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Studies on arbuscular mycorrhiza (AM) in the Alentejo (Portugal) using pea mutants resistant to AM fungi as a control tool for field conditions

Ph.D. thesis

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presented by
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Nasce um Deus; outros morrem.
A verdade nem veio nem se foi: o erro mudou.
Temos agora uma outra eternidade
E era sempre melhor a que passou.

Cega, a Ciência a inútil gleba lavra.
Louca, a Fé vive o sonho do seu culto.
Um novo Deus é só uma palavra:
Não procures nem creias: tudo é oculto

(Fernando Pessoa)

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Variables and dimensions

ΔE	saturation deficit
DAE	days after emergence
DAP	days after planting
DAS	days after sowing
h	hours
d	days
E	Einstein
PAR	photosynthetically active radiation
T	temperature
FC	field capacity
WHC	water holding capacity
w/w	weight ratio
v/v	volume ratio
dm	dry matter
FW	fresh weight
TSL	total shoot length
TGW	thousand grain weight
HGW	hundred grain weight
HI	harvest index
no.	numbers

Statistics

±	standard deviation (SD)
CV	coefficient of variation
n.s.	not significant
n.d.	not determined
n.dec.	not detectable
(n)	sample
SD	standard deviation (±)
HSD	honest significant difference
LSD	least significant difference
r	Pearson coefficient of correlation

General

AM	arbuscular mycorrhiza
AMF	arbuscular mycorrhizal fungi
AP	alkaline phosphatase staining
A-Tab.	table in appendix
CAL	calcium-acetate-lactate extraction
CEC	cation exchange capacity
EM	ectomycorrhiza
Fig.	figure
Tab.	table in text
TB	trypan blue staining
rel.	relative
conc.	concentration
conduc.	conductivity
∅	diameter
BNF	biological nitrogen fixation
BNR	binucleate *Rhizoctonia*
ENMP	Estação National de Melhoramento de Plantas, Elvas (Portugal)
GMT	Greenwich Mean Time
IAT	Department of Agronomy, Institute of Plant Production and Animal Health in the Tropics and Subtropics, University of Göttingen (Germany)
MPN	most probable number
myc $^{-(1)}$	non-mycorrhizal (AMF resistant) isoline/mutant
myc $^{+}$	mycorrhizal isoline
nod $^{-}$	non-nodulating isoline
fix $^{-}$	non-fixing isoline
N	nitrogen
P	phosphorus
per. comm.	personal communication
rpm	revolutions per minute
TDR	time domain reflectometry
UE	University of Évora (Portugal)
WUE	water use efficiency
var.	variety

Experiments

MPN 93	MPN test with safflower and chickpea 1993 in the climate chamber
MPN 94	MPN test with safflower 1994 in the climate chamber
SAF 94	open air monolith experiment with safflower 1994
SAF 95	open air pot experiment with safflower 1995
SAF 95 a	safflower AM bioassay 1995 in the climate chamber
PP 95	pea pot climate chamber experiment 1995
PF 94	pea field experiment 1993/94 at site Évora
PF 95	pea field experiment 1995 at site Évora
PF 95 a	pea experiment 1994/95 at site Elvas
PF 96	pea field experiment 1996 at sites Évora, Portel, and Mitra
PF 96 a	pea evaluation field experiment 1995/96 at site Elvas
PF 96 b	pea field experiment 1996 at site Mitra
PP 97 a	pea germination bioassay 1997 in the incubation oven
PP 97 b	pea pot inoculation experiment 1997 in the greenhouse
PP 97 c	pea pot fungicide experiment 1997 in the greenhouse
PP 97 d	pea pot N and P fertilization experiment 1997 in the greenhouse
PP 97 e	pea pot P fertilization experiment 1997 in the greenhouse

