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Symbiotic microorganisms, a key for ecological success and protection of plants

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Abstract

Plant-associated microbial diversity encompasses symbionts, protecting their host against various aggressions. Mycorrhizal and rhizospheric microorganisms buffer effects of soil toxic compounds and soil-borne pathogens. Endophytic bacteria and fungi, some of which are vertically inherited through seeds, take part in plant protection by acting directly on aggressive factors (mainly pathogens and herbivores) or by enhancing plant responses. Plant protective microbial symbionts determine the ecological success of plants; they drastically modify plant communities and related trophic webs. This review suggests approaches to improve the inventory of diversity and functions of in situ plant-associated microorganisms. **To cite this article:** *M.-A. Selosse et al., C. R. Biologies 327 (2004).*

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Résumé

La diversité des microorganismes symbiotiques : une clef pour la réussite écologique et la protection des plantes. Parmi les microorganismes associés aux plantes se trouvent des espèces bénéfiques, qui protègent leurs hôtes contre les agressions. Ainsi, les microorganismes mycorrhiziens et rhizosphériques protègent des composés toxiques et des pathogènes du sol. Certains endophytes, bactéries et champignons, parfois transmis par les graines, protègent la plante en agissant directement sur les facteurs d'agression (pathogènes et herbivores surtout) ou en stimulant les défenses végétales. Ces symbioses protectrices déterminent le succès écologique des plantes ; elles modifient communautés végétales et réseaux trophiques. Cette synthèse aborde la nécessité et les moyens d'étudier la diversité fonctionnelle des microorganismes associés aux plantes in situ. **Pour citer cet article :** *M.-A. Selosse et al., C. R. Biologies 327 (2004).*

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“Mutual aid is met with even amidst the lowest animals, and we must be prepared to learn some day [...] facts of unconscious mutual support, even from the life of microorganisms”
P. Kropotkin, 1902 [1].

1. Introduction

Physiologists have often considered the plant as a single organism, without encompassing the diversity of its microbial symbionts. As a result, the latter’s crucial roles were sometimes overlooked. Among the various reasons for this are the fact that the presence and diversity of these microbial symbionts may be hidden because of their small size and their inclusion within substrates. Similarly, this trend is reinforced by the search for plant models that can be cultivated axenically under laboratory conditions and which are therefore more convenient for experimental purposes. For example, although more than 90% of terrestrial plants associate with soil fungi to form mycorrhizae [2], the choice of non-mycorrhizal models, such as *Arabidopsis thaliana*, overlooks the common importance of fungal partners. In recent decades, however, a rising interest in plant-associated microbial life, both in ecology and plant physiology, has reduced this bias. We will summarize here mutually beneficial (mutualistic) interactions between plants and microorganisms that live in close physical association, i.e. that live in symbiosis. Major emphasis will be laid on the protective effects of microbial symbionts on plants. This review aims (i) to provide an overview of recent advances in plant protection by symbiotic microorganisms at time of writing (November 2003), (ii) to demonstrate the ecological implications of this protection, and (iii) to suggest some descriptive and experimental approaches required to analyse such symbioses further.

2. Mycorrhizae: from trophic to protective effects

Plants associate with many soil microbial symbionts that improve their nutrition. The most widespread association is mycorrhizal symbiosis, involving soil fungi and plant roots [3], a symbiosis supposed to be ancestral and to date back to the colonization of land ecosystems [2]. In a mycorrhiza, the plant receives water and mineral nutrients collected in soil by the fungal partner [3]. Some plants also fulfil their nitrogen requirements by contracting associ-

ations with N₂-fixing prokaryotes, such as legumes with *Rhizobiaceae* (see below), and several Rosids with Cyanobacteria and Actinomycetes [4]. In every case, host-plants provide carbon to their symbionts as a reward. However, since symbiosis can be defined as a reciprocal enhancement of fitness, a bilateral nutrient flux is not exclusively required and any protective effect of one partner toward the other, enhancing the latter’s survival or reproduction, is sufficient to establish a symbiotic relationship. The extent to which protective effects contribute to mycorrhizal symbiosis remains controversial among ‘mycorrhizologists’. It has been claimed that, in temperate ecosystems, plants with highly branched root systems (therefore well able to explore soil) mainly benefit from protection by the fungus [5], while others, having more reduced root systems, may profit more from nutrition by the fungus (even if protective effects coexist, they may not be the primary benefit for such plants).

