Best Production Practice of Arbuscular Mycorrhizal Inoculum

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Introduction

The approach of Best Agricultural Practice (BAP) requires solutions with regard to the core factors of sustainability namely ecology, economy and social demands (Feldmann, 2007). This is true for the biotechnological product „arbuscular mycorrhizal inoculum“ as well.

The product quality assesses whether a product is ‘fit for purpose’. The product must meet or exceed the customer’s requirements. It is the customer who sets “quality standards” in terms of his expectations. Customers may define different quality characteristics for mycorrhizal inoculum: formulation, handling, weight, safety, functionality or others. *Socio-Economic Impact Analysis* and adequate *Environmental Risk Assessments* carried out along with *Life Cycle Assessment* are measures for energy, material flows and impact estimates associated with all stages of a product from inoculum producer to the customer. Finally, the *Cost-Benefit Analysis Process* (on the basis of the *Business Ethics Assessment*) involves monetary calculations of initial and ongoing expenses vs. expected return.

All these instruments should be common standard in a company producing inoculum for national or international market. Only the application of all instruments of concurrent quality control procedures results in a traceable and reliable supply chain

with the consequence of reliability of the whole product chain as basis for sustainability. This pre-requisit prevents the customer from buying some expensive, non-effective instead of high quality AM inoculum.

The basis of production of high quality AM inoculum is the understanding of biological principles of population biology of AM fungi. But we shall not go into too much detail when documenting this biological basis, working hypotheses or procedures chosen. Here it is described how to design an inoculum of an AMF generalist and how to produce it in large scales (> 8 x 10^9 spores/year).

On basis of *working hypotheses* four steps are demonstrated: a) the *planning phase* of the inoculum production is outlined. Furthermore tuning of inoculum is described in b) the *analytical phase* the immanent functional variability of inoculum is tested. In c) the *adaption phase* is scheduled, how to extend the abilities of inoculum. Finally, in d) the *upscaling phase* of inoculum production is described. The paper will be completed by exemplifying concurrent quality control procedures.

Working hypotheses

1. *A plant does not aim for colonization by mycorrhizal fungi.* It depends on the host and fungus genotypes, their coherence and on favourable environmental conditions to develop a mycorrhiza (Allen, 1991). This offers the possibility to choose a large amount of suitable host/fungus combinations for inoculum production. The relevant taxonomic units for specifity phenomenons are plant variety and fungal strain.
2. *The process of colonisation by mycorrhizal fungi means stress for the host and the AM symbiosis is a parasitism/mutualism continuum*. This can easily be observed after inoculation of young seedlings expressing growth depression. Following the stress theory of Stocker (1947) this can be overcompensated after the “alarm phase” resulting in a desired host response (e.g. better growth) and can be stabilised by product exchanges (e.g. carbonhydrates vs. water and nutrients) with concurrent mutualism. We balance the developing parasitism/mutualism continuum (Johnson et al. 1997, compare Feldmann, 1998b) by nutrients and irrigation to favour the fungal sporulation.

3. *Mycorrhizal effectiveness is related to the ecological situation of host plants but it is no relevant measure during inoculum production*: Fungal sporulation is a cost intensive process for the plant. In inoculum production we favour and force the host plants to allocate as much carbonhydrates as possible to the fungus.
4. *Ecological characteristics of AM inoculum can be designed by pre-adaption processes* (Feldmann & Grotkass, 2002). This offers the possibility to substitute expensive, time consuming screenings of isolates with a short shelf life. Biological basics to be considered are the problem of ecological niche of AMF and hosts, limiting factors of plant growth and phenotypic plasticity of both.
5. *Phenotypical characteristics of inoculum are often expressed on the basis of fungal population composition* (Feldmann, 1998b). This requires stepwise multiplication cycles during upscaling procedure, concurrent quality control and advanced mixing techniques considering phenotypic variation of mycorrhizal fungi.

The planning phase: define what you need!

Achievements which an inoculum should fulfill, which organisms are involved and the purpose of the product dominate the planning phase and result in a biological data sheet. Following our experience, the design of an inoculum for very general use is possible, but requires much more attention, knowledge and experience than a more limited design. As an example, the planning phase for an inoculum to be used in ornamentals in greenhouses (as developed for German gardeners earlier, Feldmann et al., 1999) is presented:

The *Biological Data Sheet* (BDA) should contain specifications about host and fungus (Tab. 1). Details concerning the desired target plants are fixed. The evaluation of this commercial BDA should lead to discussion of general inoculum characteristics including desired carrier materials, formulations and acceptable pricing (maximum price). In our example it was decisive, that the fungus would colonize a range of different ornamental plant species within one-two weeks (between potting the first and second time) and developing growth responses within eight weeks after the second potting (before sale to the customer). This inoculum had to tolerate high dosages of fertilizer.

Tab. 1: Biological data sheet for inoculum design and planning

Parameter	**Macrosymbiont (Host)**	**Microsymbiont (AMF)**
Species, varities/strains	*Heliotropium arborescens* cv. "Marine", *Bidens ferulifolia* cv. "Goldmarie", *Brachycome iberidifolia; Chrysanthemum* cv. "Maja Bofinger", *Lobelia erinus* cv. "Cobalt Blue", *Lantana camara* cv. "Feston Rose", *Sutera cordata* cv. "Snowflake", *Sanvitalia procumbens* cv. "Gold Braid", *Pelargonium cv.* "Butterfly", "Leuchtkaskade", "Grand Prix", *Verbena x hybrida cv.* "Imagination" and "Romance"	*Glomus etunicatum*
Range of intended use	Ornamental greenhouses; mycorrhization from January to March	
Desired mycorrhizal effect	shortening of the „standing period“ of ornamentals in greenhouses, earlier flowering and therefore earlier sale. Longer flowering under balcony conditions: Underlaying effect: quicker growth	
Care requirements after installed (water weekly, water once, plant protection etc.)	temperature: 14-21°C; fertilizer: max. 2g/l x week of NPP 18/12/18; use of diverse pesticides, sprayed and in the irrigation water	
Natural occurrence (where, how common)	not relevant	cosmopolitan, not in sterilized substrates
Habitat preferences	warm, moderate	very variable
Strategy type/successional stage (stress-tolerator, competitor, weedy/ colonizer, etc.)	r- and k-strategists	r-strategist; strain selected for rapid colonisation; generalist
Potential ecological main factors under natural conditions	-	high tolerance to high fertilizer concentrations
Associated species	no	no
Material provided by/ collected as/from	plants available from production companies	gene bank
Propagation	cuttings	DIPP with two steps (medium scale)
Soil, substrate or medium requirements for cultivation	Humus-rich, moist soil, with pH 5.0 to 6.5 (mildly acidic).	to be adapted to pH 5.0
Specific growth, spread; or lifespan conditions (later host plant)	Introduction very early after preparation of cuttings or seedlings; three changes of users: seedling company, nursery, selling company, consumer	
Desired carrier material for plant material or propagules; technical requirements	Standard soil, potted	expanded clay particles (1-2mm diameter, broken), maximally 0.5% of later pot volume (defined by plant producer); suitability for automatic potting
Restrictions or guidelines	free of Oomycetes, free of weed seeds, free of fertilizers	
Maximum price	0.12 Euro/cutting in average	0.01 Euro/cutting in average
References (own experiences)	Feldmann et al., 1999; Weissenhorn and Feldmann, 1999	