Experimental factors

Fri	FRISSON
P2	isogenetic mutant P2 of variety FRISSON
M1 or m	mycorrhizal
M0 or nm	non-mycorrhizal
P100	P fertilization at a level of 100 (44) kg ha^{-1} P_2O_5 (P)
P300	P fertilization at a level of 300 (131) kg ha^{-1} P_2O_5 (P)
N100	N fertilization at a level of 100 kg ha^{-1} N
N450	N fertilization at a level of 450 kg ha^{-1} N
W0	drought stress
W1	well watered
IL	isoline
IN0	without inoculum
IN1	with inoculum
R	*Rhizobia* inoculum
Rhizoc.	*Rhizoctonia* inoculum

1 General introduction

Mycorrhiza, a symbiosis between plants and mutualistic soil fungi, is undoubtedly of extraordinary importance in plant production, plant and soil ecology, and plays a key role in what is generally nowadays called "sustainable agriculture" (BETHENFALVAY & LINDERMAN 1992; GIANINAZZI & SCHÜEPP, 1994). The *International Bank of Glomales* (IBG, former *Banque Européenne des Glomales* BEG, 1993) points out that: "The study of plants without their mycorrhizas is the study of artifacts" and "The majority of plants, strictly speaking, do not have roots; they have mycorrhizas." Numerous papers and reviews, the scientific journal *MYCORRHIZA*, valuable text books (e.g. HARLEY & SMITH, 1983; SAFIR, 1987; ALLEN, 1991; ALLEN, 1992; BRUNDRETT et al., 1996; SMITH & READ, 1997; VARMA & HOCK, 1999; KAPULNICK & DOUDS, 2000; VAN DER HEIJDEN & SANDERS, 2002), international symposia (ICOM), and helpful web pages such as of CSIRO, IBG, INVAM, IPB, MYCORRHIZA INFORMATION EXCHANGE, SERG, and SYLVIA (all 2002) underline the fact that studies on mycorrhiza are becoming increasingly fashionable (VLEK et al., 1996).

However, the research suffers from a dilemma of high expectations, that are often not met by indisputable and consistent results (ALLEN, 1992). It cannot be claimed that there is a good balance between promised progress, clear understandings, and successful applications, such as the use of AMF inocula as a biofertilizer (MILLER & JASTROW, 1992). The modest outcome may be related to a widely spread research strategy of simplifying complex field situations by factorial pot experiments and the subsequent transfer back to field conditions (JOHNSON & PFLEGER, 1992).

But at least a new species of scientist has been created: the mycorrhizaist (MILLER & JASTROW, 1994), also called mycorrhizologist (MORTON et al., 1999). Several types of mycorrhizologist were found, such as mycocentric, plantcentric, soilcentric, genocentric, anthropocentric, and ecocentric. Although these centrisms were mixed up in some cases, they underline the complexity of the research subject and perhaps the occasional difficulties of communication among the respective groups, all of which are certainly relevant.

1.1 Types of mycorrhizas

There are several types of mycorrhizas, all defined by a fungal mycelium growing outwardly into the soil, which extends the root system of the associated plant. Thus, the

mycorrhizal fungi colonize not only plant roots but also soils by creating a crucial linkage between these two. Ectomycorrhiza (EM) which is formed by most trees and shrubs in the temperate zone with *Basidomycotina* and (few) *Ascomycotina* has been the subject of research since the last century (FRANK, 1885) but covers only 3 % of spermatophytes (STRASBURGER, 1998). Their root surface is characteristically sheathed in a fungal mantle and the Hartig net which is formed by hyphae between epidermal and cortical cells. Transitions from ecto- to endomycorrhiza called *ectendomycorrhiza* are possible, especially in *Pinus* and *Larix* species (MOLINA et al., 1992). Endomycorrhizas are characterized by a hyphal growth into the root cortex, attributes of the ericoid mycorrhizas of *Ericales*, the endomycorrhizas of *Orchidaceae*, and the arbuscular mycorrhiza (AM). In contrast to the latter, the first two are highly host plant specific. Further differentiation as the monotropoid mycorrhiza of achlorophyllous *Ericaceae* and of EM in arbutoid and pyroloid mycorrhiza were regarded by MOLINA and co-workers (1992) as pedantic.