Mycorrhizal fungi have protective effects on their host that result partly from better nutrition [6], but also involve more specific interactions. Mycorrhizae have long been reported to counteract soil-borne pathogens, as suggested by comparison of mycorrhizal *versus* non-mycorrhizal plants [7,8] (note, however, that the non-mycorrhizal state is abnormal for most species and represents a biased situation). Hence, it has been suggested that mycorrhizae are involved in a ‘prophylactic-like’ effect [9]. Direct antibiotic properties of fungal partners have been proposed, but not convincingly demonstrated, to act, so that the most favoured nutrition-independent effects are (i) microbial competition and (ii) modification of plant physiology. In the competition model, the mycorrhizal fungus is depleting nutrients in the rhizosphere and plant tissues, as measured in some studies [10], thus limiting the settlement of pathogenic intruders. In plant physiological modification models, fungal symbionts elicit plant defences that, although allowing mycorrhizal colonization, prevent pathogenic aggressions (and perhaps limit excessive growth of symbionts). Biochemical molecular markers for plant defence (pathogenesis-related proteins with anti-microbial activities, phytoalexins, wall-reinforcing lignification...) are elicited in several model mycorrhizae [11,12]. Protection operates not only in mycorrhizae but sometimes also in other distant plant organs, a process called ‘systemic resistance’ [13]. However, it is not

demonstrated that such a ‘pre-immunization’ alone accounts for plant protection. At least roots also benefit from direct competitive interactions between mycorrhizal fungi and pathogens. Last, it should be noted that reciprocal negative interactions exist, i.e. spoliation of plant photosynthates by parasites can affect the mycorrhizal interaction [14].

Mycorrhizae also protect against soil abiotic stresses [3], such as toxic molecules, but here again, nutritional influences are difficult to exclude. In calcareous soils, root protection is well documented [15]. In addition to increased mobilization of phosphorus and iron, two elements with low solubility in calcareous soils, a true protection against Ca^{2+} is clearly involved. Ectomycorrhizal fungi possess an active Ca^{2+} efflux and precipitate it as inert calcium oxalate, thereby detoxifying calcium. Similar processes are likely to confer resistance to aluminium, which is mobilized in acidic soils, and various heavy metals from contaminated soils [16]. Moreover, ectomycorrhizal fungi can tolerate and adapt to heavy metals [17]. In addition to reducing the concentration of toxic compounds, mycorrhizal fungi can buffer stress reactions entailed by these compounds, e.g. heavy metal-induced oxidative stress [18]. Mycorrhizae are also involved in protection against water stress [19], mainly thanks to drought avoidance, via better access to water through hyphae. But in some cases, a protective drought tolerance is promoted by modification of plant physiology, i.e. osmotic modifications in roots [20] and decrease of lethal water potential [19].

Another well-studied trophic symbiosis is the association between most Legumes and Rhizobiaceae (for review, see [4] and [21]), forming nodules where the bacteria reduce and assimilate atmospheric N_2 , thus improving plant nutrition. In addition, Rhizobiaceae produce phytohormones, which can influence plant development and also act as biocontrol agents, with suppressive effect against a wide range of fungal pathogens [22]. However, mycorrhizae and nodules are but the tip of the iceberg of associations between plant and soil microorganisms.

3. Other rhizospheric symbionts and their protective roles

Numerous microorganisms live in the portion of soil modified or influenced by plant roots, the so-

called ‘rhizosphere’ [23]. Among these microorganisms, some have positive effects on plant growth [24], constituting the ‘plant growth-promoting rhizobacteria’ (or ‘PGPR’, such as *Azospirillum*, *Agrobacteria*, *Pseudomonas*, several Gram+ *Bacillus*, etc.). PGPR are involved in free N_2 fixation (for review see [25]), but their level of contribution to nitrogen cycling remains poorly documented [26], and they likely produce phytohormones and vitamins. Although their role was recognized more recently than for mycorrhizae or nodules, many rhizospheric microorganisms also contribute to plant protection. They provide useful experimental models since genetic investigations, which remain poorly developed for elucidating mechanisms of plant protection by mycorrhizal fungi, have been often used for rhizospheric microorganisms, mainly bacteria, to identify critical genes.