Furthermore, the price had to be very low because the user was not the one to expect the benefit, but the buying interest was based on added value regarding Inner Quality of plants.

At this point you have to decide for starter inoculum. The more detailed knowledge on the isolates is available the easier it is to select AMF from the own gene bank, in situ conservation area (Fig 1 and 2; Feldmann & Grotkass, 2002), to isolate fungi or to buy them.

The analytical phase: make your AMF isolate a strain and describe its abilities!

Following our example, a mycorrhizal fungus was needed which covered a broad range of environmental conditions and rapidly formed symbioses with several hosts. The growth conditions were variable, but rather common and not extreme, only the pH lower than 5.5 and the velocity of colonization might create problems during the colonization phase. In such a case a fungus is helpful which is already adapted to rapid colonization, e.g. by living together with short-living plants like *Anagallis arvensis* or others and at the same time is widespread covering the cited pH conditions in the soil. From our gene bank we chose *Glomus etunicatum* known to meet these pre-conditions.

It is important to recognize that only single spore cultivation can guarantee what we call a „strain“. Strains are derivates of single spores, „isolate“ means only that single spore or spore populations were collected from the field.

In literature a huge amount of examples for positive mycorrhizal effectiveness in scientific experiments are published. Most AMF users do not apply AMF strains, but inocula on the species level, undefined AM fungi or mixtures of different sources. Experiences show that offering host plants any mycorrhizal symbiont results in some effectiveness if the plant has a need for symbionts. The decisive point is that the predictability of effectiveness is of crucial importance for commercialisation: no plant producer will buy and introduce AM inoculum if the outcome is not to some extent predictable. Only the description of the inoculum, possibly followed by selection processes or mixing procedures offers the possibility to prepare inoculum with desired characteristics and predictable effectiveness. On that background we recommend to develop a strain from field collections or to look for a starter inoculum with exactly described content.

Fig 1: Cool storage of defined AM strains in a gene bank of an inoculum producer

Fig 2: In situ conserved genetic potential of AMF to tolerate salt stress at natural sites with salt vegetation

Fig. 3: Step one of the single spore multiplication on trap plants (here: *Tagetes spec*.)

We start with the description of phenotypic variability in test plant populations resulting from the cloning process of single spores when developing a strain.

Here, the microsymbiont was represented by single spore descendants of a taxonomically not distinguished *Glomus* spec. (probably *Glomus etunicatum*, Tab. 1, compare Feldmann, 1998b). The spores were produced on *Petroselinum crispum* in sand and used for inoculation after extraction from the soil by wet sieving and decanting techniques (Schenck, 1984). For inoculation, single spores were separated with micropipettes and placed near the rhizosphere of the host plant. Sand was used as substrate and the plants were kept in 2.5 ml plastic tubes first (Fig 3) and transferred to 25 ml plastic tubes after two weeks under controlled greenhouse conditions according to Feldmann et al. (1998a): illumination by SON-T AGRO 400 Philipps lamps ($360\mu E \cdot m^{-2} \cdot s^{-1}$), 14h/d; 60-80% relative humidity; 18-20°C night, 22-26°C daytime; irrigation below field capacity; fertilisation once per week with 1% pot volume of a commercial fertiliser solution (1g fertiliser/l solution), pH 5.5. The fertiliser contained 15% N (10% nitrate, 5% ammonium), 7% P_2O_5, 22% K_2O, 6% MgO, 0.03% B, 0.05% Mn, 0.01% Zn.

For the analysis of AM phenotype frequencies we chose test plants with a broad ecological niche and easy to cultivate: *Anagallis arvensis* and *Plantago lanceolata*. The selected plant species occur on arable lands, on open, sandy or rocky habitats or wasteland and even polluted areas. They can be found on soils with pH between 4.5 and 8.0. Soils may be poor or rich in nutrients, variable temperature and light is tolerated. The ecological niche of these plant species covers most of the factors important for agricultural and horticultural practice, both are intensively colonized by mycorrhizal fungi (Weissenhorn and Feldmann, 1999).

For inoculation, single spores were separated with micropipettes and placed near the rhizosphere of the host plant. At that time cuttings (*Anagallis arvensis*) had a root system of approximately 6-7cm length and the upper plant parts were at homogeneous developmental stage (i.e. the variation of shoot length, leaf number and leaf size was not larger than 5%).

Plants were inoculated with single spores and plant fresh weight was measured after eight weeks of culture (C1). After that, from three colonized host plants of significantly different fresh weight each time ten single spores were isolated and

inoculated to new host plant individuals. After another two months the fresh weight was measured (C2). All sub-strains of C2 deriving from one single spore (C1) were mixed and 15 single spores each isolated from this mixed population and afterwards inoculated. The third propagation cycle was carried out within the next two months.

Mycorrhizal colonization was qualitatively determined after clearing the roots in 10% KOH for 15min, neutralisation with HCl, three times washing and staining for 25min in 0.05% trypan blue in lactic acid / glycerin (10:1 vol/vol). For estimating the degree of colonization the whole root system was used.

