Symbiosis, 25 (1998) 131–143
Balaban, Philadelphia/Rehovot

The Strain-Inherent Variability of Arbuscular Mycorrhizal Effectiveness: II. Effectiveness of Single Spores

FALKO FELDMANN
Institute for Applied Botany, University of Hamburg, Marseiller Str. 7, 20309 Hamburg, Germany; Corresponding address: Konstantin-Uhde-Str. 13, 38106 Braunschweig, Germany, E-mail. c.boyle@tu-bs.de

Received May 20, 1997; Accepted September 17, 1997

Abstract

In standardized test systems the effectiveness of the symbiosis between the arbuscular mycorrhizal fungus (AMF) *Glomus etunicatum* Becker and Gerdemann, strain HH13, and the host *Petroselinum crispum* Hoffm. (parsley) was characterized on the single spore/single plant level. In three multiplication cycles 307 populations of 15 sub-strains were developed and the symbiotic effectiveness measured using shoot fresh weight as parameter. As a result, (a) Within a spore population derived from one single AMF spore, positive, neutral and negative effectiveness could be detected – independent of the original effectiveness of the "mother spore". (b) The overall variation of the original effectiveness became enhanced with the number of multiplication cycles. (c) Already after three multiplication cycles, distinct sub-strain characteristics could be identified classifying the new populations as positive, neutral or negatively effective. (d) Leaving the single spore level, the inoculation of populations seemed to result in a canalization of the host phenotype. In such cases, not the potential variation spectrum of effectiveness was found, but preferably neutral effects were expressed. The importance of the multinucleic characteristic of spores of *Glomus etunicatum* for the development of variations in effectiveness of the symbioses for the host are discussed.

Keywords: Arbuscular mycorrhiza, effectiveness, *Glomus etunicatum*, *Petroselinum crispum*, single spores, genotypic diversity, biodiversity

Presented at the Second International Congress of Symbiosis, April 13–18, 1997, Woods Hole, MA

0334-5114/98/$05.50

1. Introduction

Strains of arbuscular mycorrhizal fungi (AMF) are commonly treated as an integrated whole of distinct characteristics (Dodd and Thomson, 1994). They are considered more or less efficient in modifying plant growth parameters such as yield, biomass or content of secondary plant products. Because of a "phytocentric" approach and difficulties in measuring metric parameters of symbiontal effects to the AMF, the "effectiveness" of a symbiosis is normally defined as the quantitative output of a fungus-mediated response of the host alone. The supposedly typical characteristic of a fungal strain to cause certain effects is the most important parameter in screening processes of mycorrhizal isolates (Sieverding, 1991).

Several attempts have been made to select strains with the quantitatively best effect on the plant hosts, i.e. the highest "effectiveness". However, till the present, not one strain has been identified which could be called consistently "very effective". Effectiveness is not an exclusive strain characteristic but the result of specific host/AMF interactions under the modifying influence of environmental factors. This complexity severely impairs the possibility to predict effects and effectiveness of concrete symbiontal combinations – the largest handicap to the consequent commercial use of the mycorrhizal technology today.

We know today that the genotype of the host on the variety level, under the influence of ecological factors, determines the dependency of a plant on the mycorrhizal symbiosis as Azcon and Ocampo (1981) demonstrated with thirteen wheat cultivars. Varma and Schuepp (1994) reported that one fungal species (*Glomus intraradices* Smith and Schenck) showed positive, neutral and negative effects depending on host species, host variety and environment.

On the other hand, the genetic constitution of the AMF on the strain level is of the same importance for the effectiveness of the symbiosis: inoculations of several *Hevea* genotypes with AMF strains of *Glomus etunicatum* resulted in different degrees of resistance enhancement of rubber tree leaves against biotrophic pathogens (Feldmann et al., 1989; Lieberei et al., 1989).

Strain characteristics are known to be pre-formed by adaptation of AMF species to different habitats. Stahl et al. (1990) describe variation between populations from dissimilar environments, and suggest that physiological differentiation could occur as result of gradual micro-evolutionary changes. In their estimation, adaptation of AMF is a time-consuming process.