For example, some rhizospheric microorganisms buffer abiotic stresses by modifying the root environment: exopolysaccharide-producing bacteria can, for example, alter the soil structure and porosity, thereby protecting against water stress (excess or deficiency), buffering temperature variations and allowing better root penetration in soil [27]. They can also degrade adverse compounds, such as xenobiotics and aromatic molecules [28], and constitute a major factor for life in extreme soil conditions.

Protection against pathogens mediated by PGPR is well documented and involves various processes [29]. The proposed mechanisms parallel these of protection by mycorrhizal fungi. First, competition can limit the growth of pathogens [30]: for example, antagonistic abilities of some PGPR *Pseudomonas* spp. correlate with production of Fe^{3+} -chelating siderophores [30], which reduce availability of iron, a limiting resource in the rhizosphere. Similarly, the well-known suppressive effect of non-pathogenic *Fusarium oxysporum* strains [31] is likely explained by a competition with pathogenic strains for rhizospheric carbon as well as for space and infection sites, on the root surface or even after infection has started [32]. Second, direct interactions between symbionts and pathogens also reduce the latter’s virulence. For example, this has been reported for plant-protective *Trichoderma* spp. that are mycoparasites on several phytopathogenic fungi [33] and produce extracellular enzymes degrading the phytopathogenic cell walls [34]. Production of antimicrobial substances, probably selected by competition for the

plant resources, also acts [35], as demonstrated by mutant strains that simultaneously lack toxin production and protective effects. This accounts for the decline of a major wheat disease, the ‘take-all’ (*Gaeumannomyces graminis* var. *tritici*), for which a spontaneous reduction in fungal virulence (about 30%) is observed after some years of wheat monoculture. This decline depends on establishment of a protective soil microflora, mainly rhizospheric *Pseudomonas* spp. [36] producing several phenazine antibiotics with antagonistic effects [37]. Phenazines, which allow competitive survival of the producing *Pseudomonas* spp. in soils [38], reduce the severity of take-all by impairing fungal growth. Third, besides competition and direct interaction, some PGPR strains activate an induced systemic resistance (ISR) response [25,39]. ISR is elicited after contact with rhizospheric agents [40, 41] and involves production of elicitors or analogues of plant hormones taking part in signalling during systemic resistance, such as salicylic acid [42,43].

Protective effects of competition, direct antibiosis and enhancement of plant defences often act synergistically in the rhizosphere [44]. This mirrors the microflora from animal digestive tracts, which benefits its host by similar means. Animal gut microflora and rhizosphere microflora both buffer environmental aggressions, and lead to avoidance of these aggressions or, less commonly, to tolerance phenomena.

4. Shoot and leaf endophytic protective microorganisms, and their transmission

Microbial symbionts not only occur in soil and roots, but also in all other plant tissues. In the last decade, a diverse endophytic microflora, including fungi [45–48] and bacteria [25,49–51], was isolated or identified by DNA-based methods from various symptomless parts of plants. This microflora includes latent or newly incoming pathogens [52], saprotrophs [53] (and sometimes contaminants!), but also symbionts [46]. In 1977, C.L. Wilson wrote that “considering the varied beneficial fungus-root associations (mycorrhizae) that have evolved, it is reasonable to suspect that beneficial fungus-leaf associations (‘mycophyllae’) may have also evolved. To my knowledge, none have been described” [54]. As progressive awareness of their existence developed, it became obvious at the end of the 80’s that “endophytes may be as common

as are mycorrhizae [sic]” [55], and as relevant to plant physiology as well. Since many of these associations, although regularly present, are not obligate for plants, endophyte-free (E–) individuals can be easily obtained by antibiotic or fungicide treatment. This allows comparison of (E–) to symbiotic (E+) plants, sometimes in an identical genetic background. Endophytes are mostly supposed, but rarely shown, to fix free N₂, such as *Azoarcus*, *Herbaspirillum* and *Acetobacter* [56], or to produce vitamins required for plant growth [57]. But many of them display protective effects, as we will exemplify.

