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Directed Inoculum Production of arbuscular mycorrhizal fungi – the state of the art

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Summary

Since the mutualistic nature of arbuscular mycorrhiza (AM) had been discovered, an enormous amount of experiments elucidated the huge potential of mycorrhizal technology. Nevertheless, the predictability of mycorrhizal effectiveness (i.e. the quantitative measure of a pronounced effect) in agro-ecosystems and plant production systems remained low. On this background, Directed Inoculum Production (DIP) had been developed based on quantitative genetics. During the inoculum production process the ecological niche of an arbuscular mycorrhizal fungus (AMF) is considered, the effective ecological niche widened and population engineering included in large-scale inoculum production. This is done in order to place effective mycorrhiza products on the market. This article reviews briefly the historical development and the idea of DIP and its performance in the last 25 years. Recent and current scientific explanations for the variability of mycorrhizal effectiveness and future demands of research are outlined.

Introduction

In September 1985, 100 years after the description of the symbiotic nature of mycorrhizal fungi, an international symposium on "Mycorrhiza and Stress in Plants" was organised by the Society for Applied Botany in Hannover, Germany (Dehne, 1986). The impact of this symposium was huge. Several research groups in Germany and Europe became aware of the status quo of mycorrhizal research and decided to develop it on the background of their specific fields of interest.

The applied botanist, late Prof. Dr. Reinhard Lieberei (*04.07.1948, †06.03.2019) initially compared physiological changes in roots colonized by arbuscular mycorrhizal fungi (AMF) using the cyanogenic tropical rubber tree *Hevea brasiliensis* (Lieberei and Feldmann, 1990). Basing on the findings he induced a research group starting its work in Amazonia, Brazil (Lieberei et al., 1989) in order to evaluate the agricultural potential of mycorrhiza in tropical plant production (Feldmann et al., 1990). They found mycorrhizal effects in agro-ecosystems of perennial plants like reduction of plant loss during plantation, better nutrition of host plants and even enhanced resistance of leaves against biotrophic fungi due to mycorrhizal colonization. All these findings resulted in the chance to re-cultivate abandoned degraded areas in the Amazon region (Feldmann et al., 1995). The observation of negative mycorrhizal population dynamics induced by management practices (Feldmann et al., 2002) could be overcome by appropriate inoculum production techniques previously adapted to tropical demands (Feldmann and Idzcak, 1991).

After return to Germany, we had to learn that under European agricultural and climatic conditions cultivated plants expressed a very different responsiveness to mycorrhizal inoculation. We observed all principal effects of the mycorrhizal symbiosis (nicely reviewed by Koide and Mosse, 2004), but the predictability of mycorrhizal effectiveness as the quantitative measure of an effect remained low – a major constraint for mycorrhizal products to be placed on the market as biostimulants in Europe. The lack of predictability was in the 90ies of the last century one of the major reasons why companies had to quit after short time on the market (Feldmann, 2003).

What is behind the variability of mycorrhizal effectiveness? What could an inoculum producer do to develop a product with stable characteristics? To answer these questions, we brought together the knowledge on environment-fungal genotype/plant genotype interactions published over decades, focused on a few fungal species only (e.g. *Claroideoglomus etunicatum,* formerly *Glomus etunicatum, Rhizoglomus irregularis,* formerly *Glomus irregularis*, *Rhizoglomus intraradices,* formerly *Glomus intraradices*; Wijayawardene, 2020) and analysed the AMF/host interrelationships in a special test system (Feldmann et al., 1998c) based on quantitative genetics (Feldmann, 1998b). Out of these studies an inoculum production procedure was developed, described in detail as "Directed Inoculum Production Process" (DIP) (Feldmann and Grotkass, 2002). The differentiation between the qualitative mycorrhizal "effect" and its quantitative measure "effectiveness" is of major importance at this point. DIP is targeted to increase the predictability of effectiveness towards an economically satisfying process with ensured quality (Alten et al., 2002). Consequently, we published the description of a best practice procedure for inoculum production (Feldmann et al., 2009).

This paper reviews the historical development of DIP and the main reasons for variable mycorrhizal effectiveness, and furthermore, the quantitative genetic situation in our mycorrhizal inoculum as starting point for population engineering in plant cultivation systems. All empirical findings, which led to DIP, are discussed on the background of newest outcomes of mycorrhiza workgroups from all over the world. Finally, definitions like "strain", "isolate", "genotypes" or "genetic population" of AMF fungi are discussed explaining "adaptation" processes within the inoculum.

In honour of late Prof. Dr. Lieberei we focus on the findings of our group initiated by him.

Variability of mycorrhizal effectiveness is the variability of mycorrhizal phenotypes

Selling AMF inoculum on the market means to provide mycorrhizal propagules (spores, mycelia, colonized roots, and associated microbiomes of the production system) to users growing different plant species under several, often changing environments. From these propagules, the AMF has to colonize the roots of the hosts. The symbiosis should modify the plant's physiology in a way, which results in a practical advantage for mycorrhizal plants in comparison to non-mycorrhizal plants.

The effects desired by plant growers are very different and reach from higher fresh produce, better rooting, more intense flower-

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ing, prolongation of flowering, higher seed production, survival in drought periods or under suboptimal fertilization situations and much more (FELDMANN, 1998a).