In contrast, Feldmann et al. (1998) observed that long term cultivation of AMF strains on parsley (*Petroselinum crispum*) led to a decrease of effectiveness of those strains to that host. This indicates rapid adaptation mechanisms which act within sporulation cycles and need a maximum of

months or a few years to affect changes of physiological strain characteristics. Such mechanisms must lie within populations. The agents must be elements of the populations, the single spores.

Nearly nothing is known about the variability of the AMF genotypes represented by single spores within experimental, commercial or natural AMF populations. Because of the host genotype/AMF genotype specific interrelationships, that knowledge must be assumed as a prerequisite for understanding the phenomenon of "effectiveness". Other population biological aspects are of low significance to explain the effectiveness of the symbiosis: the degree of root colonization by AMF is no adequate parameter for the mathematical modelling of effectiveness (Alvarez-Santiago et al., 1996); no dose/response interrelationship between propagual number and resulting effectiveness exists. We know that competition between spores of a population occurs during the colonization process (Bowen, 1987) – but it is not known what this means for the effectiveness of a symbiosis.

From the genetic point of view, effectiveness of a fungal strain under defined environmental conditions is the manifestation of fungus-mediated changes in the plant phenotype. In standardized testing systems using preselected plant material, these changes can provide information about the set of genotypes within the fungal population itself.

Consequently, we turned to the single spore level and tested our hypothesis that there is a significant variability within the genetically fixed potential of single spores of an arbuscular mycorrhizal population which leads to the expression of distinct host phenotypes in the symbiosis.

2. Materials and Methods

Plant and fungal material

Maternally defined seed material of *Petroselinum crispum* Hoffm. cv. "Mooskrause" was used for all experiments. The seeds were grown in sand for 35 days, a subpopulation of equally sized seedlings with developed primary leaves and second ramification of the root system was selected, transplanted to pots of 2.5 ml volume, and inoculated with a single spore of *Glomus etunicatum* Becker and Gerdemann. After 14 days substrate, spore and plant were transfered to a pot of 50 ml volume. The plants were harvested and evaluated 60 days after inoculation.

The original AMF strain was HH13 of *Glomus etunicatum*. The strain was cultivated for six years on several hosts under greenhouse conditions following the method of Feldmann and Idczak (1992).

Plant cultivation

The plants were grown in climate chambers under the following conditions: light of SON-T AGRO 400 Philipps lamps (360 µE m^{-2} s^{-1}), 14 h/d; 60–80% relative humidity; 18–20°C at night, 22–24°C during the day; irrigation below field capacity; fertilization once per week with a 10% pot volume of a nutrient solution (1g Flory 9/l; pH 5.5).

Experimental layout and analysis

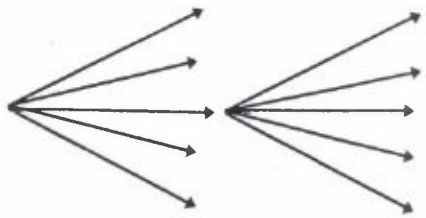
The original population of *Glomus etunicatum* HH13 contained spores of three dissimilar colour-classes and of variable size with different frequencies (Feldmann et al., 1998). Spores of type 2 and 3 had no colonization success within the test period. By selecting the spore type 1 (white, containing 247±40 nuclei) for the further inoculations, we were able to enhance the rate of successful symbiosis from 50% in multiplication cycle 1 (C1) to 71% in C2 and 72% in C3, respectively.

Fifty single spores were isolated from the original strain HH13 and each spore inoculated to a single plant (C1). Successful symbioses were obtained from 25 spores. The criterion for the success of a symbiosis was the formation of new spores by the AMF. From multiplication cycle C1 on, the derived populations were called "substrains". Five single spores of each substrain were inoculated to host plants in two following multiplication cycles (C2 and C3, Table 1).