A well-studied case, the grass endophytes, involves fungal symbionts from the genus *Neotyphodium* (*Clavicipitaceae*) [58]. Here, a large array of alkaloids protects the plants *via* their toxic and deterrent effects on herbivores [59]. Note that similar alkaloids are well known in the related plant pathogenic Ergot (*Claviceps purpurea*), but grass endophytes produce symptomless infections. In a comparison between (E+) and isogenic (E–) ryegrass (*Lolium multiflorum*), the number of parasitic aphids per plant was reduced by about one-fourth [60] (see below), and cattle pasturing on (E+) grasses lost one-third of their fertility and milk production (‘fescue toxicosis’, [61]). In addition, *Neotyphodium* infections also entail plant protection against nitrogen starvation or water stress [62], but, here, the mechanism is still far from clear. Besides grass endophytes, many other symptomless endophytes in plants have shown protective effects: leaf infection of *Theobroma cacao* (*Malvaceae*) by non-systemic endophytic fungi correlates with improved local reduction of damage by *Phytophthora* spp. [48]. Other endophytes protect against abiotic adverse factors, as exemplified by the grass *Dichanthelium lanuginosum* from Californian geothermal soils (temperature fluctuating from 20 to 50 °C), endophyted by a *Curvularia* species. (E–) plants are no more thermo-tolerant [63]: they grow normally at low temperature but die above 40 °C, while (E+) plants can survive up to 65 °C. Endophyte protection is also reported among algae of the tidal zone, which endure many stresses (thermal, hydric, photic) at low tide: fungal endophytes positively act upon algal resistance to stress [2] or grazing avoidance [55]. In many cases, like the two previous ones, the protective mechanisms remain ill understood, although profound modifications of host morphology and metabolism by endophytes are likely

involved [2,64]. However, in contrast with mycorrhizal associations, very little is known of the physiology of endophytic associations.

Many symbiotic endophytes are related to pathogenic taxa, from which they probably arose [58,59,65], and indeed, a continuum of interactions exists [66]. In *Colletotrichum magna*, a necrotic pathogen on cucurbits, mutant strains were obtained that infect their hosts without symptoms and protect them against other pathogens [67], probably by eliciting plant defences [68,69]. The fact that one of these strains is mutated in a single (yet not characterized) gene [67] suggests that protective strains can frequently emerge in evolution of pathogenic species [65]. Co-evolution of plant lineages with such protective endophytes, e.g. *Colletotrichum*-related, has still to be investigated.

Many symptomless endophytic bacteria [57] and fungi (such as grass endophytes [58]) are vertically transmitted: the lack of visible symptoms correlates with the lack of horizontal (plant-to-plant) transmission. Neither hyphae nor spores emanate from infected plants, and endophyte survival depends upon colonization of the host seeds. This feature stabilizes the symbiosis, as the endophyte fitness is tightly linked to that of the host plant. It creates a selective pressure favouring every mutation that improves host fitness, while any cheating strain, i.e. less rewarding for its host than the other endophytes, is counter-selected. In addition, vertically transmitted symbioses avoid spread of cheating strains, as they are unable to colonize other plants by horizontal transmission. These maternally inherited protective effects constitute an interesting feature in view of selection and use of bioprotection for crop production.

5. Microbial protection and plant ecological success

We have hitherto focused on the relevance of symbiotic protection to plant physiology, but recent work also emphasizes that such symbioses have further implications at the ecosystem level, by way of – or as a side effect of – plant protection. A striking piece of evidence for this, also illustrating that hidden symbionts should not remain ignored, comes from the tall-fescue (*Festuca arundinaceae*) history. In 1943, the tall-fescue cultivar ‘Kentucky 31’ was released and soon became one of the most widely grown forage

grasses (15 million ha currently), because of its ease of establishment, long growth and grazing season, as well as its broad adaptation to various, even poor, environmental conditions. However, livestock productivity on Kentucky 31 pastures remained unexpectedly low [61]. It was soon discovered that the selected agronomic characteristics of this cultivar relied on the presence of a highly toxic and stress-protective endophyte, *Neotyphodium coenocephalum* [58]. Kentucky 31 now persists as an invader of natural ecosystems, threatening several native species and entailing 800 million dollars in costs every year to US agriculture. Recent field experiments demonstrated that the competitive ability of (E–) fescues was insufficient (i) to maintain them at constant biomass level over years in field conditions, and (ii) to displace other plant species competitively, as control (E+) fescues do [70]. An endophyte, representing less than 0.1% of host aboveground biomass, can therefore account for a species survival and invasiveness in ecosystems.