The mycorrhizal efficiency (MEI) index was estimated according to Bagyaraj (1994):

$$MEI = \frac{\text{weight of inoculated plant} - \text{weight of uninoculated plant}}{\text{weight of inoculated plant}} \times 100$$

Statistical evaluation of the data was carried out by the one-way analysis of variance (ANOVA) for the respective factor with a significance level at 5%.

Following these methods it was observed that the inoculation with single AMF spores from cloned strain show a variability of effectiveness from slightly effective to medium to highly effective (Fig. 4, C1). The multiplication of single spores from sub-populations with distinct effectiveness conserved the characteristics in the next propagation cycle (C2), though, the variability of effectiveness increased after a further propagation cycle (C3). Distinct characteristics of the sub-populations did no longer exist after C3.

The reproducible response of the clonal host under standard conditions caused by AMF descendants of single spore isolates verified the existence of genotypic differences in the initial spore population. The slight variability of effectiveness during the first propagation process reflects the still existing variability of the plant material and experimental errors. If the variability of effectiveness observed in C1 would have been a result of phenotypic plasticity of only one fungal genotype, the same variability would have had to occur in C2.

After the second propagation cycle the distinct characteristics of the genotypes start to become modified because there is an increase of variability in effectiveness in C3 (Fig. 4). The basic mechanism for the enhanced variability in effectiveness of

genotypes still remains unclear. Host gene / AMF gene adaptations are as possible as high mutation rates of the fungus.

For practical application, the findings are of special importance: if genetically fixed characteristics of AMF spores are stable for only one or two propagation cycles, AMF inoculum production should not be based on past inoculum charge but on fresh spore material from stock cultures. This complicates up-scaling in inoculum production, because slight differences as shown for the effectiveness of C3 (Fig 3) can create considerable changes in effectiveness of an inoculum produced with this method (Feldmann et al., 1999).

Basing on this system a wide range of parameters can be tested: velocity of colonization, salt content, heavy metal stress (Feldmann and Grotkass, 2002). Phenotypic variability exists in every case and we recently describe by quantitative molecular genetical methods which percentage of the variability might be genetically fixed and heritable.

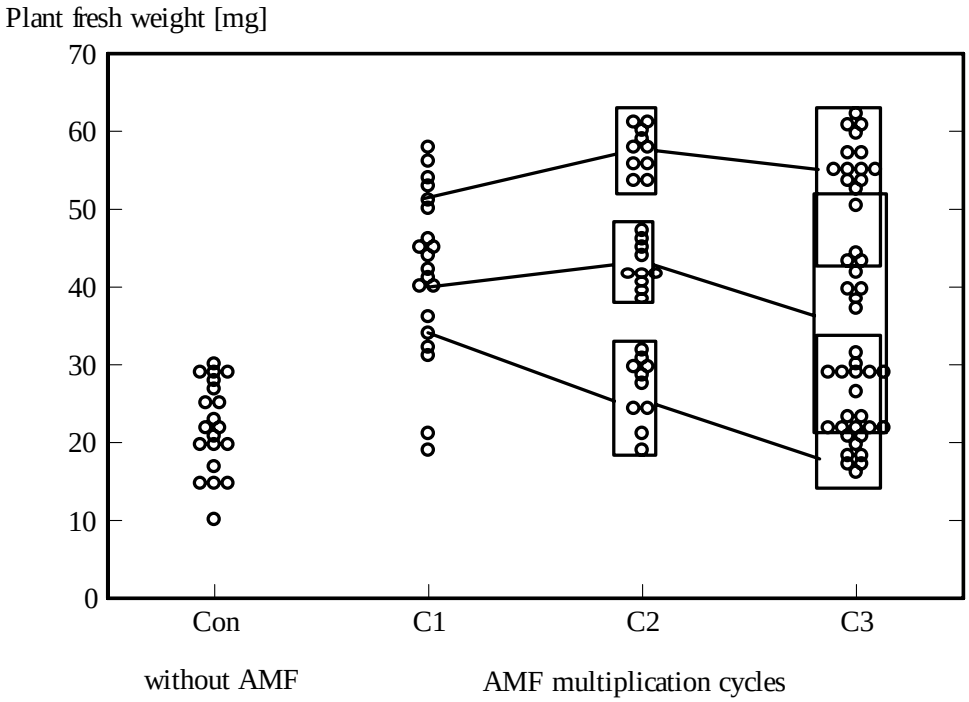

Figure 4. Mycorrhizal effectiveness of AMF single spore descendants (*Glomus spec.*) on the biomass of *Anagallis arvensis.* See distinct sub-population characteristics in C2 and overlapping effectiveness in C3.

The adaption phase: direction instead of screenings

The best sub-strains of C2 (Fig. 5) for adaption of inoculum to lower soil pH (or P-content, stressors like drought, salt or heavy metals and others) are chosen.

Ten cuttings of *Anagallis arvensis* per treatment in three parallel repetitions are grown until they develop a considerable root system (conditions as above). Before inoculation the soil is infiltered with nutrient solution of changed pH (pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) until the run off has the same pH like the infiltered solution. For inoculation approximately 100 spores are transferred into the substrate near the roots of *Anagallis arvensis*. After 21 days the plants are carefully extracted from substrate, the roots washed to remove old spores and planted to a larger pot (50ml) with fresh substrate. The time period may be chosen following your requirements. Here it is short because of the need to select „rapid“ genotypes in addition to pH tolerable ones. The plants remain in that pot for approximately another four weeks until sporulation of the fungus. Plants are harvested, shoot fresh weight determined and mycorrhizal status of roots analysed. The substrate of all treatments is pooled and called C1. After that step 1 the first propagation cycle is repeated three times (C2, C3, C4) with pH treatments and the colonization of the test plants analysed 21 days after inoculation.

An analogous experiment with different phosphate concentrations in the substrate at pH 5.5 was carried out (5ppm, 15ppm, 30ppm, 60ppm, 90ppm, and 120 ppm) and was already published (Feldmann and Grotkass, 2002).

At extreme soil pH the colonization of the host plants initially may be low (Fig. 5). But the percentage of spores within the tested inoculum, able to colonize under extreme conditions can be enhanced by separate propagation and later mixing the freshly produced spores. Consequently, the effectiveness of the tuned inoculum is enhanced under extreme conditions, as compared to the initial start inoculum. This is a further indication for the existence of different genotypes within a strain and an important step on the way to direct the inoculum production process successfully.