Table 1. Multiplication scheme and inoculation success of single spore isolates: development of substrains

	Single spore multiplication cycle		
	C1	C2	C3
Inoculated single spores (n)	50	125	445
Successful symbiosis (n)	25	89	307
Surviving sub-strains (n)	25	21	15

Multiplication scheme of single spores →



At the end of each multiplication cycle the plants were harvested, the fresh weight of root and shoot determined, and the newly developed spores retained in the substrate at room temperature for the next cycle of multiplication. The root was stained with 0.05% Trypan blue and the degree of root colonization (%) estimated using the whole root system. Changes of the host biomass were used to characterize the effectiveness of the symbiosis. Deviations from the standard reaction of non-mycorrhizal control plants led to the term "positive or negative" effectiveness.

In order to accommodate the large amounts of data the Box/Whisker and Dot Plot method was chosen. In a Box/Whisker and Dot Plot graph, descriptive statistics and distribution functions are unified. All values of host fresh weight were divided into 40 classes. Each dot represents the phenotype of one single plant inoculated with one single spore irrespective of the degree of root colonization. The box width reflects the size of the sample. The middle line of the box shows the median of the measurement, the bottom line and the top line the lower quartile or the upper quartile, respectively. The lower and upper whisker show the lower and upper adjacent value. This is equal to the quartile minus 1.5 times the inter-quartile range. Any values below or above this are outliers and are plotted individually.

3. Results

Increase of symbiontal phenotype variation

Within a population of AMF the characteristics of single spores led to positive, neutral and negative effectiveness of the symbiosis (Fig. 1). The variation of symbiontal phenotypes increased in sequential multiplication cycles. The percentage of positive symbioses increased from multiplication cycle C1 (28%) to C2 (45%) and C3 (44%), while the percentage of symbioses within the standard reaction of non-mycorrhizal plants (no effectiveness) decreased from 48 to 28%. The number of negatively effective symbioses increased slightly from 24% in C2 to 28% in C3. The median of all measurements remained within the standard reaction of non-mycorrhizal plants.

Shoot and root of *Petroselinum crispum* seedlings were influenced to a different extent (Table 2). The average of shoot fresh weight of inoculated plants was enhanced by the AMF while the root fresh weight remained within the standard reaction of non-mycorrhizal plants. This caused a change in shoot/root ratio, i.e. mycorrhizal roots were more efficient in supporting the

shoot than non-mycorrhizal roots were. The variability of shoot fresh weight increased nearly fourfold, the variability of root fresh weight more than twofold.

The average degree of root colonization and the variability of colonization rates increased from C1 to C3, though in C3, the classes of effectiveness (positive, neutral and negative) only slightly corresponded with certain, narrow ranging degrees of root colonization (Fig. 2). AMF single spores with neutral effectiveness were found to colonize from 1–80%, negative effectiveness occurred with 1–40% root colonization. For a positive effectiveness the threshold value was higher than 20%, while the maximum reached 100% root colonization.

Table 2. Range statistics of host phenotype variation expressed as fresh weight after sequential multiplication cycles (C1–C3) of single spore populations (*Glomus etunicatum* on *Petroselinum crispum*)

Parameter	Cycle	Sample size	Average	Standard deviation	Variance	Min.	Max.
Total FW (g)	C1	25	32.2	5.6	30.8	23.0	41.1
	C2	89	35.7	7.1	49.9	21.4	54.7
	C3	307	35.5	9.3	85.7	14.3	59.2
Shoot FW (g)	C1	25	17.7	3.2	10.1	12.7	23.2
	C2	89	19.8	4.7	22.3	12.6	30.1
	C3	307	20.5	6.1	36.8	8.0	32.6
Root FW (g)	C1	25	14.4	2.6	6.8	10.3	18.7
	C2	89	15.9	3.1	9.7	8.5	24.6
	C3	307	15.0	3.8	14.3	6.2	26.8
Shoot/root ratio	C1	25	1.23	0.12	0.02	1.05	1.75
	C2	89	1.26	0.26	0.06	0.92	1.99
	C3	307	1.37	0.27	0.07	0.90	2.07
Colonization (%)	C1	25	36.2	12.5	156.5	14.0	62.0
	C2	89	37.3	23.9	569.0	1.0	91.0
	C3	307	42.6	26.2	686.7	1.0	100.0
Total FW (g)	Control	75	33.2	2.1	4.3	29.1	36.0
Shoot FW (g)		75	17.5	1.1	1.2	15.4	19.2
Root FW (g)		75	15.8	1.2	1.4	13.7	17.6
Shoot/root ratio		75	1.11	0.06	0.01	0.99	1.21

FW = Fresh weight.