1.2 Specific characteristics of arbuscular mycorrhiza

Beside EM, the greatest scientific attention is paid to AM, not least because their host plants are among the most important agriculture crops. In contrast to the other mycorrhiza types which are distinguished by a more narrow host range, some EM fungi and, regularly, the AM fungi are regarded as non-host specific (MOLINA et al., 1992). However, the host specificity may be closer under natural or field conditions than in pot cultures (SMITH & READ, 1997). Arbuscular mycorrhiza is the most common belowground symbiosis (SMITH & READ, 1997) and occurs ubiquitously due to its great potential of ecological adaptation from the alpine and boreal zones to the tropical forests and grasslands (SCHÖNBECK, 1980; BONFANTE-FASOLO & SCANNERINI, 1991).

The term “arbuscular mycorrhiza“ expresses morphologically the symbiosis of vascular plants with fungi of the order *Glomales* (*Zygomycotina*) which represents the norm of about 90 % of the families of all phyla of land plants (GIOVANNETTI & SBRANA, 1998) including ferns and some mosses (SMITH & READ, 1997).

These obligate symbiotic fungi persist in soils as thick-walled spores of diameters from 10 to 1000 µm which are rich in storage lipids and nuclei (SYLVIA, 1998). The infection - or better, because of its mutualistic nature, *colonization* - of plant roots arise from spores and other vegetative propagules which germinate in the rhizosphere. Stimulated by plant exudates, the formation of appressoria, a thickening of hyphae in the epidermis, manifests the

recognition between the two symbionts. Subsequently, the fungal hyphae are allowed to penetrate the plant root.

Having once ramified, the hyphae form characteristic dichotomously, highly branched arbuscules inside of root cortical cells. The normal *arum*-type is much more common than the *paris*-type distinguished by extensively coiled intracellular hyphae. The arbuscules emerge continuously but are relatively (< 15 d) short living. They are the only and, unfortunately, merely ephemeral, taxonomically decisive feature of AMF. This interspecific interface consists of the modified plant plasma membrane or periarbuscular membrane and the fungal cell wall. The increased surface area serves both symbiotic partners for an extensive bi-directional metabolic exchange. However, this is proved more for the transfer of P from fungus to plant than for carbohydrates in the opposite direction in which also normal internal hyphae seemed to be involved (SMITH & READ, 1997).

Particularly, the enhanced uptake of phosphorus (BOLAN, 1991) and other less mobile nutrients such as zinc, copper, and ammonium is well established as the main fungal benefit to the plant (SMITH & READ, 1997). It is mediated by an external mycelium (∅ 20-30 µm) spreading out, simultaneously to the formation of arbuscules, into the surrounding soil up to several centimeters. Fanned out in fine (∅ 2 µm) absorptive hyphae, the fungus can exploit much smaller soil pores than the non-mycorrhizal plant by roots and even root hairs (JACOBSON, 1999). The external mycelium may have a considerable effect on soil aggregation. In this process, hyphal exudates (LINDERMAN, 1994), especially glomalin, a glycoprotein with N-linked oligosaccharides, seem to play a key role (WRIGHT & UPADHYAYA, 1998; RILLIG et al., 1999). The transfer of P as polyphosphates via the mycelium exceeds the speed of P diffusion through the soil solution by many magnitudes. The surface area of roots loses importance for P uptake with progressing depletion, and consequently, diffusion speed becomes the limiting factor (HINSINGER, 2001). However, hyphae can grow much faster than roots into soil zones not depleted of P (JACOBSON, 1999) and facilitate the P uptake with their much higher surface area. Hot spots of nutrient rich patches can be reached more easily (CUI & CALDWELL, 1996), although studies on capture of organic N were not consistent (HODGE, et al., 2000).