Under variable environmental conditions probably the physiological status of the host is the main factor that expresses dependency or independency on mycorrhizal fungi. Therefore, *Directed Inoculum Production Process* (DIPP) will especially be

successful, if the relationship between later target plants and desired target mycorrhizal effect is clearly defined before the inoculum production starts.

In summary, there is a possibility to influence the genotype composition of an AMF population by directed processing of the inoculum production. Abiotic environmental factors can be used to select and canalize AMF genotypes. But the chosen plant species with its specific mycorrhizal dependency seems to have special importance for the result of the process as well. Finally it has to be pointed out, that inoculum adaptation to stressors (salt, heavy metals) lasts only one to two multiplication cycles (Feldmann and Grotkass, 2002).

The upscaling phase: one further step only!

As mentioned above, strain, resp. inoculum characteristics are stable minimally one, normally two or –depending on the desired effect – three multiplication cycles.

Mass production of AMF means the production of up to several thousands of litres inoculum containing approx. 80.000 infection units per litre. Inoculum is normally produced in pots of different sizes with one or two, sometimes four host individuals. Without nutrient limitation the growth of the host plants in pots is quite homogeneous due to limited space for root development. Therefore, differences in AMF effectiveness of sub-populations were rarely observed or interpreted as result of the genetic differences between host individuals. The AMF action and the host growth were found to be different in larger plots without space limitation of root development. We calculate on the following basis: one single plant individuum (here: *Zea mays*) produces, depending on the substrate type, in six liters approximately 400,000 – 1,600,000 spores. It depends on the quantity of starter inoculum how long it will take. One should inoculate not less than 1 spore/ml and not more than 10 spores/ml starter inoculum to the seedling initially.

The mass inoculum production should be carried out in greenhouses in ground beds. Up to 50 host individuals are involved in the AMF multiplication in one unit. The ground should be covered with foil, permeable for water but not for soil fauna. The seedlings are planted with a spacing of 50cm x 50cm. Light, temperature and nutrients should not limit the growth of the host. Select a host variety which is susceptible to mycorrhiza under optimal growing conditions and let the plants grow for up to five months in the substrate under optimal conditions.

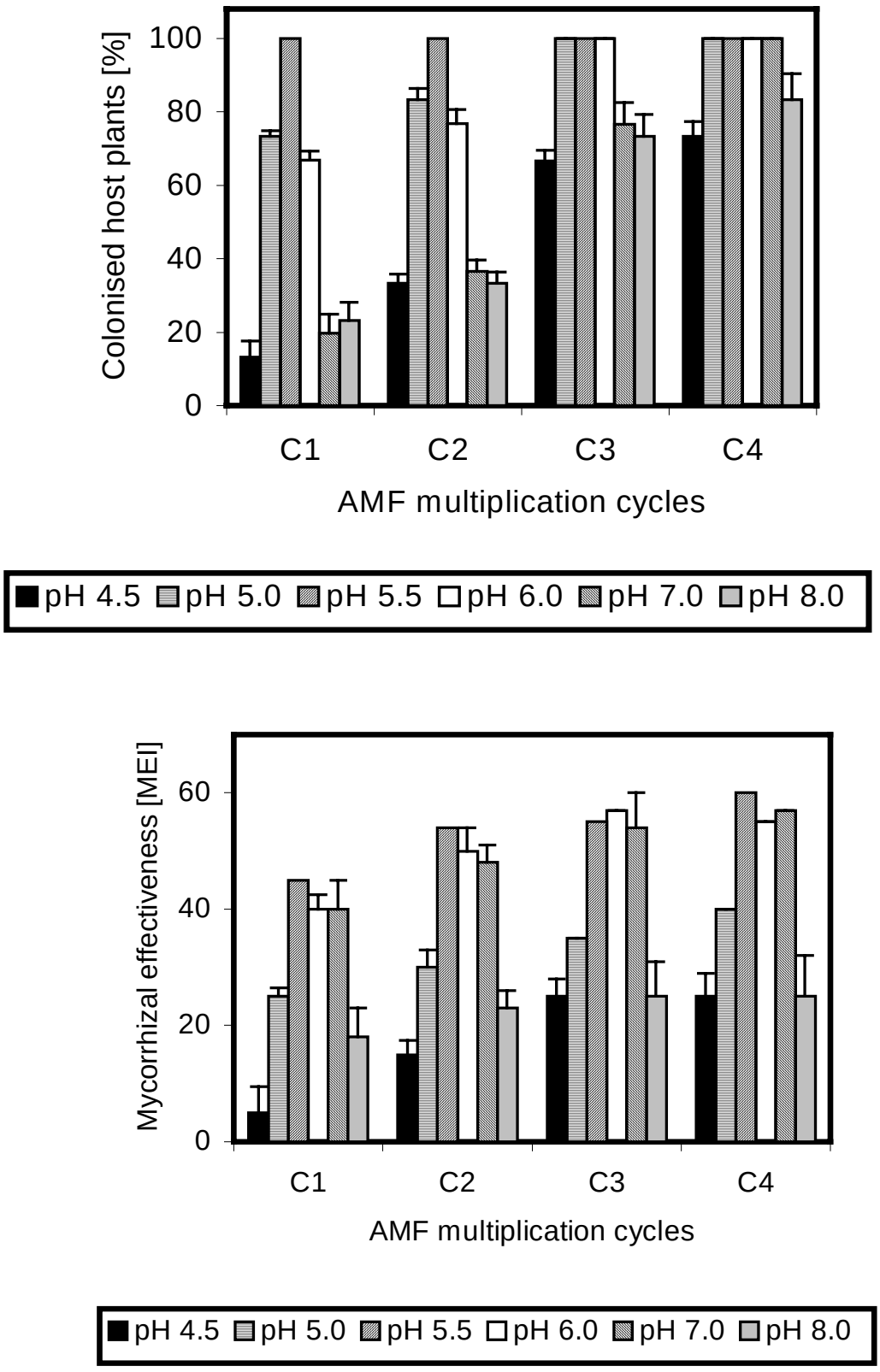

Figure 5. Root colonization ability and mycorrhizal effectiveness of AMF populations (*Glomus spec*. GK 12 on *Anagallis arvensis*) with technically modified genotype composition (Selection factor „soil-pH“, details see text).Bars: SD

At the end of the inoculum production the host plant has to be dried within less than two weeks without removal of green plant parts. Observe the fungus sporulating in this time period: As soon as the spore number is not longer varying, the process is over and drying of the inoculum can start.

