all field conditions (soil type, water content, nutrient level, climate, etc.). On the other hand, plant roots closely interact with the native fungal partners that may have genetically recorded beneficial traits for adapting to the local environment. The specific phenotype (functionality) of native AMFs may be conferred by accessory genes that are not shared by all members of a species [64]. However, due to the complexity of the genetic basis of AMF individuals, it is difficult to understand which AMF genes are really functional in the roots. In a model plant (e.g., rice), the thin root cortex would allow us to isolate the genetic information of AMF individuals in the form of the infection unit. Furthermore, rice roots are technically suitable for detecting metabolic activities by means of vital staining. Furthermore, transgenic rice producing fluorescent molecular markers (e.g., phosphate transporter, GFP) is available to assess the functionality of the AMFs in situ. Thus, future studies should focus on the functionality of field AMF individuals, with emphasis on the genetic information and the dynamic functionality.

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Conflict of interest

The author declares that they have no conflict of interest.

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