The AMF economic threshold of effectiveness as the quantitative outcome of an effect desired by an inoculum user can vary depending on the effect observed: root colonization only already supports the development of cucumber plants in presence of *Meloidogyne hapla* in greenhouses with soil infestation (FELDMANN et al., 2008). Only 5% more stem thickening is sufficient for significantly earlier budding of trees (FELDMANN et al., 1990), 10% less plant losses can economize *in vitro* production of *Baptisia tinctoria* (SCHNEIDER et al., 2008), 20% more tubers are desired in production systems of potato in China (CHENG et al., 2008). In bell pepper, cotton and marigold biotic stress reduction of only 10% already showed significant economic net benefit for the producer (LONG et al., 2008).

The variability of effectiveness becomes clear during the selection of fungal isolates belonging to different AMF species. As an example, BALTRUSCHAT (1993) tested 21 AMF isolates on two strawberry cultivars in order to identify the "best isolate". He repeated the experiment in a short time after another under changed light conditions in the greenhouse and found very different rankings of the isolates' effectiveness (Fig. 1). Exactly this variability of effectiveness is a marketing problem for inoculum producers. They simply cannot guarantee a certain quantitative outcome of the symbiosis after inoculation with their product. The results reasoned to accept the impossibility to find "the best mycorrhizal isolate" for inoculum production in those days. Consequently, it was necessary to identify reasons for variability of effectiveness and options to reduce it.

The example shows that host plant genotype/AMF genotype/environment interrelationships are relevant for the quantitative outcome of the symbiosis. From a quantitative genetical point of view the quantitative outcome of the symbiosis, the effectiveness, reflects the phenotype of the mycorrhizal symbiosis. The results of BALTRUSCHAT (1993) indicated that there was a phenotypic plasticity in an inoculum with a huge potential to react to changes in the environment. But at this point we did not yet understand which part of the reaction was induced by the plant and which by the fungal partner.

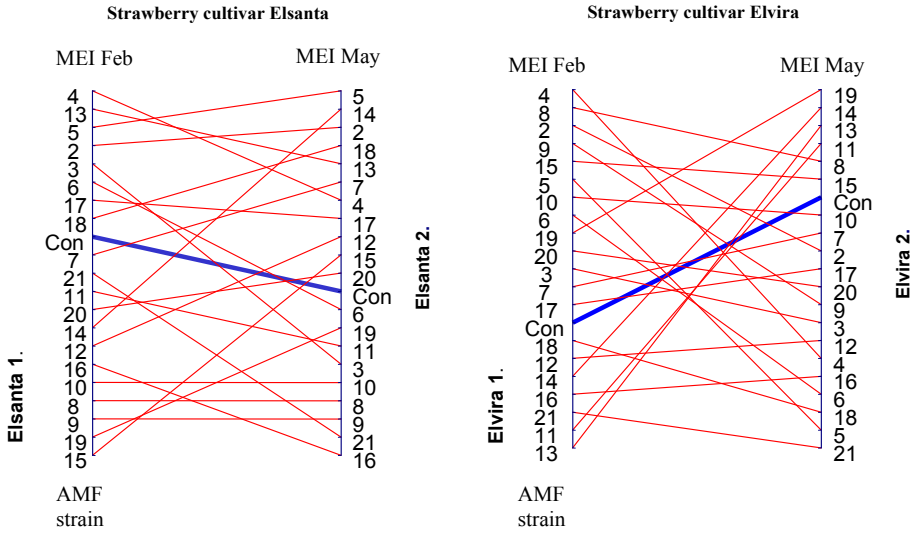
Environment-induced changes of mycorrhizal effectiveness reflect the host dependency of plants on AM symbiosis

In natural ecosystems mycorrhizal species have relationships with heterogeneous plant communities of different species frequencies and changing abundances, seasonal variations, long-term changes, and successions over time are observed (ALLEN, 1992). Host species can be colonized by various AMF species at the same time or subsequently in a seasonal succession (GANGE et al., 1993). Hosts are interlinked with each other forming a widespread fungal internet, in which nutrients are exchanged between host individuals and host species (NEWMAN, 1988). It is hard to estimate mycorrhizal effectiveness *in situ* under natural growth conditions. However, recently, there were interesting results indicating within-species ("strain") differences of mycorrhizal phenotypes in plant communities:

SAVARY et al. (2018) found that within-species differences in *Rhizoglomus irregularis* did not strongly influence the performance of individual plants or the structure of the overall plant community. However, the evenness of the plant community was affected by the phylogeny of the fungal isolates, where more closely-related AMF isolates were more likely to affect plant community evenness in a similar way compared to more genetically distant isolates. This study underlined the effect of within AMF species variability on plant community structure. While differential effects of the AMF isolates were not strong, a single AMF species had enough functional variability to change the equilibrium of a plant community in a way that is associated with the evolutionary history of the fungus. This observation implies the differential effectiveness of the fungal partner at a given site.

Furthermore, the mycorrhizal effectiveness can be different under changing environmental conditions. This phenomenon was explained by variable "dependency" on symbioses of hosts and was already observed by STAHL (1900). The dependency of hosts under certain environmental conditions is quantified by their responsiveness to AMF colonization. The idea behind is the action of the "law of the minimum", a principle already developed in agricultural science in 1828 by Carl Sprengel (GRÖGER, 2010).