The plant community seems in part structured by its symbionts. This is also substantiated by microcosm experiments manipulating the diversity of mycorrhizal fungi. Using a plant community from a calcareous grassland, including mycorrhizal and non-mycorrhizal species, Van der Heijden and co-workers demonstrated that it was influenced in two ways [71]: (i) non-mycorrhizal species, which are abundant in the absence of mycorrhizal fungi, often decrease in biomass when such fungi are present; (ii) the biomasses of each species differ when different fungal strains are present. This is partly mediated by nutrient exchanges, but protective effects should cooperate. In response to different mycorrhizal inocula, drastic modifications of the plant’s shape and reproduction strategy (vegetative versus sexual, seed-based) are reported to occur and to modify the plant’s competitive abilities, e.g. for *Prunella* spp. of this calcareous grassland community [72]. Elsewhere, it has been shown that some fungal host-preference does exist in field conditions [73,74], even between closely related *Poaceae* [74], therefore linking more tightly epigeous diversity to hypogeous diversity. Given this host-preference, the hypothesis that monoculture could lead to changes and depletion in the fungal community was addressed. Microcosm experiments clearly established that monospecific plant populations do affect mycorrhizal fungal

diversity, and thus affect installation of newly arriving plant species [75].

As plant protection partly relies on herbivore deterrence, an effect on food chains based on the (E+) plants can be expected. It was demonstrated on a simple model web, involving (E+) or (E-) ryegrass, their sap-sucking aphid parasites (two species), and two levels of hymenopteran parasitoids, namely various aphid parasites and hyperparasites living on parasitized aphids [60]. As mentioned above, aphid number was reduced by one fourth on (E+) plants. In terms of parasitism rate, aphid parasites were only slightly affected, while hyperparasites were two times less frequent on (E+) plants, leading to disproportionate decrease in secondary parasitism [60]. Moreover, parameters describing this food web were altered on (E+) plants, with a reduction in number of parasites shared by the two aphid species and a decrease in connectance in the web (ratio of number of observed trophic links to the maximum possible links). So plant symbionts, including protective ones, help to shape plant communities and ecological interactions.

6. Final considerations and prospects

Isolation methods have long biased our view of microbial diversity; analyses of environmental DNA samples by molecular approaches have profoundly changed our perception of microorganisms in a variety of ecosystems, such as soil [76] and the rhizosphere [23]: 90 to 99% of microorganisms are not cultivable using standard techniques [77]. Knowledge of plant-associated microbes has also benefited from DNA-based detection, e.g. for endophytic bacteria, which are often not cultivable [49,51].

A striking, recent study focused on a well-studied ecological niche, the roots (with the grass *Arrhenatherum elatius* as model), using newly designed fungal-specific primers for PCR amplification of the small ribosomal subunit RNA gene [79]. In this work, an unexpected fungal diversity was revealed where all known fungal phyla were represented. The diversity within these phyla was high, with a large proportion of unknown species [78]. The phylogenetic reconstructions performed also highlighted the presence of unknown, deep branch-forming clades within the Basidiomycota and Ascomycota. But, as pointed out by the authors [78,79], it is only possible to specu-

late on the possible roles of most of the fungal diversity found, and on its relevance for plant physiology. Clearly, much remains to be done in the description of plant-associated microbial diversity, and financial support (as currently in France by the 'Institut français de la biodiversité' or the 'Bureau des ressources génétiques') is crucially needed for such purposes.

Another main goal is to know which fraction of this microbial diversity is involved in a recognized environmental process (such as plant protection), that is, to take into consideration ecological functions of diversity. Few methods are available to analyse in situ the functional diversity of microorganisms (at least from the plant's point of view) with the need to avoid any preliminary cultivation or isolation step. Among these, classical methods such as electron microscopy or fluorescent in situ hybridisation (FISH) [80,81] have been successfully used. This imaging-based strategy allows us to check for the presence of organisms detected by molecular tools [82] and can also suggest whether a microorganism is biotrophic (preserving the host's tissue), necrotic or even dormant (e.g., forming spores). Nevertheless, this method focuses on a particular known organism. A more refined approach is based on modification of density of DNA [83] or, more powerfully, of RNA [84,85]. Microorganisms physiologically interacting with plants are detected by providing ^{13}C to the plant in situ, and then fractionating ^{13}C -RNAs from ^{12}C -RNAs co-extracted from roots. Libraries are generated through reverse transcription followed by PCR amplification. As RNAs well reflect the current nucleotide pool, this allows preferential detection of microorganisms using photosynthates, avoiding dormant species. This novel approach is well suited to analyse the functional diversity of both known and unknown microorganisms.