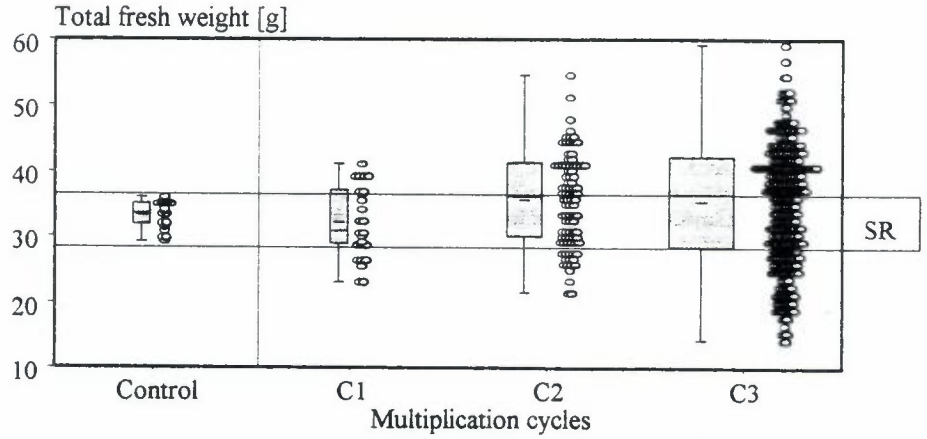


Figure 1. Increase of symbiontal phenotype variation in sequential multiplication cycles of single spore populations (*Glomus etunicatum* on *Petroselinum crispum*). SR = standard reaction of non-mycorrhizal plants.

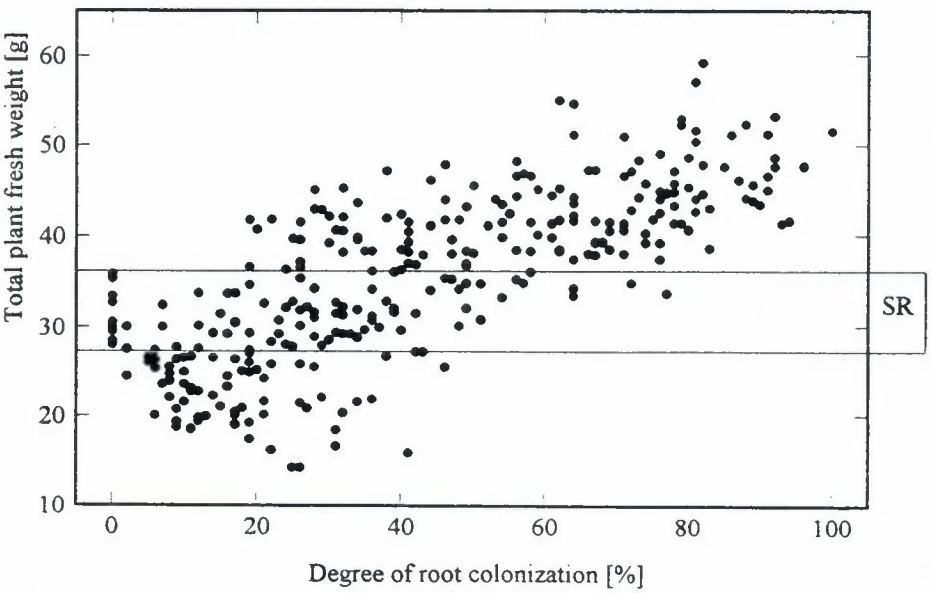


Figure 2. Interrelationship between biomass and AMF root colonization of *Petroselinum crispum* seedlings after inoculation with single spores of *Glomus etunicatum* in multiplication cycle C 3. SR = standard reaction of non-mycorrhizal plants.