Mycorrhizal dependency of a host is genetically fixed (AZCON and



Ranked **M**ycorrhizal **E**ffectiveness **I**ndex (MEI)
First experiment in February (Feb), repetition in May (May)
AMF: **A**rbuscular **M**ycorrhizal **F**ungi; Con: without mycorrhiza

Fig. 1: Variability of mycorrhizal effectiveness on fresh weight after inoculation of two strawberry cultivars in two subsequent repetitions (ranked from „best" isolate (top) to „worst"). The mycorrhizal effectiveness index (MEI) is the percentage difference between mycorrhiza inoculated and not inoculated plants (Con). Numbers are the isolate numbers used for inoculation. Ranking below Con means fresh weight reduction, above Con fresh weight increase of mycorrhiza inoculated plants. The figure was drawn based on data derived from BALTRUSCHAT (1993).

OCAMPO, 1981). The degree of mycorrhizal dependency is expressed on individual level as a gradient within the host's fundamental ecological niche and relevant environmental conditions. If a plant cannot explore a resource in its actual ecological niche without mycorrhiza, ROMELL (1939) speaks of "obligate mycotrophy". He assumes that effectiveness is then always positive. To his opinion, "facultative mycotrophy" leads to a shift of actual niche characteristics, too, if mycorrhiza is developed: in a facultative symbiosis mycorrhiza would either increase the acquisition of a limiting resource (e.g. P) or decrease it (e.g. carbon gain under low light). ALLEN (1992) supports this hypothesis.

From our point of view, the cited hypothesis is applicable to the practice of plant production with some considerations, which have to be kept in mind. First, advanced plant production normally bases on long selection processes of plant cultivars grown without consideration of mycorrhizal fungi. The plants are, therefore, selected to be independent on mycorrhiza. Furthermore, long term experiences normally led to optimized procedures and growing conditions during plant cultivation. The actual ecological niche of a cultivated, intensively screened and selected plant cultivar, therefore, should make nearly optimal use of resources without any mycorrhiza. This circumstance leads to the impression that it is stress, which creates some mycorrhizal dependency of useful plants in production systems. Deviations from the optimal growing system, suboptimal periods, and temporary depletion of resources, upcoming disease and unknown limiting factors open the window for use of mycorrhiza in advanced plant production. If primary selections are used, wild collections tested or even plant varieties with well-documented host characteristics are produced, at least facultative mycotrophy can be assumed. Higher degree of plants' mycotrophy, low extent of their genetic selection and suboptimal growing conditions in tropical and subtropical agriculture lead to much better situations for mycorrhizal use than under conditions of the Northern hemisphere.

A practical complication to classify a host as facultatively mycotrophic or obligate symbiont is the multifactorial nature of an ecological niche. Important effects of mycorrhiza might be masked because of not influenced limiting factors existing in a cultivation system. In Fig. 2 we show the ecofactor strength on a scale from zero to one hundred. A plant can explore the resources necessary for optimal growth without restrictions if the ecofactor strength is at maximum. Reduced ecofactor strength opens the opportunity for mycorrhizal dependency. The plant can be responsive to mycorrhizal colonization if the symbioses can increase the ecofactor strength. Because mycorrhizal fungi might influence only specific ecofactors, the law of the minimum leads to apparent or hidden mycorrhizal effects if the limiting factor is not influenced by the mycorrhizal symbiosis. For instance, a host might be obligatory dependent on mycorrhiza with respect to the survival under heavy metal stress and mycorrhiza overcomes this stress. But because of light deficiency it may not grow better than non-mycorrhizal plants even if the heavy metal stress has been overcome by mycorrhization in mycorrhizal plants. The result would be a hidden effect of heavy metal stress reduction if the light deficiency would not be reduced.

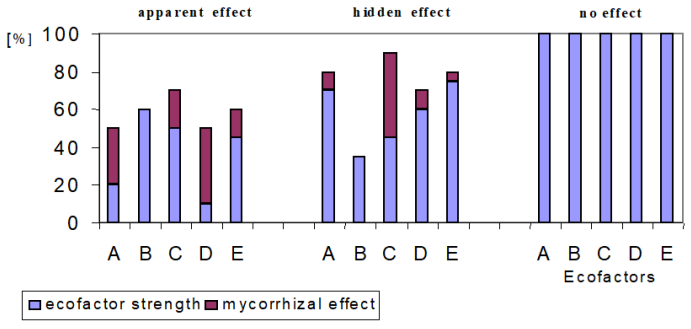


Fig. 2: Ecofactor strength and apparent, hidden and no effect of mycorrhizal symbioses. Mycorrhizal effects might be hidden by not influencing the limiting factor (Explanation see text).

Concluding, our ability to predict mycorrhizal dependency of a host under specific conditions depends on the knowledge of stress tolerance characteristics and growth limiting factors of that host. Additionally, to host dependency, the AMF are interacting with the environmental factors, too. Adaptations of AMF to environmental conditions occur (READ, 1999) and are well documented. Even environment mediated selection on AMF species takes place. If environmental conditions change drastically, a loss of AMF species can be the consequence. For instance, in plant production systems, the AMF communities are negatively influenced by management practices (KÖNIG et al., 2014). Such selection pressure by cultivation methods leads to stress tolerant AMF communities, which might be of low effectiveness (FELDMANN et al., 2002). In horticultural practice, in nearly all potting substrates used in plant production, AMF are normally completely missing if not added (FELDMANN, 1998a). In these systems, especially the soil and sometimes many more parameters (e.g. in greenhouse production) are optimised for the plant to be produced. This leads to the observation that the same host is differently colonized by AMF under different environments.

