Directed inoculum production – shall we be able to design AMF populations to achieve predictable symbiotic effectiveness?

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Introduction

All over the world there are efforts to include the arbuscular mycorrhizal technology into the 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), and the 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 the 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 has to work successfully and must provide advantages to the target plant. „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, there is only a low predictability of the quantitative aspect of an effect (i.e. the effectiveness) a mycorrhizal symbiosis might have in practice. In fact, the AMF effectiveness following artificial inoculation ranges from positive to negative (Varma and Schuepp, 1994) in an mutualism-parasitism continuum (Johnson et al., 1997).

To deal 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 horticultural and agricultural practice, especially in moderate climates. Industrial interest on the use of AMF in plant production processes had never been sustainable (compare Feldmann, 1998a).

At present, there are two fundamental questions to be answered for the understanding of the basis of mycorrhizal effectiveness:
a) The „mycorrhizal dependency“ of a host is geneticly fixed (Azcon &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 basing on practical experiences and not on the knowledge of the basic mechanisms for host dependency.
b) The AMF inoculum was thought to be geneticly homogenic 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 (Feldmann et al., 1998; Feldmann, 1998b).

In those experiments it remained unclear whether the mutualism-parasitism-continuum based on the action of different AMF genotypes or show genetic differences between individual host seedlings, i.e. the reaction norm of the host population to a geneticly homogenous 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 this report we focus 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 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 phenotypical characteristic of a symbiosis raising from the inoculation of single spores must be reproduced when descendants of these spores are inoculated to homogenic 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 present a procedure to handle potential genetic differences of an inoculum by canalizing the variability of effectiveness via the Directed, technical use of abiotic and biotic selection factors during the inoculum production process. This procedure, called „Directed inoculum production process“ (DIPP) increases 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 charcteristics.

Material and Methods

AMF genotype frequencies: For our studies 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).

The microsymbiont is represented by single spore descendants of a taxonomically not distinguished *Glomus* spec. strain GK12 (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). Sand was used as substrate and the plants were kept in 25ml-plastic tubes under controlled greenhouse conditions according to Feldmann et al. (1998a): illumination by SON-T AGRO 400 Philipps lamps (360µE $\cdot$ 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.

The experiments were carried out between March and July 1996 at the Institute for Applied Botany, University of Hamburg, and the Institute for Microbiology (Technical University, Braunschweig, Germany), and between 1997 and 1999 in the Institute for Plant Cultivation, Solkau, Germany).

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

For the first experiment plants were inoculated with single spores and the plant fresh weight was measured after eight weeks of culture (C1). After that, from three colonized host plants of significantly different fresh weigth 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.

In a second experiment (variation of soil pH and P-content) we used the spore population tested in experiment 1. Ten cuttings of *Anagallis arvensis* per treatment in three parallel repetitions were grown until they developed a considerable root system (conditions as above). Before inoculation the soil was 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 had the same pH like the infiltered solution. For inoculation approximately 100 spores were transferred into the substrate near the roots of *Anagallis arvensis*. Exactly 21 days later the plants were carefully extracted from the substrate, the roots washed to remove old spores and planted to a larger pot (50ml) with fresh substrate. They remained in that pot for another four weeks until sporulation of the fungus took place. Then the plants were harvested, the shoot fresh weight determined and the mycorrhizal status of the roots analysed. The substrate of all treatments was unified and called C1. After that step 1 the first propagation cycle was repeated three times (C2, C3, C4) with all 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).

Mycorrhizal analysis: 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%.

Natural and experimental adaptation of AMF inocula to environmental stress: *Plantago lanceolata* was found at salty sites as well as at heavy metal polluted areas but was sensitive to both stresses in preliminary tests. It therefore was chosen for this experiment as host plant.

Mycorrhizal populations were isolated from the North Sea salt marshes near Neufelder Koog, Germany. They contained three morphologically different spore types at a minimum. After the isolation the spores were stored in sand at room temperature for nine months. In previous tests 0,5% NaCl content in the irrigation water was proofed to allow the best mycorrhizal effectiveness on the host plant *Plantago lanceolata*.

AMF populations from mine spoils near Oker, Germany, were extracted in the same way. Soil from the same site was suspended in water (1kg in 2l) for 24 hours, decanted into a sieve of 45µm mesh width, collected and used as heavy metal stock solution in the irrigation water without previous determination of the heavy metal content. The pure heavy metal solution strongly inhibited the growth of the non-mycorrhizal test plant *Plantago lanceolata.* The best mycorrhizal effectiveness was found at 30% concentration of the stock solution.

Directly before the experiment an estimation of the most probable number of propagules (Feldmann and Idczak, 1994) in the test inoculum was carried out.

The effectivness of the natural AMF populations was compared with that *Glomus* spec. GK 12 before and after the selection process shown in experiment 2.