The interrelationships between root colonization and effectiveness of single spores indicated very few correlations between fresh weight parameters and

degree of root colonization (Table 3). Only in the case of positive effectiveness did a significant positive correlation between total biomass and colonization exist. Because of the strong correlation between shoot and root fresh weight in any case, and the fact that the average root fresh weight was not influenced by root colonization (Table 2), the conclusion must be drawn that changes in the physiological status of the mycorrhiza (i.e. the fungus/root-compartment) should be the reason for neutral and negative symbiontal effectiveness, e.g. by changing source/sink interrelationships.

Proceeding with the characterization of the variability increase within the strain HH13 from C1 to C3, we compared the original (C1) and subsequent (C2 and C3) effectiveness of AMF single spore populations. We found that in each case the whole spectrum of variability including positive, neutral and negative effectiveness can evolve from a single spore of any original effectiveness (Fig. 3). It was not possible to infer the previous effectiveness from the present or to predict the future effectiveness.

Up to here we evaluated all derivates from the initial 25 successful symbioses in C1 together. Focusing now on each line of derivates which developed from those single spores in C1 (called "sub-strains"), we could observe that the sequential differentiation of variability resulted in C3 in 15 remaining sub-strains (10 were extinct in C2 and C3). Each of these sub-strains showed large variability of effectiveness in C3. Nevertheless, the variation within a sub-strain was a maximum of 50% of the total variation (Fig. 4). Therefore, three groups of sub-strain populations could be recognized. The median of those groups was localized within the standard reaction of non-mycorrhizal plants (predominantly neutral effectiveness), above (mainly positive effectiveness), and below the standard reaction (mainly negative effectiveness).

Table 3. Pearson Product Moment Correlation (95% confidence interval) between biomass (fresh weight, FW) and degree of root colonization by AMF (DRC)

Pair of variables	Shoot FW /root FW	Total FW /DRC	Shoot FW /DRC	Root FW /DRC
Standard reaction of control plants	0.69	–	–	–
Positive effectiveness of AM combinations	0.70	0.76	0.81	0.53
Neutral effectiveness of AM combinations	0.81	0.42	0.47	0.32
Negative effectiveness of AM combinations	0.92	0.26	0.29	0.23

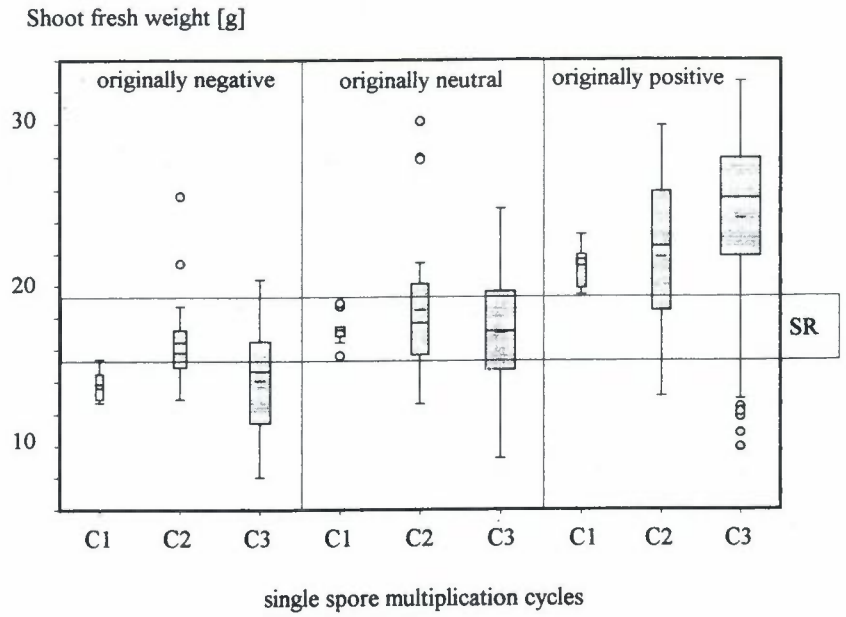


Figure 3. Comparison between original and subsequent effectiveness in sequential multiplication cycles (C1–C3) of AMF single spore populations. SR = standard reaction of non-mycorrhizal plants.