Nevertheless, the prediction of effectiveness in production systems is much easier than in nature. Here, the widely controlled practical conditions and non-mycorrhizal treatments as comparisons in tests facilitate the application of the mycorrhizal technology for users and inoculum producers. The more practical experience in plant cultivation a grower has, the better he can predict success of mycorrhizal symbiosis in his growing system because the difference between performance of actual niche and fundamental host niche defines the maximum expression of mycorrhizal effectiveness in the symbiosis.

Selection phenomenons between the symbionts

At this point, it is necessary to introduce some population genetical definitions regarding AMF inoculum.

As "isolate" we define descendants of mycorrhizal spores isolated from certain sites (proveniences) whether taxonomically determined or not. Isolates often are morphologically identical spores but not descendants of a single spore.

All spores produced from one single spore we call "strain". In our studies, we developed descendants of single spores of strains and called them "sub-strains".

The expression "population" is used to describe all taxonomically defined descendants of one AMF species in a given inoculum. Population refers, here, to pure strains in the inoculum production system or to mixtures of strains of the same species. If AMF species are mixed in an inoculum or the relations in situ to other, e.g. naturally occurring AMF are studied the expression "AMF community" is used.

As shown in the example of Fig. 1, AMF isolate characteristics change with changing environment. But in a given stable environment further specificity phenomenons occur. For instance, a preference of partners forming the symbiosis can be observed, which leads to the support of one specific host being planted in dual-culture (FELDMANN, 1998a; CROLL et al., 2008). Such an observation could be obtained in a maize field as well, where symbiotic weeds could favour the cultivated plant. In contrast, non-mycorrhizal weeds reduced maize fresh weight (FELDMANN and BOYLE, 1999). Consequently, we could find a competition of mycorrhizal isolates for the host plant resulting in the exclusion of certain AMF species in mycorrhizal communities from colonizing the root system of a test plant.

Such specificity phenomena with enormous consequences for symbiotic effectiveness were postulated even on the level of AMF popu-

lations of one single strain (FELDMANN, 1997): We produced inoculum of a *Claroideoglomus etunicatum* strain over four years on two maize cultivars, "Badischer Landmais" and "Felix". After two years, the effectiveness on the host plant decreased on "Felix" while this was not observed on the other cultivar. The shift of effectiveness continued over four years in case of *Zea mays* var. "Felix" could be reproduced in a further experiment and could be demonstrated to depend on the plant genotype. After the fourth year, we changed the inoculum produced on the maize cultivars. The low effectiveness of inoculum produced on "Felix" in the fourth year was improved over three subsequent years by var. "Badischer Landmais", while the highly effective inoculum produced on "Badischer Landmais" lost its effectiveness on "Felix" (Fig. 3). Because "Badischer Landmais" is an old maize cultivar and "Felix" a modern hybrid the maintenance of higher effectiveness correlates with higher genetic heterogeneity of the host. Whether this was the cause could not be proved here. However, if – in case of high host dependency – genetic heterogeneity of host would guarantee stable effectiveness of AMF, the influence of biodiversity of host and fungal communities could have an important impact on symbiotic relevance in natural ecosystems and production systems.

These observations were a clear indication for us that host genotype/AMF genotype selection processes take place with relevance for the effectiveness of the symbiosis. Therefore, we developed a functional genotyping test system to study such processes.

	Zea mays cultivar	
Multiplication cycle	**Badischer Landmais**	**Felix**
I	35	41
II	30	38
III	31	16
IV	36	9
Exchange of host/inoculum		
V	20	25
VI	44	17
VII	36	3

Fig. 3: Mycorrhizal Effectiveness [%] of *Claroideoglomus etunicatum* on *Zea mays* (varieties Badischer Landmais and Felix) over seven years of subsequent inoculum production. (The inoculum was exchanged after the fourth year; derived from FELDMANN, 1997).

"Functional AMF genotype" in inoculum of arbuscular mycorrhizal fungi

Effectiveness of AM symbioses, measured e.g. as fresh weight, number of flowers, survival under stress and so on normally is a continuous or metric character of a host plant. While genes individually associated with pronounced phenotypic effects (oligogenes) control the production of discontinuous, qualitative characters, polygenes individually have a small effect but are the basis of metric traits. Oligogenes segregate clearly and are easily subject to Mendelian analysis, polygenes are not. Each character is always determined by a series of genes, and depends on allelic and non-allelic gene interactions (FUTUYMA, 1998).

For instance, physiologically induced effects are an important drivers of mycorrhizal response and colonization, in particular hormonal balance and respiration patterns, which concentrate response from both genomic and bio/abiostimuli effects (BEDINI et al., 2018; MERCY et al., 2017). Up to date, all attempts to link quantitative trait loci (QTL) with a given response pattern failed, because there are likely multifactorial components that regulate the symbiotic partners. Here, we have clearly to keep in mind that we are studying the phenotype of the host to describe the action of fungal genotypes. The host is, therefore, the "principal environment" for the biotrophic microsymbiont, while all the other abiotic and biotic factors can be seen as "secondary environment". If we want to argue to observe the function of AMF genes, we have to make sure that the principal and the secondary environment is as stable as possible. Consequently, we designed the quantitative genetic analysis as „common garden experiment", i.e. we standardised both environments as much as possible, including the plant material (FELDMANN et al., 1998c).

From the host's point of view, the micro-symbiont is as well the principal environment, and all other environmental parameters belong to the secondary environment. In our experiment, the secondary environment was standardized. Changes of the host phenotypic performance of polygenic characters should, therefore, be modified by the heterogeneity of the principal environmental factors, the AMF. If we, then, inoculate with one spore only, we should be able to observe the action of a certain AMF spore phenotype resulting from a standardized principal and secondary environment and its related genotype and epigenetics. To ease the communication, we, therefore, speak of "functional AMF genotypes" from here on.