Concerning the effectiveness it can be observed that the unlimited root growth of hosts in plots may lead to further seggregation of pre-selected strains with high effectiveness into new sub-strains with neutral to high effectiveness during mass production (Fig. 6). Nevertheless, more than 90% of the inoculum caused positive growth response in the host (*Zea mays*) during inoculum mass production. This quota seems to be reproducible and would make it economically feasible to select sub-strains with special effectiveness and to discard eventual sub-populations of lower effectiveness after mass production.

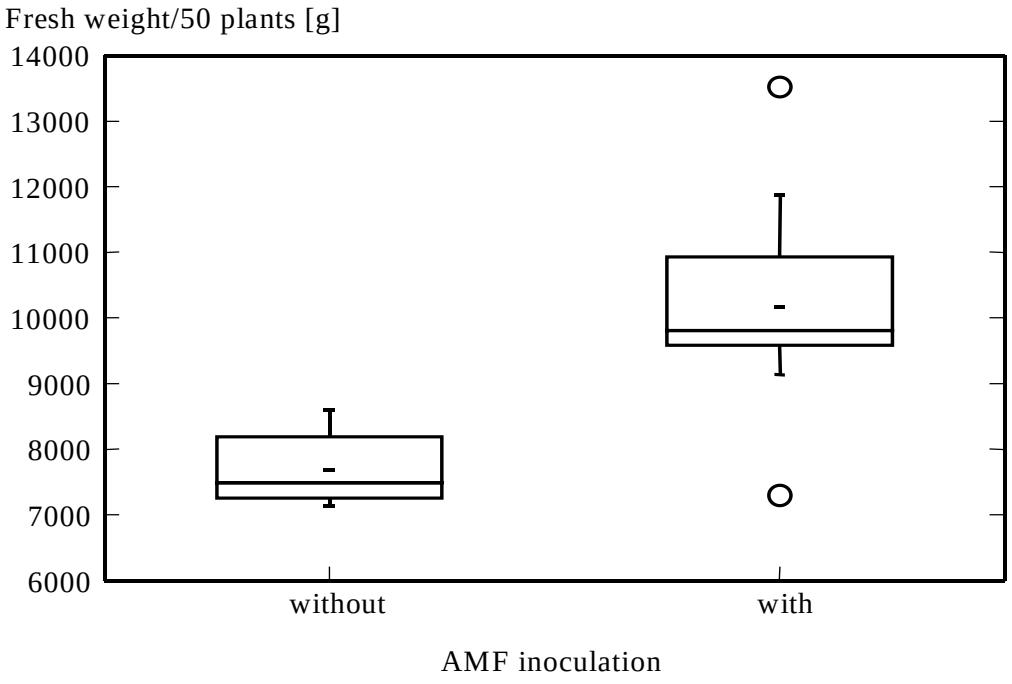

Figure 6. Variability of effectiveness of an AMF strain (*Glomus* spec.) during inoculum mass production in plots with 50 host individuals (*Zea mays*).

Concurrent quality control: you should know what is going on!

Samples are taken every three to four weeks to measure the principal components in relation to the developing symbiosis. Soil nutrient analysis is carried out by

commercial labs, faunistic and microbiological analysis by molecular genetical analysis (DNA multiscan®) The Most Probable Number of infective propagules is measured only at the end of the production according to Feldmann and Idczak (1994).

All results are analysed together in a multi-variet plot to investigate whether negative interrelationships developed (Fig. 7). Basing on this analysis modifications of the system are processed. Be aware that we are not speaking about the final quality control procedure for the inoculum before liberated to the market (compare von Alten et al., 2002), but process control of the production process.

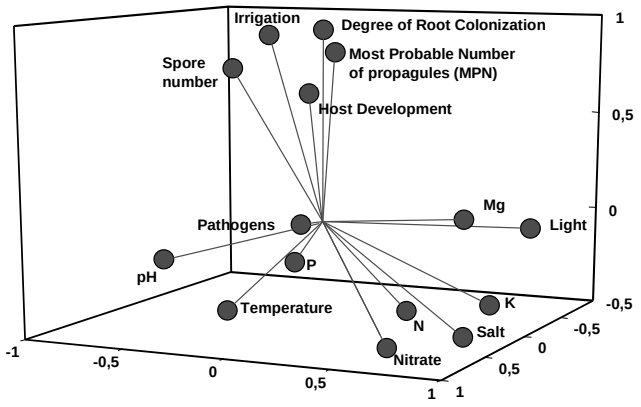

Figure 7. Principal component factoring of growth conditions

Testing the inoculum in practice: did it achieve the desired effects?

The inoculum was introduced to the plant production process under practical conditions. Later plants were sold or studied until their death caused by the end of the growing season at their growing site (balconies, gardens). Mycorrhizal plants were sold in average 5 days earlier preferentially (Fig. 8). This effect was observed in ten of fifteen plant cultivars tested, reflecting the achievement of the desired effects (better flowering or better growth). The effect was measured as a multi-factorial trait by the customer. Out of a mixed plant population in a plant shop customers selected plants without knowing that AMF were used. If they preferred AM plants the desired effect was called ”realised” (Fig. 9).

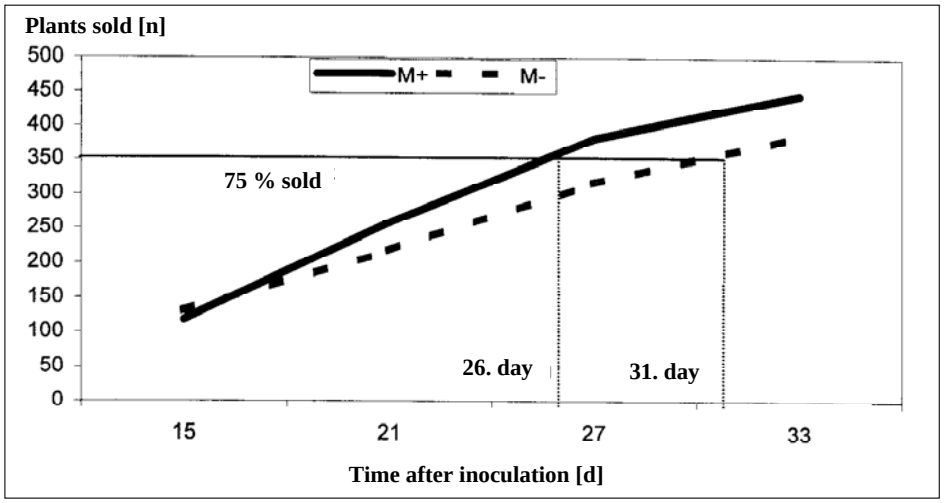

Figure 8. Preferential sale of mycorrhizal versus non-mycorrhizal plants (the action was economically senseful and the desired effect achieved after selling 75% of the plants in a shorter time period (Figure from Feldmann et al, 1999.)