Stability of effectiveness after selection by stressors: In the fourth experiment we inoculated *P. lanceolata* seedlings with an inoculum quantity of approximately 100 propagules of *Glomus* spec. GK12. This inoculum had been pretreated with salt stress and genotypes had been preselected as shown above. After that we propagated the AMF populations on *P. lanceolata* in three subsequent propagation cycles without stressors and measured the MEI again under stress conditions.

Mass production of inoculum: In contrast to the single spore isolates used above, for the mass production a commercial inoculum was chosen, which already had been used by several companies and in research projects (e.g. Feldmann et al., 1999). The inoculum contained morphologically different spore types and showed variable banding patterns after PCR analysis of single spores (Hildebrandt and Hutter, 2000,

pers. communication). The method of the inoculum production followed the detailed description of Feldmann et al. (1999). Mass production means the 160,000 fold multiplication of the initially inoculated AMF.

Predictability of AMF effectiveness with and without DIPP: To demonstrate the impact of a Directed inoculum production process (DIPP) in our lab we designed many experiments with the same AMF strain but different host species, different inoculum quantities, environmental conditions, scales and effects. In practice a threshold value of MEI >30 must be exceeded to create interest by a potential customer in the mycorrhizal technology. The positive outcome of an inoculation was called „predicted“ if that MEI value was clearly passed under conditions plant producers use.

Results and Discussion

Genotype differences in an AMF inoculum: The inoculation with single AMF spores from a commercial inoculum showed a variability of effectiveness from slightly effective to medium to highly effective (Fig. 1, 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.

Nevertheless, 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 like in C1.

After the second propagation cycle the distinct characteristics of the genotypes start to become modified because there is an increase of variability of effectiveness in C3 (Fig. 1). The basic mechanisms 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 the last inoculum charge but on spore material from stock cultures. This complicates the scaling-up in inoculum production, because slight differences as shown for the effectiveness of C3 (Fig 1) can create considerabel changes in effectiveness of an inoculum produced at that base (Feldmann et al., 1999).

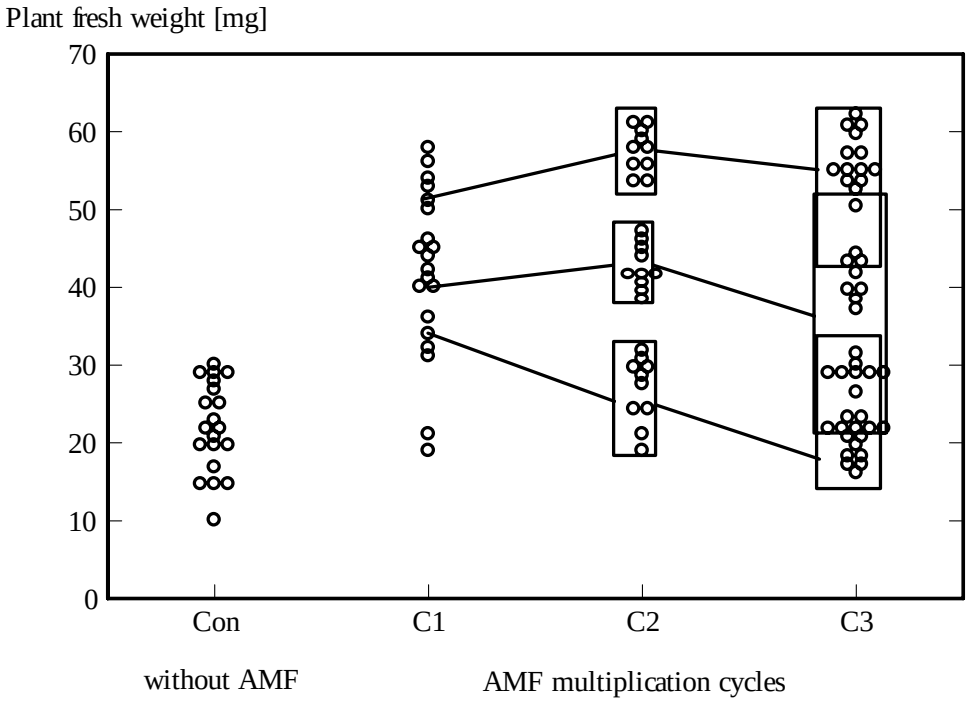


Figure 1. Mycorrhizal effectiveness of AMF single spore descendants (*Glomus spec.* GK 12) on the biomass of *Anagallis arvensis.* See distinct sub-population characteristics in C2 and overlapping effectiveness in C3.

The relationship between AMF population composition and effectiveness: The initial AMF spore population of *Glomus spec*. GK 12 (compare Fig. 1) contained spores with different effectiveness on biomass production of *Anagallis arvensis* under standard conditions. In the next test we inoculated *Anagallis arvensis* cuttings with approximately 100 spores of that initial inoculum and varied the soil factors „pH of soil solution“ and „phosphate concentration of soil solution“ in independent test systems. Because we did not select spores of a characteristic genotype for the test, the differences in colonization of the sub-populations under different environmental conditions did not reflect the reaction norm of one genotype; in contrast, the colonization pattern of the population under changed environmental

conditions is able to prove the existence of different genotypes of the initial population.

