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Crystal ball

Combinatorial reprogramming of lipid metabolism in plants: a way towards mass-production of bio-fortified arbuscular mycorrhizal fungi inoculants

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Summary

Arbuscular mycorrhizal fungi (AMF) are among the most ancient, widespread and functionally important symbioses on Earth that help feed the world. Yet, mass-production of clean (i.e. *in vitro* produced), safe and robust inoculum at affordable costs remains a critical challenge. Very recently, Luginbuehl et al. (2017) found that plants supply lipids to the symbiotic partner, thus '*providing the AMF with a robust source of carbon for their metabolic needs*'. Hence, engineering plants for enhanced delivery of lipids to AMF could represent an innovative avenue to produce a novel generation of high-quality and cost-effective bio-fortified AMF inoculants for application in agro-ecosystems.

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil microorganisms forming a mutualistic symbiosis with an estimate of 72% of terrestrial plant taxa. They are key players in agro-ecosystems, improving plant nutrition (Smith and Read, 2008) and increasing their resistance/tolerance to biotic and abiotic stresses (Pozo *et al.*, 2013; Plouznikoff *et al.*, 2016). Unfortunately, overuse of chemical fertilizers and pesticides and intensive agricultural practices have recklessly decreased mycorrhizal diversity in modern agricultural systems (Sosa-Hernández *et al.*, 2019), a situation which is exacerbated under climate changes believed to impart dramatic effects on plant–soil–AMF interactions by altering the community structure, abundance, composition and functional activity of plant-associated microbial taxa (Sergaki *et al.*, 2018). Because of that, applying agricultural practices favouring/restoring AMF diversity or introducing selected mass-produced AMF inoculants into soils have become key challenges of the next big revolution in the development of sustainable agricultural systems.

Towards engineered *in vitro* AMF production systems: a novel generation of AMF mass-produced inoculants

The large-scale production of AMF is not possible in the absence of a suitable host and most production systems are conducted on substrate-based (e.g. in greenhouse) or substrate-free (e.g. in hydroponics, aeroponics) conditions which often require large surfaces and cannot guarantee the absence of unwanted microbes. Promising alternatives are the production of AMF on transformed root organ cultures (ROC) which correspond to a successful established Petri plate culture of AMF with Ri T-DNA transformed root organs on a gelled medium. Different sub-production systems have been derived from this basic ROC system for mass-production, by cultivation of root organs and AMF in small containers (Declerck *et al.*, 2005), in airlift bioreactors (Jolicoeur *et al.*, 1999) or in bioreactors containing solid support elements [US Pat. No. 5554530 (1996)]. In parallel, *in vitro* culture systems based on autotrophic plants have been developed with shoots developing outside the Petri plate while roots and AMF were associated inside the Petri plate on gelled medium (Voets *et al.*, 2005). *In vitro* cultivation on root organs or whole plants are the only systems that could guarantee the propagation of contaminant-free material, making them prior candidates for

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large-scale production of high-quality controlled inoculum. However, these systems remain technologically costly, are limited to a restricted number of AMF species and production remains low as compared to *in vivo* systems. There is thus a need for the development of innovative technologies allowing the production of high density, safe and robust spore inoculum with reduced costs.

Several attempts have been conducted to tackle this challenge with the AMF *Rhizophagus irregularis* by using dual-compartment culture systems (Rosikiewicz *et al*., 2017) or by the addition of stimulating chemical molecules to compensate for lack of microbial associates and trigger gene activation for secondary metabolite production. The addition of fatty acids (myristate) in the growth medium was recently shown to induce hyphal branching, colonization ability and sporulation in the asymbiotic stage of *R. irregularis* (Kameoka *et al*., 2019). The addition of chemical molecules is, however, very costly, but suggests that engineering fatty acid metabolism in host plants may be an innovative approach for a novel generation of *in vitro* AMF produced spores.

The prospective to engineer plants–microorganisms interaction has been conducted successfully in different studies but these synthetic biology approaches have been focused only on filamentous fungi such as *Aspergillus niger* and *Penicillium chrysogenum* (Guzmán-Chávez *et al*., 2018). Currently, there is no research on the use of AMF colonized plants in synthetic biology to improve the symbiotic establishment and large-scale spore production. Moreover, AMF are multinucleated organisms not amenable for engineering (Dubey *et al*., 2019). Technological advances need to focus on synthetic biology with AMF colonized plants. Lipid engineering in plants is expected to fuel the AMF with high value carbon sources, leading to a novel generation of high quality and quantity *in vitro* produced AMF inoculants.