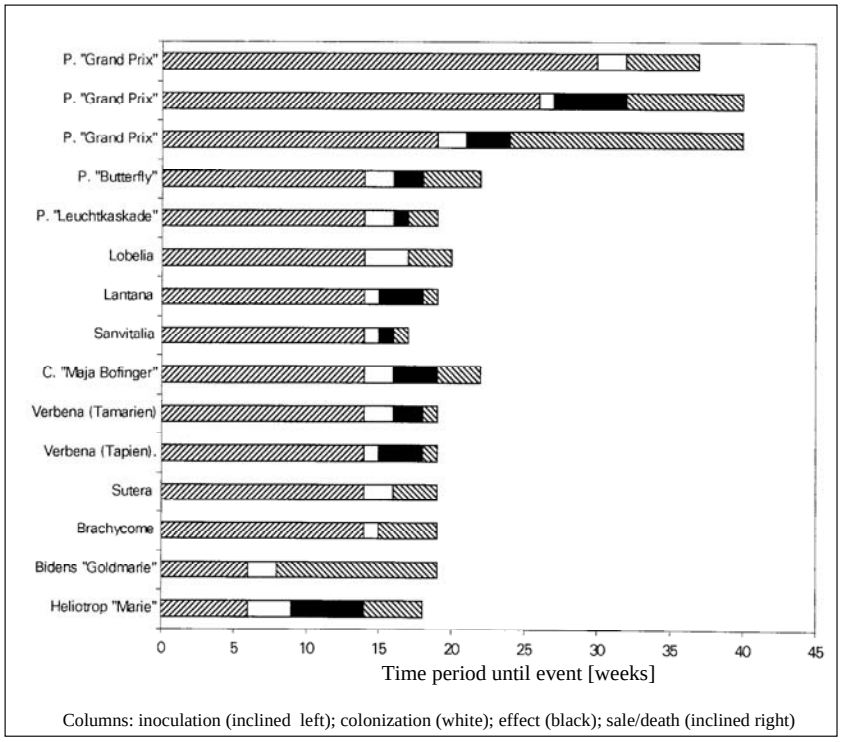

Figure 9. Effectiveness of directed AMF inoculum in practice: black columns show the time period until the AM plants were preferentially sold to customers out of a plant population mixed with non-mycorrhiozal individuals. (Figure from Feldmann et al, 1999).

To demonstrate the impact of *Directed Inoculum Production Process* (DIPP) many experiments with the same AMF strain but different host species, different inoculum quantities, environmental conditions, scales and effects were designed. In practice a threshold value of MEI >30 must be exceeded to create interest of a potential customer in mycorrhizal technology. The positive outcome of an inoculation was called „predicted“ if that MEI value was clearly passed under commercial conditions of plant producers.

Table 4. Increase of predictability of mycorrhizal effectiveness with *Directed Inoculum Production Process* (DIPP). „Constant environments“ are greenhouse or growth chamber conditions, field or garden experiments were carried out under „variable environments“

Inoculum production	Experiments [n]		Predicted success [% experiments]	
	environment		environment	
	constant	variable	constant	variable
with DIPP	16	35	87,5%	68,6%
without DIPP	59	41	52,5%	36,6%

DIPP was introduced to the plant production and optimized in the company IFP since 1996. Defining „predictability of AMF effectiveness“ as quantitative value for the frequency of expected host growth response to symbiosis we can compare experiments before and after the introduction of DIPP. The results (Tab. 4) showed that predictability could be clearly increased. DIPP is a promising way to provide guaranteed thresholds of effectiveness.

Discussion and Perspectives

All over the world there are efforts to include arbuscular mycorrhizal technology into processes of plant production. Benefits caused by arbuscular mycorrhizal fungi (AMF) are used in the weaning stage of in vitro cultivated plants (review see Lovato et al, 1995). Inoculation of seeds, seedlings, cuttings or completely developed plants (Chang, 1994) is recommended. The introduction of AMF to target plants is carried out under greenhouse conditions (Miller et al., 1986), in nurseries (Nemec, 1987) and in the field (Thompson, 1994). One single AMF species can be inoculated to dicotelidons, monocotelidons and ferns (e.g. Feldmann, 1998a). Furthermore, the

same AMF species can be used in humid tropics (Sieverding, 1991) and in temperate climates (Baltruschat, 1993).

In spite of such a spectrum of different environmental and cultivation conditions there is one unique expectation in case of an AMF inoculation: the developing symbioses have to work successfully, must provide advantages to the target plant and fulfill the customers requirements. „Symbiontal effectiveness“ is a multifactorial phenomenon. Host and fungal genotype both together influenced by abiotic and biotic environmental conditions express the phenotype of the specific, relevant symbiosis. „Positive effectiveness“ in agricultural or horticultural sense is judged as a „positive response“ of the host under perspectives for the plant growth, yield or stress tolerance.

There are several possibilities to influence the phenotype expression of the symbioses in practice, e.g. deciding the time of inoculation with respect to the developmental stage of the host, quantifying the inoculum potential or changing the culture conditions.
Nevertheless, before introduction of *DIPP* there was only a low predictability of the quantitative aspect of an effect (i.e. the effectiveness) a mycorrhizal symbiosis might have in practice. In fact, AMF effectiveness following artificial inoculation ranged from positive to negative (Varma and Schuepp, 1994) in a mutualism-parasitism continuum (Johnson et al., 1997).