At extreme soil pH the colonization the host plants initially was low (Fig 2a). But the percentage of spores within the tested inoculum, able to colonize under extreme conditions could be enhanced by seperate propagation and later mixing the freshly produced spores. Consequently, the effectiveness of the meliorated inoculum was enhanced under extreme conditions, as compared to the initial start inoculum. This was 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.

Changes in the phosphate concentration in the soil, did not allow conclusions on a comparable adaptation process (Fig 2b). Under optimal and luxury P-supply the test plants probably did not depend on the symbiosis: neither the percentage of colonized plants nor the inoculum effectiveness could be optimized.
Under variable environmental conditions probably the physiological status of the host is the main factor that expresses dependency or independency on mycorrhizal fungi. Because of this the directed inoculum production process probably will especially be successful, if the relationships between later target plants and desired target mycorrhizal effect are 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 characteristic seems as well to have special importance for the outcome of the process.

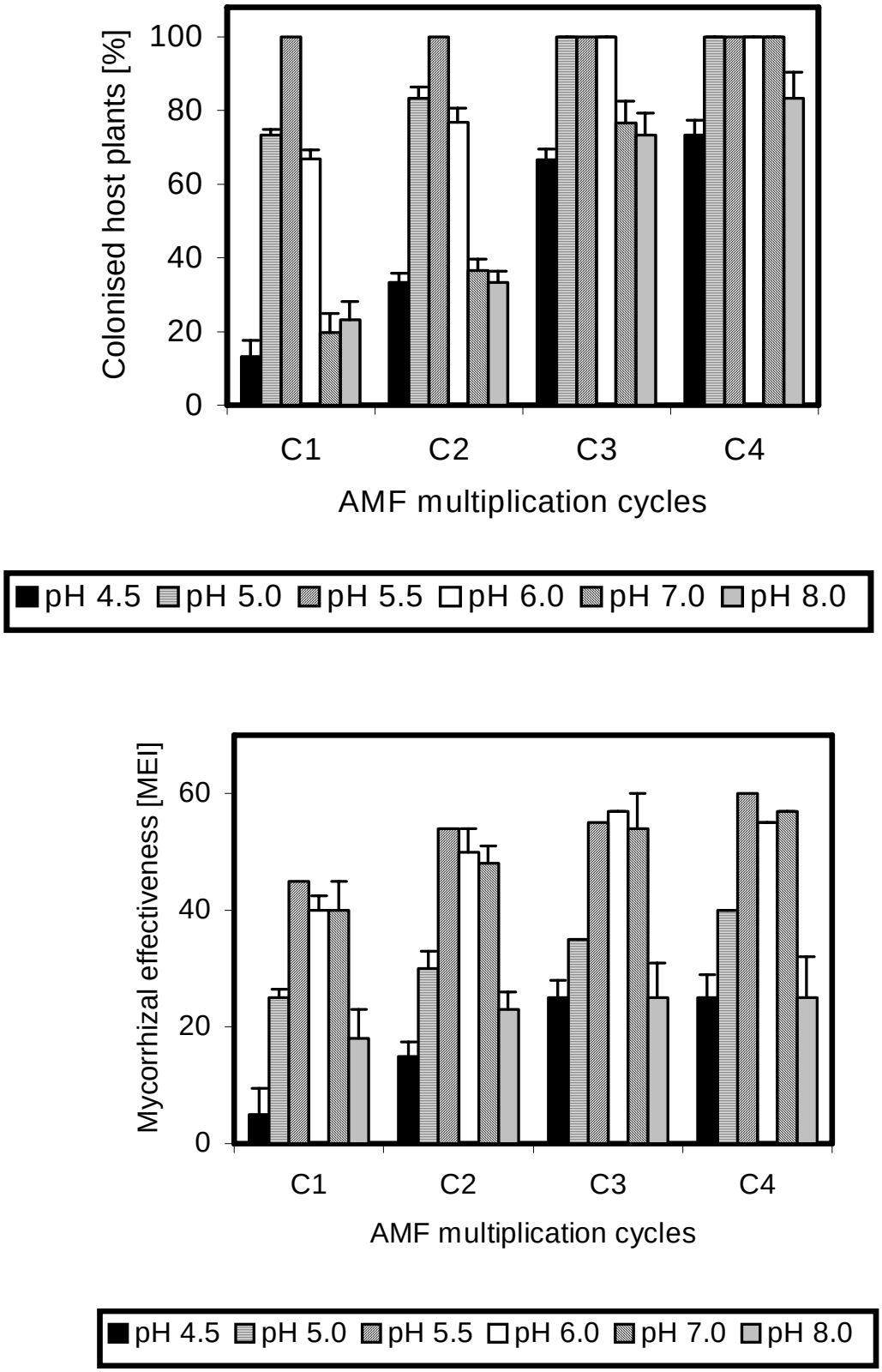
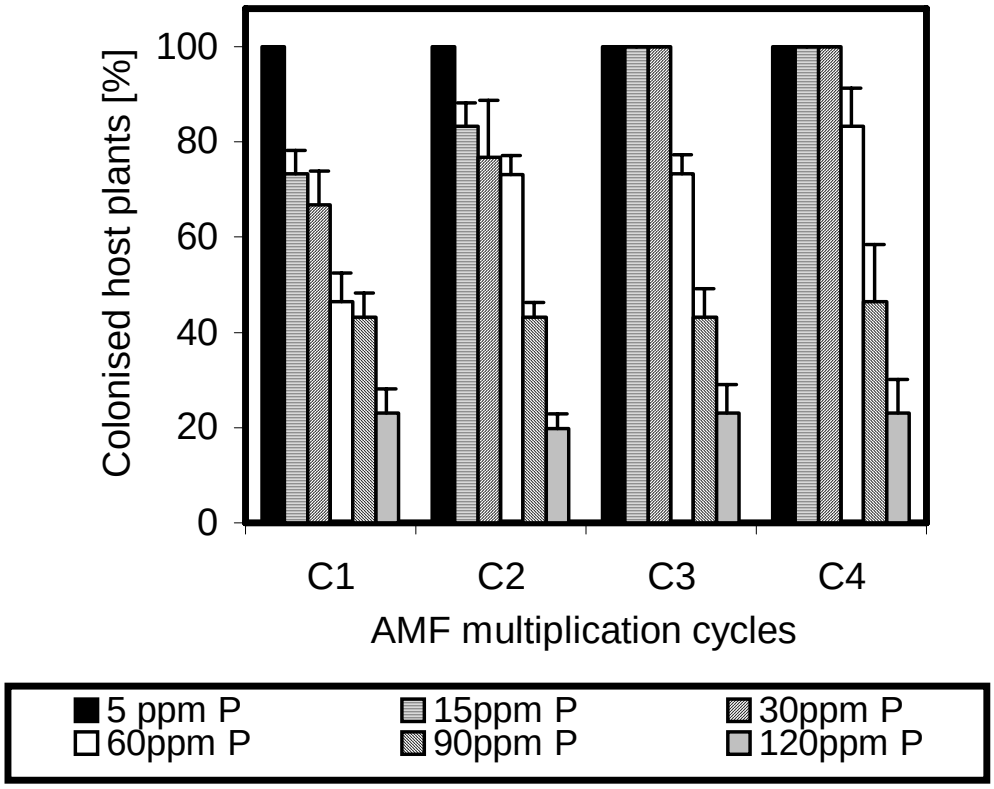