We exemplarily worked with the metric trait „host plant fresh weight" as a result of the expression of a set of non-allelic, quantitative polygenes under the influence of a standardized secondary environment and the tested functional AMF genotype. The variation observed should be due to AMF induced variation at several or many loci, each with a rather slight contribution to the variation in host phenotype. Because of the standardization of the environment for the host, the total phenotypic variation should result of host gene – AMF gene interactions. Against this background, an inoculum of identical functional AMF genotypes, including epigenetics, should have a nearly identical reaction norm under a given environment, including the host.

We started with the experiments with the development of strains of an initial *C. etunicatum* spore population multiplied over subsequent multiplication cycles. Single spores of sub-strains of each multiplication cycle were tested for their effectiveness on *Petroselinum crispum* as outlined in FELDMANN (1998c) and FELDMANN (1998b). It could be shown that the inoculation with single spores increased the variance of *P. crispum* fresh weight drastically, while the standard reaction of non-inoculated plants did not change significantly (FELDMANN, 1998b).

The spread of effectiveness values reached from negatively effective over neutral effectiveness to positive response. In case of positive effectiveness, the plant response was positively correlated with the degree of root colonization. Effectiveness of spores with neutral effectiveness and negative effectiveness did not show this correlation. High root colonization was not a guarantee for positive effectiveness. Even when descendants of a single spore ("strain") were compared, within this strain positively, neutrally and negatively effective substrains could be derived (Fig. 4, data derived from FELDMANN, 1998b).

Testing multispore inocula of these defined sub-strains for their effectiveness in subsequent multiplication cycles, originally negative strains appeared neutrally effective, neutrally effective strains remained neutrally effective and positively effective strains lost their positive effectiveness after two multiplication cycles (FELDMANN, 1998b).

By these data, we could demonstrate the existence of different functional AMF genotypes within an inoculum of a defined AMF strain for the first time.

In a next step, we estimated the "heritability" of the trait from one multiplication cycle to the next. A statistical measure of variation is the variance of measures. The proportion of the phenotypic variance that is genetic variance is the heritability of a trait. Here, we calculated the genetic component of variation by the correlations of effectiveness between spore descendants in the subsequent multipli-

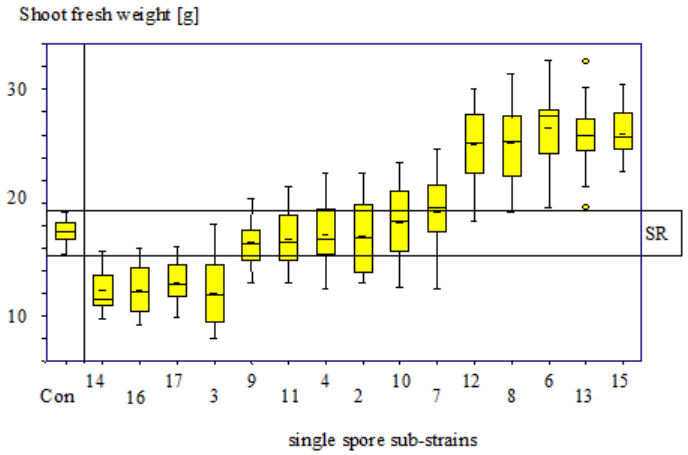


Fig. 4: Increase of variance of fresh weight of *Petroselinum crispum* by mycorrhizal strains after three subsequent multiplication cycles (data derived from FELDMANN, 1998b).

cation cycles C1/C2 and C2/C3. There was no correlation between spore effectiveness in C1 and C2 calculated from the slope (R=0.41), but a significant "heritability" of spore characteristics between C2 and C3 (R=0.80). These calculations were supported by the observation of distinct strain characteristics described above. Finding no heritability for the trait between the first multiplication cycles might be due to the particular spore population studied having not yet sufficient genetic variation to show because of the small sample size (25 observations in C1 and 50 in C2).

The next question to be answered was the clarification whether quantitative or qualitative interaction exist between host and functional AMF genotypes. Therefore, we repeated the experiment with a second host, the clonally multiplied host *Anagallis arvensis* (data derived from FELDMANN and GROTKASS, 2002).

In *Anagallis arvensis* clones, the inoculation with single AMF spores showed a variability of effectiveness from slightly effective to highly effective (C1). The multiplication of single spores from sub-strains with distinct effectiveness conserved the characteristics in the next propagation cycle (C2) indicating a purely quantitative interaction of functional AMF genotypes with the "new" host. The variability of effectiveness increased after a further propagation cycle (C3) but followed the pattern of quantitative interactions (see more details in FELDMANN and GROTKASS, 2002).

Genetic homogeneity of plant material seems to "conserve" strain characteristics better than selected seedlings. "Heritability" of strain characteristics on *Anagallis* clones were calculated 0.94 between C1/C2, 0.86 between C2/C3 and 0.88 between C1/C3.