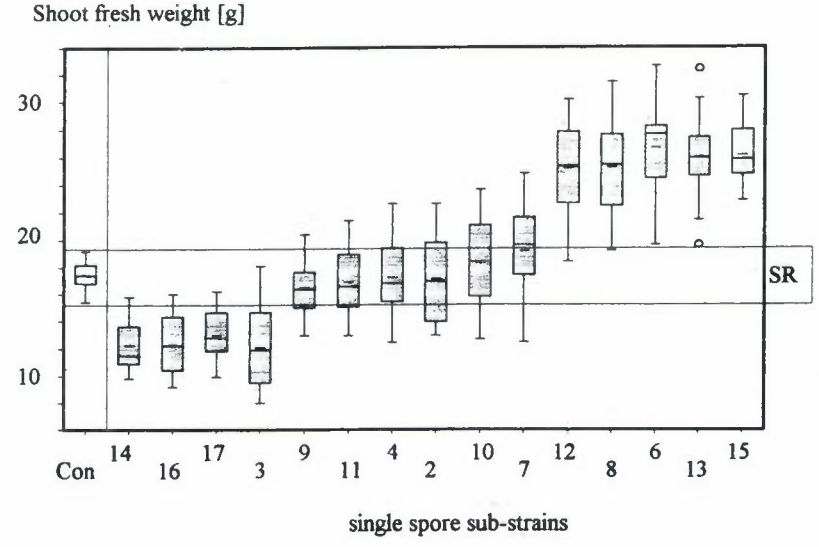


Figure 4. Effectiveness of single spore sub-strain populations in the multiplication cycle C3. SR = standard reaction of non-mycorrhizal plants (Con).

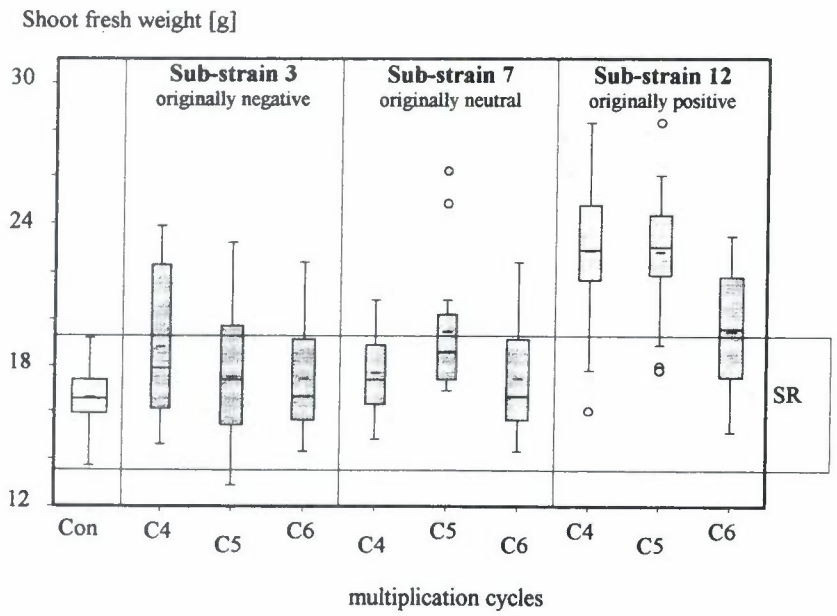


Figure 5. Host response after inoculation with AMF populations (100 spores of sub-strain C3/3, C3/7, C3/12). SR = standard reaction of non-mycorrhizal plants (Con).