Lastly, the tall-fescue history demonstrates that ecological experiments are needed to take into account all features of symbiotic associations. Analysis of symbiotic versus non-symbiotic plants can be performed under simple laboratory conditions to assess the role of a given partner. But studies in more realistic environments, such as microcosms or field plots, are also needed, especially in view of applied uses of a symbiotic association for biocontrol or plant protection. Only such conditions ensure that a given microbial strain can persist without being outcompeted or 'diluted' through introgression with resident

strains. Introduced plant beneficial strains have been successfully tracked in situ [86], suggesting that plant-associated microflora can be managed, but considerable uncertainties exist regarding the long-term implications of such practices. Caution should also be exercised regarding the potential disturbance of the indigenous microbial community and non-target populations following such inoculations, especially for genetically modified strains containing an extra-copy of a gene involved in the beneficial trait (antibiotic or phytohormones) [87–89]. Several studies have already revealed that diversity of bacterial and fungal communities can be affected by the inoculant strain(s) [86,90–92]. Nonetheless, depending on assessment methods, time of sampling and type of partners (plant/inoculant), such an impact is not always recorded or is non-significant [93–95]. Consequently, the extent of this interference should be monitored in the long term with additive inoculation events (additive effect) or without (remnant effect), together with a tracking of the inoculated strain. Moreover, besides a global structural approach, more refined investigations should be developed to identify a potential positive stimulation of native beneficial microbes or disturbance of native symbiotic relations [96]. To be exhaustive, the impact on microbial functions must be investigated.

Studies of integrative diversity in conjunction with functional diversity, at the physiological and ecological levels, are important challenges for future researches: one could hope that they will benefit from both field or microcosm experiments and DNA-based diversity surveys.

Question / Answer – Question / Réponse

Question de M. Blouin (laboratoire d'écologie des sols tropicaux, Institut de recherche pour le développement, Bondy)

La richesse spécifique, la diversité intraspécifique et la vitesse d'évolution des organismes microbiens ne sont-elles pas contradictoires avec le projet d'inventaire de l'effet des microorganismes sur chaque espèce de plante ?

Réponse de M.-A. Selosse

Sans doute un pur inventaire n'est-il pas très intéressant, ni très faisable ! Il vaudrait mieux se fo-

caliser sur quelques interactions modèles, par exemple rendues intéressantes par leurs implications écologiques, ou sur l'importance économique des espèces choisies. Mais il est vrai que, surtout en ce qui concerne les bactéries, les transferts génétiques horizontaux peuvent faire varier très vite le phénotype. D'ailleurs, l'environnement de la plante (rejoignant en cela aussi le tube digestif des animaux) est une niche écologique où coexistent des microorganismes variés, en grandes concentrations : c'est donc un lieu privilégié pour les transferts génétiques (J.D. van Elsas et al., *New Phytol.* 157 (2003) 525–537). Cette variabilité ne remet pas en cause la possibilité de tester l'effet physiologique et/ou écologique d'une souche microbienne donnée sur une plante donnée. En revanche, elle limite la possibilité de définir en extension des groupes fonctionnels au sein des communautés microbiennes – en ce sens qu'un microorganisme donné peut rapidement changer de groupe fonctionnel. Mais, bien que les organismes qui les constituent n'y soient pas durablement inféodés, de tels groupes existent probablement, et restent à définir. La question posée remet aussi en cause la possibilité d'utiliser à des fins pratiques les interactions protectrices : mon exposé s'intéressait peu à cet aspect appliqué et ne visait qu'à montrer des potentialités. Mais le risque qu'une souche profitable change d'effet sur l'hôte est réel : par exemple, deux souches endophytes protectrices peuvent se compléter quant à leur effet pathogène [69] et recouvrer ensemble un effet délétère sur l'hôte. Cela souligne la nécessité d'étudier les couples microorganisme/plante-hôte dans des conditions écologiquement réalistes, par exemple non axéniques.

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