Going back to the *P. crispum* test system, we started to develop an inoculum with known characteristics. We produced populations derived from sub-strains with distinct characteristics in C3 and inoculated them as populations – not as single spores. Would the host/AMF interaction further allow increase of variation? Alternatively, could a selective host maintain a certain level of effectiveness? Surprisingly, plants, which were inoculated with a population of C3 containing negative and neutral effective origins exclusively expressed neutral or positive effective symbioses in multiplication cycle C4. In the following two cycles C5 and C6 this characteristic was maintained with the majority of measurements mainly in the standard reaction range (neutral effectiveness). A certain sub-strain with a broad range of effectiveness (from negative to positive, but mainly neutral) did also not express negative growth responses but positive and mainly neutral effective symbioses. Another nearly completely positive effective sub-strain showed a trend to lower effectiveness in the subsequent multiplication cycles C4-C6.

In summary, it was obvious that the actually expressed effectiveness of the *P. crispum*/*C. etunicatum* symbiosis rapidly tended to result in neutral effective combinations, if the host was inoculated with spore populations of mixed sub-strains (FELDMANN, 1998b).

Environment/AMF functional genotype interactions

Changing the host led to quantitative host/functional AMF genotype interactions. Changing the secondary environment for both partners modified the physiological reaction norm of the principal environment for the fungus. Additionally, changed secondary environment might influence the strain characteristics directly. In order to understand more about the AMF population dynamics, we made a simple test. We let *Anagallis arvensis* grow together with a positively effective strain under different environmental conditions (pH of the substrate). The root colonization appeared low under low pH and the effectiveness in this treatment remained low. If we then multiplied spores produced under such conditions and inoculated again, we could achieve higher effectiveness under unfavourable conditions (more details see FELDMANN and GROTKASS, 2002).

The experiment showed that selection of AMF genotypes occurred under sub-optimal conditions possibly allowing only parts of the population to colonize the plant (qualitative functional AMF genotype/environment interactions). Furthermore, if this part was enhanced technologically, the inoculum could be optimized for use under sub-optimal conditions.

This was the breakthrough for the design of the Directed Inoculum Production (DIP).

The Directed Inoculum Production

As the consequence of the observations described above, we designed a very simple protocol for inoculum production (FELDMANN et al., 2009) integrating the population dynamical aspects with and without dominating environmental factors. The protocol recognizes that the relevant taxonomic units for specificity phenomenons are plant variety and fungal strain. We select only plants and plant cultivars for inoculum production showing high root colonization. As fungal partners, we use AMF from *in situ* conserved natural sites with certain environmental characteristics (e.g. drought or salt influenced). They should be highly sporulating and competitive.

This fungal material undergoes the following processing steps:

- We develop strains from single spores, resulting in sub-strains, which might cause different effects with different effectiveness on the plants involved in the production process
- We stress the plants during the first step of the production process (or longer) and select spores, which can reproduce and colonize under this pressure. Doing so, we adapt the inoculum to certain environments.
- We then mix inocula (sub-strains) with certain characteristics to populations of certain species or even communities of different species

Because the later target plants of customers are not pre-visible before inoculum production, we try to achieve a high functional AMF genetic heterogeneity in the inoculum. This can be reached by mixing different inocula from different strains and/or by producing them on diverse plants separately. The idea is that the target plant should have the possibility to choose the partner according to their dependency.

This DIP allowed us a continuous inoculum production over 25 years. The ideas were followed scientifically: BEDINI et al. (2018) presented first results of the adaptation of AMF as a tool to overcome phosphate inhibition in practical condition. Successful adaptation to high inorganic phosphate P_i would allow the use of AM fungi in conventional agriculture field, providing to crops the beneficial effects induced by the symbiosis and reducing the need of chemicals.

R. irregulare was propagated in parallel, for five generations, in presence or not of high P_i concentrations (up to 3.25 mM) in root organ cultures. Inoculation of potato with *in vitro* spores of *R. irregulare* exposed to high P_i for one generation did not highlight any effect on plant growth and root colonization. 5th generation grown under high P_i, instead, was associated with improved plant biomass and root colonization. Suggesting that in this case, at least five generations are necessary for the adaptation.

A very important example for strain adaptation was worked out by VAN BUI and FRANKEN (2018). They found that AMF in root organ cultures are able to gain heavy metal tolerance when they were grown in high heavy metal conditions. Moreover, the Zn-adapted strains could confer its higher heavy metal tolerance to plants. Plants inoculated with Zn-adapted strains showed higher biomasses than plants inoculated with a non-adapted strain. To our knowledge, this is the first research group outside of our own lab who reproduced the adaptation process with success following the DIP protocol. We hope that more such attempts will result in elucidating more details about the underlying mechanisms of the adaptation process already in use for so much years without knowing much detail about the mode of action.

Discussion

One important question needs to be discussed: what constitutes a "functional AMF genotype"?

We define it as "all genetic information of a single spore transmittable over spore multiplication cycles" This genetic information is expressed in tied interaction with the principal environment (the host) under the influence of (secondary) environmental factors.

This definition is the result of a long research process. Three decades ago, inoculum producers ignored the fact that effectiveness of symbioses resulting from inoculum was very variable because of genetical differences between propagules. They assumed not to have the right strain for the right environment or the right plant species if the inoculum failed to achieve the desired effect. AMF inoculum was thought to be genetically homogenous in a wide range because of the potentially exclusive asexual and clonal reproduction of spores (STUKENBROCK and ROSENDAHL, 2005). The assumed genetic homogeneity of AMF inoculum was the basis for all screening projects on AMF strains (BALTRUSCHAT, 1993). But the genetic homogeneity of an AMF strain does not exist (summarised in CLAPP et al., 2001).