Figure 2a. Root colonization ability and mycorrhizal effectiveness of AMF populations (*Glomus spec*. GK 12 on *Anagallis arvensis*) with technically modified genotype composition (Selection factor „soil-pH“, details see text).Bars: SD



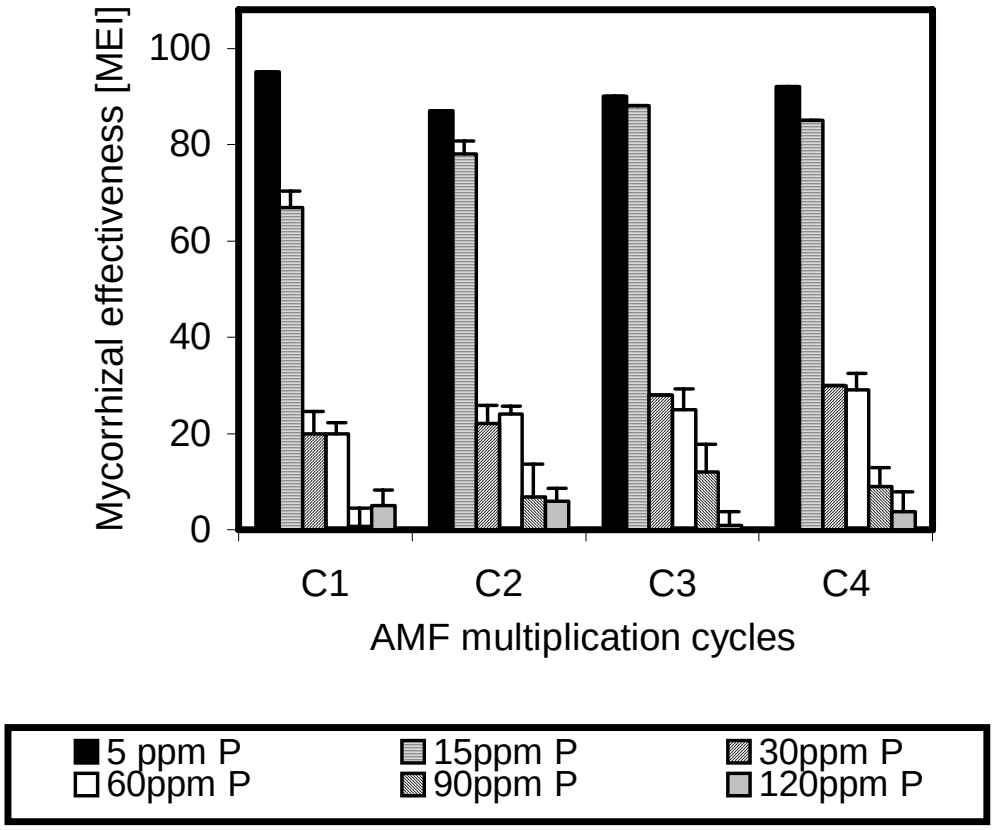


Figure 2b. Root colonization ability and mycorrhizal effectiveness of AMF populations (Glomus spec. GK 12 on Anagallis arvensis) with technically modified genotype composition (Selection factor „soil-P-concentration“, see text). Bars:SD

Natural and experimental adaptation of AMF inocula to environmental stresses: After these first experiments it was still the question whether selected sub-populations have an effectiveness comparable to ecologically highly adapted ecotypes of AMF. Important decisions concerning the inoculum production strategy can be made, e.g. whether the isolation of ecologically adapted AMF for a specific production process has to be performed or whether technical adaptation can be carried out, a much cheaper alternative.

In order to describe the relative effectiveness of a standard inoculum we compared *Glomus spec.* GK 12 before and after DIPP with AMF either naturally adapted to salt stress or another population adapted to heavy metal stress. In both stress situations the presence of mycorrhiza is favourable for the host (e.g. Rosendahl and Rosendahl, 1991; Hildebrandt et al., 1999) and is intensively studied in applied research projects of the EU (MYCOREM, EU No. QLRT-1999-0009).

In both cases the effectiveness of naturally adapted AMF populations (without previous technical propagation) was higher than the experimentally non-selected or preselected sub-populations of *G. spec.* GK 12 (Tab. 1). Anyhow, the preselection process resulted in AMF populations with 85% effectiveness of naturally occurring salt tolerant AMF and 87% effectiveness of heavy metal tolerant AMF. As we know from applying the mycorrhizal technology, differences of less than 10% effectiveness are of low significance for a plant grower. Technical preselection of sub-populations with favourable characteristics and isolation of naturally adapted AMF from natural sites are nearly equivalent with respect to obtainable effectiveness of the inoculum. Therefore the most rapid and cheaper way of start inoculum supply can be chosen by the inoculum producer.

Table 1. Comparison of effectiveness (MEI) of naturally and experimentally adapted AMF populations on the growth of *Plantago lanceolata* under abiotic stresses (salt and heavy metals, see Material and Methods). The test was repeated three times with 25 individuals per treatment. Values of one row marked with the same letter are not significantly different.

	G. spec. GK12		Native AMF
	without	with phenotype preselection [MEI]	without
Salt stress	25.6 ± 3.5	45.4 ± 2.7a	53.4 ± 3.5a
Heavy metal stress	18.8 ± 1.3	65.9 ± 13.6a	75.8 ± 7.8b

As demonstrated above the replication of inoculum can change its characteristics. It is therefore a risk to isolate adapted fungi, multiply sufficient amounts of start inoculum for mass production and store it until use. Besides this it is expensive to maintain such a gene bank that increases the cost of inoculum.

Consequently, we monitored AMF eco-types at sites with abiotic stress and leave them in their natural site for further use. The sites themselves serve as „in situ conserved gene banks“ of specially adapted AMF. Examplarily for our location, several natural sites are listed (Tab. 2). Recently, we began using unclassified AMF, but will identify them in the future using PCR-techniques.

Table 2: Ecologically differentiated areas with AMF populations in Lower Saxony, Germany („in situ conserved gene bank“ of IFP)

Vegetation type	Assumed differentiating abiotic eco-factor
Mesophyllic forest	pH >5.5, moderate water and nutrient availability
Forest on acidic soil	pH <5.0, low water and nutrient availability
Forest on chalky soil	pH > 6.5, low-moderate water availability
Bog forest	wet areas, flooded over months
Moist green lands	high ground water, high host diversity
Violo-Nardion	dry and sandy soils, low pH.
Rock debris vegetation	especially grasses and mosses and ferns as hosts, partially extreme conditions
Heavy metal vegetation	variable heavy metal content
Primary and secondary salt vegetation	salt stress
Long term agriculture	frequent disturbance, high fertilizer input
Sustainable agriculture	frequent crop rotation, bio-fertilization
Wall vegetation	dryness

Stability of AMF effectiveness after selection by stressors: Selection of sub-populations by stressors in the cited sense meant replication of the strain under conditions of stress (e.g. salt) and separation of the most effective sub-populations afterwards. Since a commercial inoculum must be free of contaminants, e.g. salt or heavy metals originating from the first inoculum production step, the second step, therefore, must involve production without stressors. Furthermore, after this second step the inoculum must still have the desired characteristics.