What would happen if populations derived from sub-strains with such distinct characteristics were now inoculated as populations – not as single spores? Would the host/AMF interaction further allow increase of variation? Or 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 (sub-strain 3, Fig. 4) exclusively expressed neutral or even positive effective symbioses in multiplication cycle C4 (Fig. 5). In the following two cycles C5 and C6 that characteristic was maintained with the majority of measurements mainly in the standard reaction range (neutral effectiveness).

Sub-strain 7 (Fig. 4) 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 (Fig. 5).

The nearly completely positive effective sub-strain 12 (Fig. 4) showed a trend to lower effectiveness in the subsequent multiplication cycles C4–C6 (Fig. 5).

In summary, it was obvious that the actually expressed effectiveness of the *P. crispum/G. etunicatum* symbiosis tended to result in neutral effective combinations, when we inoculated with spore populations.

4. Discussion

The strain-inherent spectrum of symbiotic interactions

Symbioses are classified after evaluating quantitative and qualitative "advantages" for both partners. The symbiosis between AMF and higher plants often results in advantages ("effects") for the host in comparison to non-mycorrhizal plants, such as better growth and yield or higher tolerance to stress. The decisive symbiotic advantage for the AMF is thought to be its own survival per se. The arbuscular mycorrhizal symbiosis therefore usually reflects the quantitative output of a fungus-mediated response of the host plant, seen as positive or negative effectiveness of a symbiosis. AMF/host combinations with positive effectiveness in this sense can be determined mutualistic. Negative effectiveness indicates parasitic actions of the fungus. In case of the lack of advantages for one of both partners, the host-fungus relationship is one of commensalism. Using the parsley test system, we designed experiments which demonstrated the existence of the whole spectrum of genetically fixed, potential effectiveness within a population of *Glomus etunicatum*.

Genetic diversity of AMF spores

In our tests the majority of single spores of *G. etunicatum* was able to colonize the parsley root system without respect to later effectiveness. The extreme variability of effectiveness in symbioses established from single spores goes along with a high number of nuclei in spores of *G. etunicatum* (Feldmann et al., 1998). It can be assumed that during the germination process the nuclei are liberated into the developing mycelium which contacts the host root. It is not known whether these nuclei are homocaryotic or not (Wood and Cummings, 1992). If heterocaryosis exists, already that first contact with the host and the genetic constitution of the actually involved nuclei could decide upon the nature of interaction which develops. There are genetic differences between isolates of the same AMF species manifested in genomic DNA (Lanfranco et al., 1995). Franken and Gianinazzi-Pearson (1996) described nucleotide exchanges on the 5.8S rRNA gene between clones of *Glomus mosseae*. Additionally, there are first indications that single spores of a population differ in their ITS- and 5.8S rRNA sequences (Hurek, 1997). Along with the multinucleic character,

heterocaryosis could therefore be the key to understanding of the variability of effectiveness in the presented testing system.

Hypothesizing that heterogenic nuclei in a spore and in a developing mycelium cause the variability of effectiveness opens new, important questions: (a) Are environmental factors decisive for a selection of active nuclei, which then influence the symbiontal characteristics? (b) Can the host plant as the most important part of the micro-environment select directly for certain nuclei?

On the scale of AMF species environmental differences were observed to lead to distinct characteristics of autochthonous spore populations (Stahl et al., 1990). Beyond that observation, the presented data (Fig. 5) indicate that either an intermediate "average" effectiveness of several colonizing spores is expressed as symbiotic phenotype, or a fungal genotype canalization caused by the host plant takes place. Due to the inoculation conditions, which cause competition between the spores, the selectivity of that environment (including the host) for certain spores or even nuclei populations is probable.

In future studies it has to be clarified if (a) direct interactions between the spores or nuclei in spores or (b) the selection of certain spore genotypes by the host leads to the tendency of the symbiosis towards more commensalistic combinations in subsequent multiplication cycles. The findings of Dhillion (1992) and Talukdar and Germida (1994), who pointed out the existence of preference by hosts for certain AMF species, permit speculation that selection takes place on the scale of single spores and their populations of nuclei. Shifting towards decreased effectiveness after three subsequent multiplication cycles is already documented for other AMF/host combinations (Feldmann et al., 1998).