Twenty-five years ago we analysed the interrelationship between AMF and host plants with quantitative genetical methods. A description of functional AMF genotypes as described above does of course not describe the actual genetic differences on the DNA level between strains but is focussed on active functional genes for specific interactions, including epigenetics, and transmitted characteristics of gene expressions. Nevertheless, the chosen definition reflects genotypes as targets for eco-factor actions and, therefore, gives a strict orientation for the design of the mycorrhizal technology in practice without knowing the actual physiological and molecular mechanisms.

Over more than one decade biochemical and molecular methods have been developed to distinguish between AMF species (e.g. SIMON et al., 1993), determine phylogenetic relationships (e.g. (REDECKER et al., 2000) and genetic heterogeneity (e.g. PRINGLE et al., 2000) of AMF. Heterogeneity of genes within single spores of AMF was described (e.g. SANDERS et al., 1995) by restriction fragment length polymorphism analysis (PCR-RFLP) and sequencing studies and had also been reported for the 18S rRNA genes (e.g. SCHÜSSLER, 1999). Other studies (PRINGLE et al., 2000) indicated that the level of genetic diversity in AMF rRNA genes was very high. This was supported by studies of the whole genome using other molecular techniques including randomly amplified polymorphic DNA (RAPD, e.g. WYSS and BONFANTE, 1993), M13-primed minisatellites (ZEZE et al., 1997), PCR-generated microsatellite loci (LONGATO and BONFANTE, 1997) and Amplified Fragment Length Polymorphism (AFLP, ROSENDAHL and TAYLOR, 1997). Intra- and interspecific diversity of ITS rDNA sequences was assessed by cloning and sequencing, and by small subunit (SSU) rDNA analysis (VANDENKOORNHUYSE and LEYVAL, 1998).

Different hypotheses existed about the organization of AM fungal genomes (ROPARS and CORRADI, 2015). Spores of AMF can contain more than a thousand of nuclei, which are proposed to represent the mycorrhizal „individual" (PRINGLE et al., 2000). These nuclei can be genetically different from each other. Nuclear exchange exists in some AMF fungi through anastomosis (GIOVANNETTI et al., 1999). More recently, DAUBOIS et al. (2016) could show independent mitochondrial and nuclear exchanges through anastomoses of strains from different proveniences. Furthermore, indications of recombination were found in *Glomus* populations (VANDENKOORNHUYSE et al., 2001), but were not confirmed by STUKENBROCK and ROSENDAHL (2005). Additionally, they pointed out that populations with mainly asexual reproduction are characterized by multilocus associations and identical genotypes composed of unique allels and proved this. Testing this in natural AMF populations they confirmed that all spore genotypes were unique and subdivision of genotypes existed at the experimental site within plots. The question of inter-nucleus recombinations was supported by CHEN et al. (2018), but AUXIER and BAZZICALUPO (2019) could not confirm their results.

Overall, it seems to be clear that genetically very different propagules in an inoculum meet the plant symbiont. But still in 2006, KOCH et al. had doubts that genetic differences would have an effect on the outcome of a symbiosis. In own experiments our group investigated with partners genetical differences between single spores on basis of the alternative oxidase (AOX), which is an enzyme of the alternative respiratory chain already described in different taxa, including various fungi, which decreases the damage caused by oxidative stress. The analysis of *RiAOX* polymorphisms in single spores of three different isolates showed a reduced variability in one spore relatively to a group of spores. A high number of polymorphisms occurred in introns; nevertheless, some putative amino acid changes resulting from non-synonymous variants were found, offering a basis for selective pressure to occur within the populations. Given the *AOX* relatedness with stress responses, differences in gene variants amongst *R. irregularis* isolates are likely to be related with its origin and environmental constraints. (CAMPOS et al., 2015).

We think, that "functional AMF genotypes" are constituted by more or less genetically heterogenous nuclei assemblages in all progagules involved in the formation of the symbiosis, including the coenocytium of arbuscular mycorrhizal fungi in and outside of host roots. It probably depends on the quantity of certain genetically defined nuclei and their activity pattern how the development of the symbiosis starts and continues. A selection of nuclei or activation of nuclei by plant and environment may be the basis for adaptation processes to plant properties (e.g. dependency) or environmental factors utilized in the directed inoculum production process. VAN BUI and FRANKEN (2018) state that differential gene expression could be based on the selective accumulation of particular nuclei during the adaptation (what they call "acclimatization process"). The consequence would be the expression of different alleles from one locus in the adapted and not adapted strains (EHINGER et al., 2012).

Further sequencing of transcripts and genomes of individual nuclei must clarify, if differential expression of the same allele or the occurrence of different genomes accompanies variations in adapted strains. Unfortunately, attempts to determine whether sequence diversity found in single spores originates in polymorphic genes within single nuclei or within different nuclei had long time not been successful (e.g. HIJRI et al., 1999).

In any case it became clear that genetic exchange and altered mycorrhiza-specific gene transcription occurs (COLARD et al., 2011).

Currently, indications rise that genetic variation of the symbionts regulate the physiological interaction of micro- and macrosymbiont (Savary et al., 2020). The group found a clear link between mycorrhizal fungal genetic variation and plant molecular reprogramming that reflect the evolutionary history of closely related AMF involving the production and transport of one essential currency that is fundamental to the symbiosis. While previous molecular studies have qualitatively demonstrated the switching-on of this important pathway in symbiosis, the study shows that in order to understand the regulation of the currency involved in this important mutualism, incorporate fungal genetic variation rather than using more simple experimental designs is necessary. Furthermore, dual RNA-seq revealed large-scale non-conserved genotype × genotype-specific genetic reprograming and molecular crosstalk in the symbiosis (Mateus et al., 2020).