The pool of available P may be greater for hyphal uptake than for roots, probably caused by synergistic effects with P-solubilizing microorganisms and the possible excretion of protons. As a consequence, the utilization of rock phosphates may be enhanced at certain rates for mycorrhizal plants compared to non-mycorrhizal ones (SMITH & READ, 1997). The

hyphal P uptake against a strong concentration gradient is enabled by an active ATP dependent process, as well as the two subsequent steps of mycorrhizal P transfer, hyphal transport, and transition to the plant in arbuscules (JACOBSON, 1999). Therefore, the mycorrhiza requires photosynthates from the plant not only for fungal growth and respiration, but also for processing this nutrient uptake and exudates.

Another type of hyphae, so called runner hyphae, grows fast along the root and causes secondary infections of the same root system (READ, 1992). Generally, the external mycelium has importance for spore production and as a vegetative infective propagule itself. Additionally, fragments of hyphae and previously infected (dead) roots can initiate mycorrhizal colonization. Intra- and interspecific linkage of (unevenly aged) plants is possible (MOORA & ZOBEL, 1998) and may transfer carbohydrates, N, and P (SMITH & READ, 1997; MÅRTENSSON et al., 1998). Competition strength of species within plant communities is expected to be altered as a consequence.

The efficiency of nutrient uptake in relation to the requirements of carbohydrates from the host plant, persistence in soils as well as infectivity, competitiveness, and sporulation can generally differ between the various glomalean species and isolates (WILSON & TOMMERUP, 1992; JOHNSON & PFLEGER, 1992).

1.3 Plant responses to arbuscular mycorrhiza

The macro symbionts can also widely differ in their interaction with AM. If the host's nutrition is strongly dependent on the micro symbiont and the plant cannot complete its life cycle under natural conditions without mycorrhiza, the concept of mycotrophy is considered obligate in contrast to facultative mycotrophic plants which are without mycorrhiza in some natural situation and with mycorrhiza in others (MOLINA et al., 1992). About 70 % of all angiosperms are regarded as obligate mycotrophic species (TRAPPE, 1987).

The colonization by glomalean fungi can be high for certain plants such as some cereals, however, without any consequence for nutrient uptake (BAON et al., 1993). Such phenomena demonstrate the limitation of the concept of mycotrophy and suggest a differentiation in susceptibility and responsiveness to AM under distinct conditions. PLENCHETTE (1983) proposed the "relative field mycorrhizal dependency" (RFMD) at a determined available P soil content, calculated by dry matter (dm) of mycorrhizal (m) and non-mycorrhizal plants (nm) as:

$$RFMD = \frac{dm_m - dm_{nm}}{dm_m} \cdot 100$$

This calculation is used later in the course of the work presented for the determination of mycorrhizal response.

The evaluation of mycorrhizal efficiency concerning P uptake depends on how many photosynthates must be invested by the plant and involves therefore an appropriate cost benefit analysis (KOIDE & ELLIOTT, 1989). However, also other mycorrhiza mediated plant benefits such as enhanced soil aggregation or resistance to drought, pathogens, toxic cations, and heavy metals are of agricultural interest (SMITH & READ, 1997).

Mycorrhizal plants are generally known to be more resistant against pests. AMF are considered as a kind of biological means of protection, called bioprotector by LINDERMAN (1994), not only because of the indirect effect of a better P status as discussed later in section 1.5.5 for enhanced drought stress resistance, but also because of direct effects such as competition for infection sites and nutrients. Mycorrhizal plants seem to be less sensitive to transplantation stress which has particular importance for nurseries and micropropagation from tissue cultures (HOOKER et al., 1994).