In order to ascertain the stability of effectiveness after stress selection we tested *Glomus spec.* GK 12 before and after genotype selection under salt stress (Fig 3). We found an increase of the effectiveness in C1 due to the selection of only very

effective sub-populations from the initial test population C0. In the subsequent second reproduction step (production without stressor, C2) similar results were obtained, although the variability of effectiveness increased already in the third propagation cycle without stressor (C3).

For the commercial inoculum production these data mean that minimally the first multiplication step after the selection step can be carried out without stressor without loosing the desired characteristic. These results are consistent with the findings shown in Fig. 1.

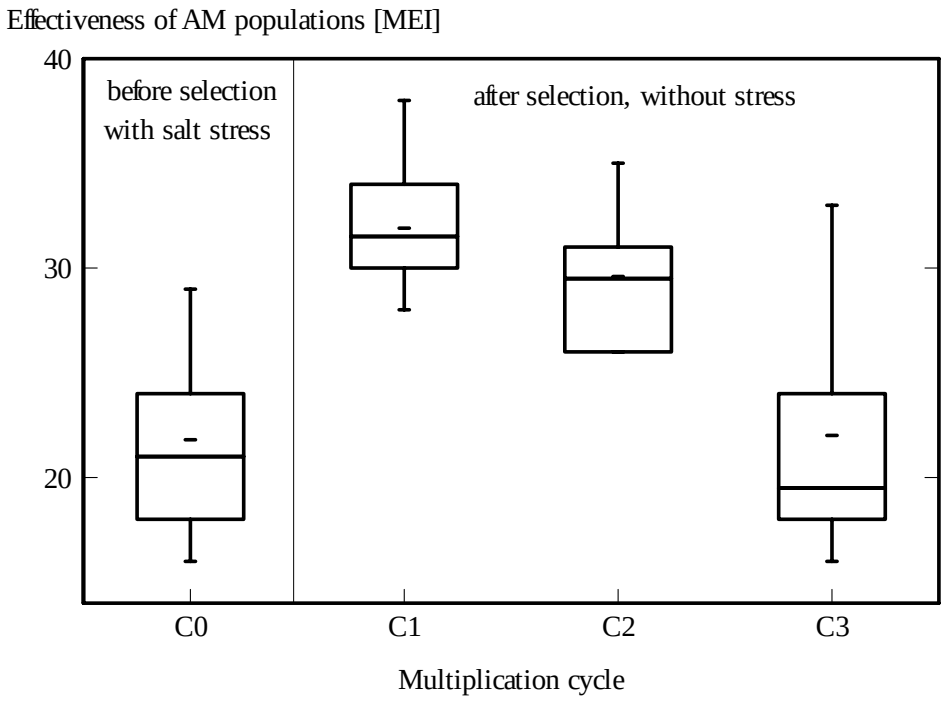


Figure 3. Stability of strain characteristics (effectiveness) before and after sub-population selection under the influence of salt stress. The mycorrhizal effectiveness index (MEI) was calculated according to Bagyaraj (1994)

Preference of AMF genotypes by host plants: Since several years it is well known that different varieties of host plants react specifically to the same AMF inoculum (Azcon and Ocampo, 1981). On one hand, this reflects the relevant mycorrhizal dependency of the host under certain conditions. But on the other hand, our data suggest that such specific interactions partially could have an AMF population biological basis.

In order to proof whether preference phenomenons (compare Dhillion, 1992) between host plant species and AMF genotypes are able to influence the inoculum characteristics we tested the inoculum of 52 sub-strains of *Glomus spec.* (morphologically similar to *G. etunicatum*) in 1998 on different host combinations (Tab. 3) and measured the mycorrhizal effectiveness index (MEI). Out of that 52 sub-strains nine sub-strains of different effectiveness were chosen and stored for a subsequent test in 1999. A mixture of all 52 sub-strains was included to the experiment in 1999. The inocula of the second year were inoculated to a further target plant (*Baptisia tinctoria*) not used as host plant during inoculum production.

In the first year an effectiveness spectrum from neutral to positive was expressed in treatments with *Zea mays* and from negative to positive effectiveness with *Tagetes erecta* as host plant cultivated alone.

Tab. 3: Influence of mass production to the effectiveness of AMF inoculum (strain *Glomus spec. GK12*, inoculated to 52 plots. Nine sub-strains of 52 selected) with respect to host and target plant species (fresh weight of host shoots). The MEI was calculated on the basis of 50 plants per plot. „T“ *Tagetes erecta*, „Z“ *Zea mays*, „T/Z“ mixed cultivation. „T, resp. Z“ Tagetes or maize cultivated alone. „MIX“ sub-strain mix.