Outlook

For a producer of AMF inoculum, DIP was a decisive step forward in enhancing the predictability of the inoculum effectiveness. In Fig. 5 the most important factors influencing mycorrhizal effectiveness are summarized. The viewpoint is strictly host oriented, because it is the effectiveness on the host we want to achieve.

The genotypes of host and fungus form the mycorrhizal host phenotype under the influence of concurrent environmental conditions. The mycorrhizal host phenotype in relation to non-mycorrhizal host plants reflects the mycorrhizal effectiveness ("dependency") with regard to the evaluated effect. Roughly summarized, variability of environmental factors causes variability of effectiveness via qualitative and quantitative changes of inoculum characteristics, root characteristics or root colonization rates.

Mycorrhizal dependency results from the host's genetically and epigenetically fixed characteristics under given environmental conditions, which define the physiological status. This includes e.g. cultivar specific pattern of growth and development, the tolerance and resistance metabolism, developmental state and root morphology. Knowledge of plant's growth, therefore, allows exact application time for inoculations with AMF. At the same time, the growth of AMF and the production of propagules is well known even in commercial scale (Vosatka et al., 2012). Overall, there is no real problem to bring both partners together to colonize the host's roots and to form efficient symbioses.

For the AMF, the micro-symbiont, there are two quantitative aspects of major importance: the inoculum potential, i.e. the number of propagules of AMF inoculum, and the AMF community and population composition. These two parameters are influenced during technical inoculum production. The other parameters cited in Fig. 5 are processing factors, which guarantee the desired coincidence of the right developmental stage of roots/plants and infective micro-symbionts. Against this background, high quality inoculum has to provide sufficient fungal material with characteristics realising desired effects with commercially reasonable effectiveness. Because the plant cultivar is defined by the target production system, the question returns, which fungus should be used. At this point, DIP is a process to adapt the fungal material to later, anticipated environmental conditions.

The next step is definitely much more complicated: to develop an inoculum with desired characteristics for a number of plants and target environments. On the one hand, the key lies in the inoculum production process. We need to find plants, which are not selecting AMF genotypes but multiply all. We need ideas how to adapt inocula to various environments at the same time. The reason are simply the costs of the inoculum.

It is mandatory to reduce the developmental costs of inoculum to be able to inoculate more propagules to a given target scenario. This would favour adapted AMF in competition situations. Still, another question is unanswered: should we breed crops for mycorrhizal symbiosis (Feldmann et al., 2013)? The future might be a completely different inoculum: ecologically optimised inoculum for large-scale use in cash crops, which are genetically adapted for use of this important symbiosis.

On the other hand, we have to learn much more about "AMF population engineering". This is mainly directed to understand a quantitative aspect of inoculum application. Changing the quote of different isolates, strains or functional genotypes within a commercial inoculum should result in guaranteed establishment of the elaborated strains under practical conditions. Furthermore, it has a qualitative aspect by influencing the functional potential of strains by environmental adaptation processes. Such qualitative population engineering will result in the option that the fundamental ecological niche of both, host and AMF, is utilized to create a widened actual ecological niche of the symbiosis resulting in advantages for the host to be explored.

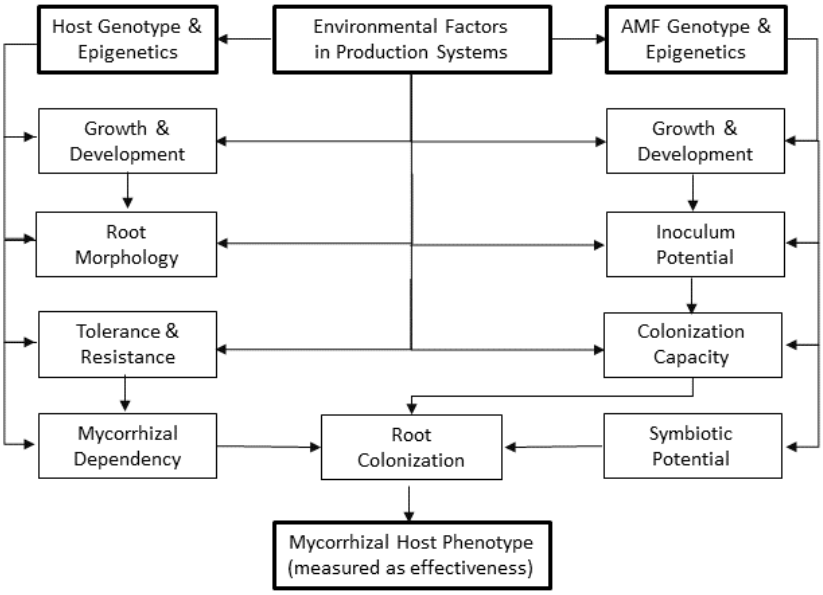


Fig. 5: Mycorrhizal effectiveness as phenotype of host/AMF interactions and population biological measures (after Feldmann, 1998a).

We worked now 25 years on engineering functional AMF genotypes in arbuscular mycorrhizal inoculum in order to get a good product for use in agriculture and horticulture. Our long way started with the simple question of late Prof. Dr. Reinhard Lieberei who asked: "Why shouldn't you be able to find out how to use them?" We are thankful for this encouraging initiating push.

Conflict of interest

No potential conflict of interest was reported by the authors.

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