Acknowledgements

I am grateful to Dr. C. Boyle for her general support and patient discussion of the difficult subject. I wish to thank my students and technicians R. Finkenwirth, E. Görtz, H. Ecks, S. Mondenschein, G. Deylitz, and S. Gaude for their assistance in routine procedures and maintenance of cultivation conditions over three years. I thank Dipl.-Biol. H. Boyle for proof-reading and correcting my English.

REFERENCES

Alvarez-Santiago, S.A., Garcia-Oliva, F., and Varela, L. 1996. Analysis of vesicular-arbuscular mycorrhizal colonization data with logistic regression model. *Mycorrhiza* **6**: 197–200.

Azcon, R. and Ocampo, J.A. 1981. Factors affecting the vesicular arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. *New Phytologist* **87**: 677–685.

Bowen, G.D. 1987. The biology and physiology of infection and its development. In: *Ecophysiology of VA Mycorrhizal Plants*. G.R. Safir, ed. CRC Press, Boca Raton, FL, USA, pp. 27–58.

Dhillion, S.S. 1992. Evidence for host-mycorrhizal preference in native grassland species. *Mycological Research* **96**: 359–362.

Dodd, J.C. and Thomson, B.D. 1994. The screening and selection of inoculant arbuscular-mycorrhizal and ectomycorrhizal fungi. In: *Management of Mycorrhizas in Agriculture, Horticulture and Forestry*. A.D. Robson, L.K. Abbott, and N. Malajczuk, eds. Kluwer Academic Publishers, Dordrecht, pp. 149–158.

Feldmann, F. and Idczak, E. 1992. Inoculum production of VAM fungi for use in tropical nurseries. In: *Methods in Microbiology: Experiments with Mycorrhiza*. A.K. Varma, J.R. Norris, and D.J. Read, eds. **24**: 339–357.

Feldmann, F., Junqueira, N.T.V., and Lieberei, R. 1989. Utilization of vesicular-arbuscular mycorrhiza as a factor of integrated plant protection. *Agriculture, Ecosystems and Environment* **29**: 131–135.

Feldmann, F., Kruse, W., Boyle, C., and Lieberei, R. 1998. The strain-inherent variability of arbuscular mycorrhizal effectiveness: I. Development of the *Petroselinum crispum/Glomus etunicatum* test system. *Symbiosis* **25**: 115–129.

Franken, P. and Gianinazzi-Pearson, V. 1996. Construction of genomic phage libraries of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Scutellospora castanea* and isolation of ribosomal RNA genes. *Mycorrhiza* **6**: 167–173.

Hurek, T. 1997. Expression pilzspezifischer Gene bei der arbuskulären Mykorrhiza. DFG-Meeting on Mycorrhizas, February 13–14, 1997, Leichlingen, Germany. p. 18.

Lanfranco, L., Wyss, P., Marzachi, C., and Bonfante, P. 1995. Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. *Molecular Ecology* **4**: 61–68.

Lieberei, R., Junqueira, N.T.V., and Feldmann, F. 1989. Integrated disease control in rubber plantations in South America. *Proceedings of the '89 Integrated Pest Management*, February 8–15, 1989, Bad Dürkheim, Germany, pp. 445–456.

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

Stahl, P.D., Christensen, M., and Williams, S.E. 1990. Population variation in the mycorrhizal fungus *Glomus mosseae*: uniform garden experiments. *Mycological Research* **94**: 1070–1076.

Talukdar, N.C. and Germida, J.J. 1994. Growth and yield of lentil and wheat inoculated with three *Glomus* isolates from Saskatchewan soils. *Mycorrhiza* **5**: 145–152.

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

Wood, T. and Cummings, B. 1992. Biotechnology and the future of VAM commercialization. In: *Mycorrhizal Functioning*. M. J. Allen, ed. Chapman & Hall, New York, pp. 468–487.