Inoculum production 1998			**Inoculation 1999 [MEI]**				
host plants	**sub-strain**	**MEI**	**Z**	**T**	**Z / T**		***Baptisia tinctoria***
Z. mays	9	43,1	31,6	28,6	44,1	12,1	31,0
	40	19,8	29,6	12,2	28,7	-7,1	25,3
	41	0,4	23,5	-36,4	24,8	-18,5	16,7
	Mix	-	22,6	0	25,4	-8,2	44,4
T. erecta	24	43,0	36,3	29,4	44,4	4,8	44,7
	32	14,6	21,5	-20	28,5	-34,2	-5,2
	45	12,1	33,0	-36,5	29,4	-23,4	56,5
Z. mays* / *T. erecta	52	39,3 14,0	40,4	-1,7	31,8	3,1	48,7
	44	32,5 -52,5	20,7	-57,9	60,5	-72,2	25,4
	22	36,3 –22,8	-31,4	-18,2	67,4	-34,8	32,4

During the next inoculum production cycle the effectiveness of the sub-strains on *Zea mays*, changed with a tendency to reach all the same value of MEI: the best sub-

strain was still the best but with decreased effectiveness while the others enhanced its effectiveness when tested on maize itself.

Produced on *Tagetes* the effectiveness of the sub-strains decreased to different degrees when tested on *Tagetes*: the better the effectiveness was before the second multiplication the smaller the decrease was afterwards.

Tagetes inoculum tested on maize resulted in positive effectiveness, but maize inoculum showed a positive effectiveness only when it had been very effective before (MEI >40). In all other cases an inoculation resulted in neutral or even negative effectiveness on *Tagetes* plants.

In a co-cultivation of *Zea mays* and *Tagetes erecta Zea* showed a high degree of competitiveness to *Tagetes* (Feldmann et al., 1999). Co-cultivation and additional inoculation with mycorrhizal fungi together enhanced the negative effect of maize on *Tagetes*.

Nevertheless, the specific results (e.g. in case of sub-strain 44 positiv on maize, negative on *Tagetes*) were reproduced when the sub-strains were tested on each host separately. When tested on co-cultivated hosts the discrepancy was even stronger than in the year before. The same could be seen when the mixture of all sub-strains was tested.

Similar eefects of the interactions seem to exist for *Baptisia tinctoria,* too. But a classification of *B. tinctoria* as „*Zea*-type“ host or „*Tagetes*-type“ host was not possible as there were no strong correlations between effectiveness on plants the inoculum was produced on and the later target plants.

Our results can be explained by a hypothesis: each host plant species could prefere the association with specific AMF genotypes within the inoculum. The physiological basis for a postulated preference would not be connected with a later (positive) effectiveness of the developing symbiosis (independent colonization behaviour, compare Feldmann, 1998b), and would be independently transmitted to the next spore „generation“.

If such AMF genotypes are able to colonize hosts without respect to later effectiveness (like shown for *Petroselinum crispum*, Feldmann, 1998b) the response of the host in a symbiotic interaction would be due to the composition of AMF populations. Sub-strain 41 / *Zea mays* would include more genotypes with negative

impact on further symbiotic interactions than, for example, sub-strain 9. If plants (e.g. maize) could control AMF genotypes with negative potential or favour AMF that guarantee mutualism, and other plants (e.g. *Tagetes*) would not be able to do so, their specific influence to the AMF genotype composition would lead to different genotype frequencies in the next spore „generation“. Consequently, inoculation of *Tagetes erecta* with sub-strains containing a higher amount of negatively effective AMF genotypes, can lead to negative growth responses of the plant. Furthermore, the postulated inability of plants like *Tagetes* to control the further replication of non-mutulistic AMF genotypes leads to inocula which are still less effective than before. With respect to the „intermediate“ response of *B. tinctoria* to the inoculated sub-strains the question arises whether there could be a continuum in the ability of host plants to control and direct the mycorrhizal influence to their physiology.

The consequence for the practice of inoculum production of all those findings is, to base predictions of inoculum effectiveness only on well known host plant species used during the inoculum production. In our case the most probable positive response of later target plants (*Tagetes* and *Baptisia*) was observed when the previous MEI exceeded 18.6 on *Zea mays* as host during the inoculum production.
For the mass production of an inoculum it is necessary to use only one host with characteristic selectivity to achieve a widely homogenous inoculum with positive effectiveness on the desired target plant species.

The constancy of AMF effectiveness within a mass production process: Mass production of AMF commonly means the production of up to several hundreds of litres inoculum containing ca. 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 normally quiet homogenous 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. Up to 50 host individuals are involved in the AMF multiplication in one of such units (Feldmann et al., 1999). Comparing plots very often larger differences in plant growth are observed than between pots.

For our company commercial AMF mass production recently means to produce more than 25.000 litres inoculum per year (with 100.000 infection units/l). Such amounts of inoculum require a start inoculum of minimally 125.000 infection units. This can be prepared genetically widely homogenous following the cited production steps.