To deal with that problem screening processes for AMF strains (Dodd and Thomson, 1994) in order to find the „best“ mycorrhizal strain (e.g. Baltruschat, 1993) or effective AMF mixtures (e.g. Sieverding, 1991) have been developed. The results of all those efforts were disillusioning. The predictability of AMF effectiveness remained too low for the sustainable use of AMF in commercial horticultural and agricultural practice, especially in moderate climates. Industrial interest in the use of AMF in plant production processes bears still no relation to potential of the technology (compare Feldmann, 1998a).

At present, there are two fundamental questions to be answered for understanding the basis of mycorrhizal effectiveness:
a) The „mycorrhizal dependency“ of a host is genetically fixed (Azcon and Ocampo, 1981) and the degree of mycorrhizal dependency is expressed on the level of an

individuum, expressed as a gradient within the host´s ecological niche and relevant environmental conditions (Feldmann, 1998a). But are we able to predict mycorrhizal dependency under specific conditions? Predicted success of the symbiosis is still based on practical experiences and not on the knowledge of the basic mechanisms for host dependency. Only if we learn to decribe the limiting factors of host growth in much more detail, predictability of effectiveness will be enhanced even more.

b) AMF inoculum was thought to be genetically homogeneous in a wide range, because of the mitotic reproduction of spores. Ignoring that, the initial inoculum multiplication was often processed using a multispore start inoculum. The assumed genetic homogeneity of AMF inoculum was the basis for all screening projects on AMF strains. But the genetic homogeneity of an AMF strain does obviously not exist: recent experiments on the variability of mycorrhizal phenotypes demonstrated that the mutualism-parasitism-continuum of mycorrhizal effectiveness is even found within one single strain of an AMF containing only single spore descendants (compare Fig. 4, Feldmann et al., 1998; Feldmann, 1998b).

In those experiments it remained unclear whether the mutualism-parasitism-continuum was based on the action of different AMF genotypes or showed genetic differences between individual host seedlings, i.e. the reaction norm of the host population to a genetically homogeneous AMF strain. It was of special importance to clarify whether different genotypes occur within an AMF strain and whether their action results in changes of mycorrhizal effectiveness.

In the results shown in this report (Fig. 4) and in earlier studies (Feldmann and Grotkass, 2002) we focussed on the second question. We assumed that spores or AMF infection units are able to colonize a host root-system without respect to their later effectiveness (Feldmann, 1998b) and that more than one infection unit of the AMF population will be successful in infecting the roots. To proof the hypothesis of different genotypes within an AMF population we therefore worked with distinct fungal units, with single spores.

To our definition a „genotype“ is a functional one, reacting to a given environment in a reproducible, predictable way for one propagation cycle of the spores as a minimum. That means that the phenotypic characteristic of a symbiosis raising from the inoculation of single spores must be reproduced when descendants of these

spores are inoculated to homogeneous plant material in a subsequent experiment. A functional description of a genotype does of course not describe the actual genetic differences between AMF units on the DNA level but is focussed on active functional genes for specific interactions. Nevertheless, the chosen way reflects genotypes as targets for eco-factor actions and therefore gives a strict orientation to practice of the mycorrhizal technology.

We here present a procedure to handle potential genetic differences of an inoculum by directing the variability of effectiveness via the technical modification of abiotic and biotic selection factors during the inoculum production process. This procedure, called *Directed Inoculum Production Process* (DIPP) increased the predictability of the qualitative and quantitative output of the symbioses. DIPP might serve as prototype for process optimizations which finally lead to the achievement of AMF inoculum with predictable characteristics.

Producing AMF inoculum is not longer a „black“ box process. Defining an AMF „genotype“ we focussed on phenotypic effects which were pronounced in the hosts by single spore inoculation and could be reproduced after replication of single spore descendants (compare Tommerup, 1988, who defined the AMF species level as AMF genotype). Nevertheless, the stability of the characteristics was very low indicating that there might be a mechanism involved which can change the strain characteristics rapidly to a certain extent. To us such changes do not occur spontaneously but triggered by abiotic or biotic ecofactors including the host itself. If we assume gene / gene interactions of host and fungus to establish and perform a symbiosis (Krishna et al., 1985; Lackie et al., 1988; Gallotte et al., 1993) and if we accept that the quantitative effects of the symbiosis depend on polygenic characters of the host, any increasing or decreasing variability of the host phenotype can be due to a large amount of mycorrhiza induced changes of the host physiology.

Of special importance is the multinuclear character of AMF spores (Peterson and Bonfante, 1994; Genre and Bonfante, 1997; Lingua et al., 1999). We still do not know how much and which nuclei of an AMF spore are active, how they are activated and which influence the heterocaryosis within a spore would have on the effects observed. Does caryogamy exist? Does a population biological process exist favouring the selection of specially adapted nuclei within the population of single spore descandants of an AMF strain? Are strain characteristics mixed under the

control of the host? Due to relative stability of AMF effectiveness after one propagation cycle there is no arbitrary exchange of information between spores of a spore population colonizing a host during this process but a competition between genotypes being controlled by the host or not.

This hypothesis means that a 100% predictability of mycorrhizal effectiveness cannot be achieved. This information is necessary for the selection of target areas, target effects, target plants, and design of inoculum.

Directed Inoculum Production Process presented here, integrates many aspects resulting from the practical extrapolation of theoretical hypothesis and is already leading to more than 85% predictability under commercial conditions. That means that we solved a general problem to an extent which probably reaches the biological limitations of the system. In future we will turn to technical applications of DIPP, e.g. in bioreactors and in vitro techniques. But to clarify the basis of mycorrhizal dependency of host plant species (compare Tewari et al., 1993; Boyetchko and Tewari, 1995) will be of special importance for the economically successful application of mycorrhizal technology in agriculture and horticulture in the future.