Combinatorial metabolic engineering strategy

Transcriptomic analysis showed that the development of arbuscules is accompanied by the activation of an AMF-specific operational unit of lipid biosynthesis and transport of fatty acids (FA) from plant cells to the FA auxotroph AMF (Fig. 1), governed by transcription factors and genes found to be absent from genomes of non-mycotrophic plants (Luginbuehl *et al*., 2017). Such information is presumed to be essential to the development of a novel generation of mass-produced AMF inoculants, giving rise to consistently increasing TAG content of spores (spore bio-fortification) that would enhance spore production as well as spore fitness to increase overall colonization efficiency. Further analyses need to be undertaken with engineered plants to characterize the key AMF-specific genes that promote TAG accumulation in fortified spores and/or increase their number. As known, gene modification analyses demonstrated that single-gene manipulations typically only have marginal effects on TAG content in vegetative tissues (Vanhercke *et al*., 2019). However, recent combinatorial metabolic engineering strategy focused on the simultaneous optimization of oil synthesis, packaging and degradation pathways have a great power with engineered TAG levels in plants. This strategy has been coined in plants as 'Push, Pull, Package and Protect' (PPPP strategy) (Vanhercke *et al*., 2019). The ultimate aim of the use of PPPP strategy is to mimic the lipid metabolic fluxes occurring in oilseeds plants into non-seed plant tissues, where carbon is efficiently shunted into TAG for longer term storage in photosynthetic sources tissues. By analogy, the PPPP strategy could be applied in the symbiotic interface to generate mycorrhized transgenic lines with boosting de novo FA biosynthesis in plant root cells, shunt more FA pool from root cells to arbuscule cells, increase transport of neutral lipids to hypha structure, and storage of neutral lipids in AMF spore (Fig. 1). Such approach could open the way to develop an engineered host plant for the growth benefits of the non-engineered AMF symbiont, thereby effectively changing host plant into a 'factory' delivering essential energy dense lipids for the production of bio-fortified AMF cells. The proposed engineering approach should, when coupled with a series of conventional enzymatic assays and continuous pulse and pulse-chase metabolic isotopic labelling (Allen *et al*., 2015), provide a holistic understanding of the redistribution of acyl groups between various pathways of membrane lipids and TAG production (Zhou *et al*., 2020) in the symbiotic interface. Furthermore, functional oriented approaches of the bio-fortified AMF spores could be applied to characterize their role in plant growth, development, physiology and stress tolerance under non-optimal field growing conditions and environmental changes. In the long term, such information could become essential to the development of the next generation of bio-fertilizer inoculants, giving rise to consistently functioning microbial communities that restore soil health and enhance agriculture sustainability and crop productivity.

Domestication of AMF 'bio-fortifed' spores

Until today, the *in vitro* cultivation of AMF is mostly restricted to species in the genus *Rhizophagus* (formerly *Glomus* for most of them) and to a few individuals belonging to the Gigasporaceae. Moreover, with the exception of *R. irregularis* and *R. Intraradices* and a few others species, biomass production remains low and for some species can even await several months to produce