But would the remaining genetic inhomogeneity of the fungal material allow the up-scaling of the inoculum production with a tolerable variability of the resulting effectivness under practical conditions?

A further segregation of a preselected sub-strain with high effectiveness into new sub-strains with neutral to high effectiveness occurred (Fig. 4). Nevertheless, more than 90% of the inoculum caused positive growth response in the host (*Zea mays*) during inoculum mass production. If this quota is reproducible, it would be economically feasible to select sub-strains with special effectiveness here a second time and to discard sub-populations of lower effectiveness after mass production.

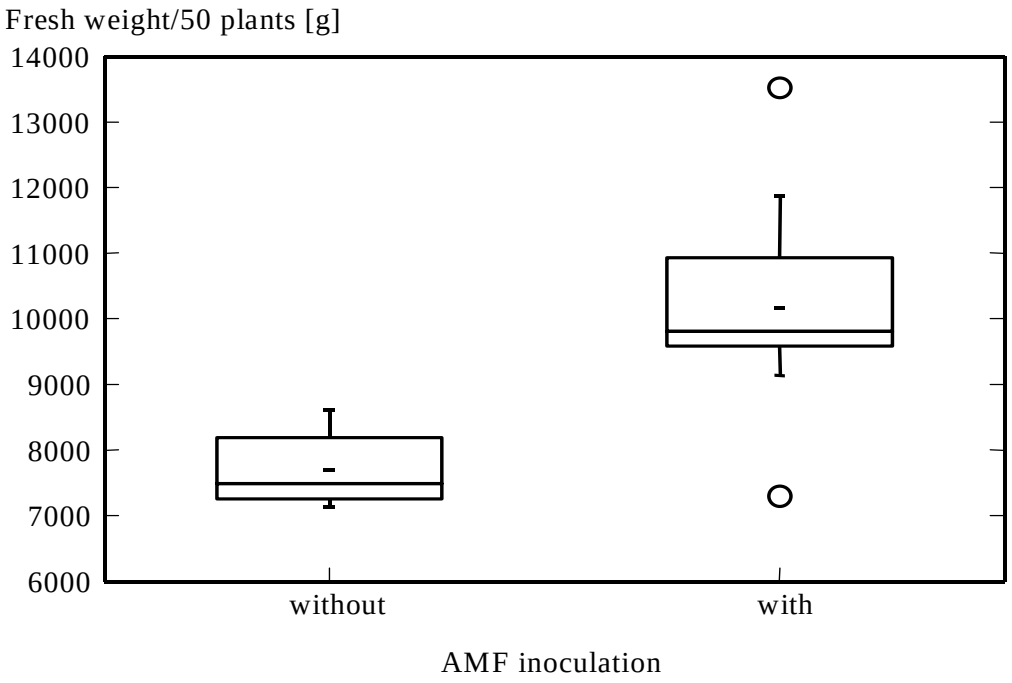


Figure 4. Variability of effectiveness of an AMF strain (*Glomus* spec.) during inoculum mass production in plots with 50 host individuals (*Zea mays*).

The Directed Inoculum Production Process (DIPP) and its influence on the predictability of inoculum effectiveness: Respecting all results we are able to provide a protocol for the direction of later AMF genotype composition included in

the inoculum production process which results in an enhanced predictability of inoculum effectiveness.

1. On the basis of a detailed description of the agricultural or horticultural problem to be solved by the introduction of AMF, the decision is made whether the isolation of specially adapted AMF from natural areas has to be carried out or an established generalist can be used.

2. Tests with standard plants like *Zea mays* and – if possible - the later target plant species directed to the target effect are carried out to show the potential effectiveness of the available fungal material. These small scale tests can be carried out in growth chambers and can include stressors. The analysis of the variability of AMF effectiveness in a given test system allows the selection of best sub-strains.

3. The third step includes the mass production. How many inoculum can be produced in this step without any loss of guaranteed stable characteristics is not well known up to now.

Table 4. Increase of predictability of mycorrhizal effectiveness with the 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%

The DIPP was introduced to the plant production and optimized in our company since 1996. Defining „predictability of AMF effectiveness“ as an 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 the predictability could be clearly increased. Despite of that we still cannot be sure what really will happen after an inoculation. To us DIPP seems to be a

promising way to provide guaranted thresholds of effectiveness which will be more than anything provided in the last ten years.

Perspectives

Producing AMF inoculum is still a „grey“ 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; Gollotte 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 the design of the inoculum.

The directed inoculum production process presented integrates many aspects resulting from the practical extrapolation of the theoretical hypothesis and is already

leading to more than 85% predictablity under commercial conditions. That means that we solved a general problem to an extent which probably reaches the biologically limitations of the system. In future we will turn to technical applications of our 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 the mycorrhizal technology in agriculture and horticulture in the future.

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