References

Allen M (1991): The ecology of mycorrhizae. University Press, Cambridge, UK, ISBN 0 521 33551 1, 180pp

Alten v H, Blal B, Dodd J C, Feldmann F, Vosatka M (2002): Quality Control of Arbuscular Mycorrhizal Fungi Inoculum in Europe. In: Gianinazzi, H., Schuepp, H., Barea, J.M., Haselwandter, K.: "Mycorrhiza Technology in Agriculture: from Genes to Bioproducts" , Birkhäuser, Switzerland, 281-296

Azcon R, Ocampo JA (1981) Factors affecting the vesicular arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytologist 87: 677-685

Bagyaraj DJ (1994) Vesicular-arbuscular mycorrhiza: Application in Agriculture. In: Norris JR, Read DJ, Varma AK (eds) Techniques for the study of mycorrhiza. Academic Press, London, pp. 819-833

Baltruschat H (1993) Zur ökosystemaren Bedeutung der VA-Mykorrhiza in Agrarökosystemen und Möglichkeiten der praktischen Nutzung dieser Symbiose. Habilitation in the FB 17 of the University Giessen, Germany

Boyetchko SM, Tewari JP (1995) Susceptibility of barley cultivars to vesicular-arbuscular mycorrhizal fungi. Canadian Journal of Plant Science 75: 269-275

Chang DCN (1994) What is the potential for management of VAM in horticulture? In: Robson AD, Abott LK, Malajczuk N (eds.) Management of mycorrhizas in agriculture, horticulture and forestry: 187-190

Dodd JC, Thomson BD (1994) The screening and selection of inoculant arbuscular mycorrhizal and ectomycorrhizal fungi. In: Robson AD, Abott LK, Malajczuk N (eds.) Management of mycorrhizas in agriculture, horticulture and forestry: 149-158

Feldmann F, Idczak E (1994) Inoculum production of VA-mycorrhizal fungi. In: Norris JR, Read DJ, Varma AK (eds.): Techniques for mycorrhizal research. Academic Press, San Diego, pp 799-817

Feldmann F (1998a) Symbiontentechnologie in der Praxis: Arbuskuläre Mykorrhiza im Gartenbau. Thalacker-Medien, Braunschweig, Germany. ISBN 3-87815-109-8

Feldmann F, Kruse W, Boyle C, Lieberei R (1998) The Strain - Inherent Variability of Arbuscular Mycorrhizal Effectiveness: I. Development of a Test System Using *Petroselinum crispum* as Host. Symbiosis 25: 115-129

Feldmann F (1998b): The Strain - Inherent Variability of Arbuscular Mycorrhizal Effectiveness: II. Effectiveness of single spores. Symbiosis 25: 131-143

Feldmann, F., Grotkass, C., 2002: Directed inoculum production – shall we be able to design AMF populations to achieve predictable symbiotic effectiveness? In: Gianinazzi, H., Schuepp, H., Barea, J.M., Haselwandter, K.: "Mycorrhiza Technology in Agriculture: from Genes to Bioproducts" , Birkhäuser, Switzerland, 261-279

Feldmann F (2007): The Concept of Best Agricultural Practice. Feldmann Publisher, ISBN: 978-3-00-021432-5, 42pp

Feldmann F, Hutter I, Niemann P, Weritz J, Grotkass C, Boyle C (1999) Einbindung der Mykorrhizatechnologie in die Heil- und Zierpflanzenproduktion sowie den Endverkauf. Mitteilungen der Biologischen Bundesanstalt 363: 6-38.

Genre A, Bonfante P (1997) A mycorrhizal fungus changes microtubule orientation in tobacco root cells. Protoplasma 199: 30-38

Gallotte A, Gianinazzi Pearson V, Giovanetti M, Sbrana C, Avio L, Gianinazzi S (1993) Cellular localization and cytochemical probing of resistance reactions to

arbuscular mycorrhizal fungi a "locus a" myc negative mutant of *Pisum sativum* L. Planta (HEIDELBERG) 191: 112-122

Johnson NC, Graham JH, Smith FA (1997) Functioning and mycorrhizal associations along the mutualism-parasitism continuum. New Phytologist 135: 575-586

Krishna KR, Shetty KG, Dart PJ, Andrews DJ (1985) Genotype-dependent variation in mycorrhizal colonization and response to inoculation of pearl millet (*Pennisetum americanum*). Plant and Soil 86: 113-126

Lackie SM, Bowley SR, Peterson RL (1988) Comparison of colonization among half-sib families of *Medicago sativa* L. by *Glomus versiforme* (Daniels and Trappe) Berch. New Phytologist 108: 477-482

Lingua G, Sgorbati S, Citterio A, Fusconi A, Trotta A, Gnavi E, Berta G (1999) Arbuscular mycorrhizal colonization delays nucleus senescence in leek root cortical cells. New-Phytologist 141 (1): 161-169

Miller JC, Rajzpakse S, Garber RK (1986) VAM in vegetable crops. Hortic Sci. 21: 974-984

Nemec S (1987) VAM in horticultural systems. In: Safir SR (ed) Ecophysiology of VAM plants. CRC Press Boca Raton, USA, pp. 193-221

Peterson RL, Bonfante P (1994) Comparative structure of vesicular-arbuscular mycorrhizas and ectomycorrhizas. Plant and Soil 159: 79-88

Schenck, N.C. 1984. Methods and principles of mycorrhizal research. The American Phytopathological Society, St. Paul, Minnesota, USA, pp. 244.

Sieverding, E. 1991. Vesicular-arbuscular mycorrhiza management in tropical ecosystems. Deutsche Gesellschaft für Technische Zusammenarbeit, Eschborn.

Stocker O (1947). Probleme der pflanzlichen Dürreresistenz. Naturwissenschaften 34, 362-371.

Tewari L, Johri BN, Tandon SM (1993). Host genotype dependency and growth enhancing ability of VA-mycorrhizal fungi for *Eleusine coracana* (finger millet). World Journal of Microbiology & Biotechnology 9: 191-195

Thompson JP (1994) What is the potential for management of mycorrhizas in agriculture? In: Robson AD, Abott LK, Malajczuk N (eds.) Management of mycorrhizas in agriculture, horticulture and forestry: 191-200

Tommerup IC (1988) Long-term preservation by L-drying and storage of vesicular arbuscular mycorrhizal fungi. Transactions Brit. Mycol. Soc. 90: 585-592

Varma A, Schuepp H (1994) Infectivity and effectiveness of *Glomus intraradices* on micropropagated plants. Mycorrhiza 5: 29-37.

Weissenhorn I, Feldmann F (1999) Perspektiven der Nutzung der arbuskulären Mykorrhiza im niederländischen Gartenbau unter Glas. Mitteilungen der Biologischen Bundesanstalt 363: 65-73.