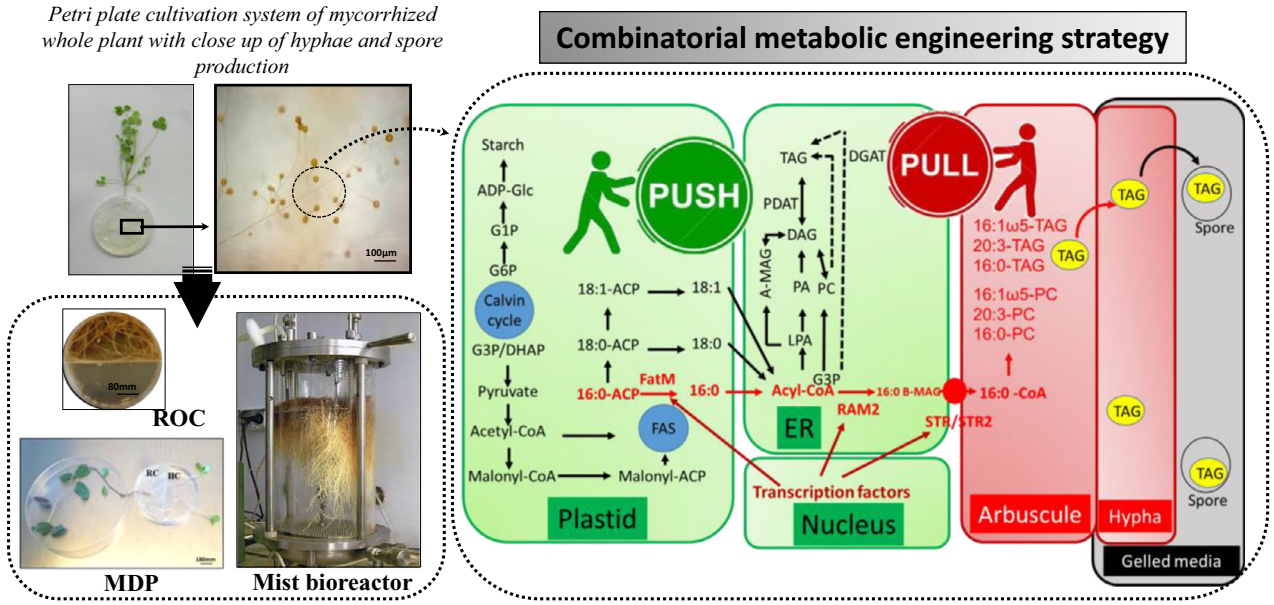

Fig. 1. Combinatorial lipid engineering strategy of mycorrhized host plants for mass-production of bio-fortified AMF in Petri plates or bioreactors using 'Push, Pull, Package and Protect' strategy (Vanhercke, *et al.*, 2019). AMF-specific operational unit of lipid biosynthesis and transport pathways are represented by red arrows and lipid biosynthesis pathways in root cell are represented by black arrows. Model of the proposed route for biosynthesis of fatty acyl groups stored in fungal triacylglycerols (TAG) was adapted from Luginbuehl *et al.* (2017). ROC correspond to Root Organ Culture (Ijdo, *et al.*, 2011). MDP correspond to Mycorrhizal Donor Plant *in vitro* culture system with (RC) root compartment and (HC) hyphal compartment (Lalaymia and Declerck, 2020). The mist bioreactor photograph was extracted from Urbańska *et al.* (2014).

only a limited number of spores (e.g. the Gigasporaceae). The reason for the failure to grow a broad set of AMF remains enigmatic, probably partly related to their life history strategy, r-strategists being presumably more prone for *in vitro* cultivation than K strategists. Similarly, the reasons for their relative limited biomass production are manifold and may be related to the limited volume of Petri plates, the short period of growth of root organs and to the limited capacity of the host to sustain high levels of spore production. Modulating growth media and volume have been proposed for mass-production and achieved for a very limited number of species in bioreactors. Similarly, moving from ROC to photosynthetically active plants have been suggested to increase the number of species grown *in vitro* but should now been demonstrated. Metabolic engineering for lipid production and delivery in whole plants or hairy roots lines combined with bioreactor technologies could represent an innovative approach either to access AMF species that until now have evaded *in vitro* cultivation (e.g. *Fulleniformis mosseae*, etc.) or for mass-production. Several studies have reported the culture of hairy roots in various bioreactor configurations for large-scale cultivation of AMF. Hairy roots have been grown in solid support elements (Fortin *et al.*, 1996), mist- and airlift (Jolicoeur *et al.*, 1999) bioreactors, which were evolved to ensure cultivation in a low-stress environment, homogenous growth distribution as well as adequate oxygen and nutrient supply. However, none of them involved the production of AMF using engineered plant host for enhancing a low-cost mass production of AMF. Hence, the development of a bioreactor could be a step forward for the optimization process of bio-fortified spores production by high lipid producing and delivery plants under strict *in vitro* cultivation conditions. This approach is likely to reduce the cost per item and labour time.

Concluding remarks

We envision that future efforts to extend the number of AMF species grown *in vitro* and to achieve mass-production of these organisms could focus on lipid engineering of the symbiotic interface. Success in this attempt will inevitably necessitate intense methodological research including combinatorial lipid metabolic engineering, selection of mycorrhized TAG-accumulating host, lipid flux analysis and spores domestication. This new

concept will offer versatile and multi-beneficial options: (i) increase TAG-based carbon sources in the AMF, with vesicles, intra- and extra-radical spores accumulating more lipids for a higher germination and root-colonization potential (bio-fortification = best quality), ii) stimulate the asexual reproduction machinery to produce more spores in Petri plates and bioreactors (biomass production = high quantity), decreasing costs of *in vitro* spore production systems (cost-efficiency = industry profitable). Ultimately, it is expected that research will transform this concept into a novel generation of high-quality and cost-effective bio-inoculant for large-scale application in agro-ecosystems.

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