Few vascular plants, in particular of the families *Caryophyllaceae*, *Brassicaceae*, *Cyperaceae*, *Juncaceae*, *Proteaceae*, *Chenopodiaceae* (GIANINAZZI-PEARSON et al., 1996) and of the genus *Lupinus* are characterized as non-hosts for arbuscular mycorrhiza. Reviews including lists of host and, the easier way, of non-host plants were found in MOLINA et al. (1992) and TRAPPE (1987).

1.4 Taxonomy of arbuscular mycorrhiza fungi

All approximately 150 species of AMF belong to the order *Glomales* which consists of two suborders (MORTON & BENNY 1990; WALKER & TRAPPE, 1993). They are apparently asexual organisms and probably of very ancient origin (350-460 million years). One, the *Glomineae* with the families *Glomaceae* (genera: *Glomus* and *Sclerocystis*) and *Acaulosporaceae* (genera: *Acaulospora* and *Entrophospora*), comprises fungi that form usually inter- and intraradical vesicles as storage compartments for lipids. Vesicles can also function as spores in some *Glomus* species (BRUNDRETT et al., 1996). Before the taxonomic revision of the *Glomales* by MORTON and BENNY (1990) the term “vesicular arbuscular mycorrhiza (VAM) fungi“ was therefore generally used. The other suborder *Gigasporineae* which consists of the two genera *Gigaspora* and *Scutellospora* do not form

any vesicle but extraradical auxiliary cells of unknown function. Hence, it is nowadays more convenient to use “arbuscular mycorrhiza fungi (AMF)“ as is done in the work presented. The, formally correct, subdivision of the two suborders of the *Glomales* into VAMF (*Glomineae*) and AMF (*Gigasporineae*) causes unnecessary confusion because all VAMF are AMF, but not *vice versa*. Conservative authors point out that the second suborder covers only about 20 % of the *Glomales* and express doubts about the general taxonomic qualities of the functional feature *arbuscules* (SMITH & READ, 1997). Furthermore, they regard VAM as a well introduced expression.

Recently, MORTON and REDECKER (1999) proposed the new family *Archeosporaceae* for outliers from *Glomus* and *Acaulospora* species. In the most actual taxonomic revision of AMF by SCHUßLER and co-workers (2001), the order of *Glomales* was replaced by the new phylum of *Glomeromycota* with the single class of *Glomeromycetes* consisting of the four orders *Glomerales*, *Diversisporales*, *Archaeosporales*, and *Paraglomerales*.

1.5 Difficulties in studying arbuscular mycorrhiza

The knowledge of the biology of these fungi still remains elusive because of their obligate biotrophy and, up to now, non-cultivability (REQUENA et al., 1999).

Research on arbuscular mycorrhiza struggles with conceptual and methodological difficulties. The high level of complexity of mycorrhizal functioning causes not only fascination but also frustration. Fungus, host, soil, climate, agricultural practice and other (micro) organisms interact to such a degree that the contribution of individual components is generally impossible to estimate, both under natural and under cropping conditions (KOSKE & GEMMA, 1992). Consequently, the identification of cause and effect becomes difficult despite some apparently convincing results obtained in greenhouse or laboratory experiments (READ et al., 1976).

1.5.1 Taxonomy

One major problem concerns the species (mis) identification demonstrated widely in the literature (KOSKE & GEMMA, 1992). Irrespectively of a quantitative variation, spores were often only identified by size, shape, color and hyphae attachment (SMITH & READ, 1997). Spore material must be necessarily obtained fresh from pot cultures because spores extracted from field samples suffer from parasitism, degradation, and other modifications (BENTIVENGA & MORTON, 1994). However, species may be disregarded which were

found in the field but not in trap pot cultures (SIEVERDING, 1991). Furthermore, an observation of the spore development over a certain time period is necessary to detect ephemeral characteristics (MORTON et al., 1999). For example, the sporiferous saccule, being either terminal or lateral, distinguishes the genera *Acaulospora* and *Entrophospora* (SCHENCK & PÉREZ, 1990), must be examined before spore formation is completed. Additional identification of internal and external fungal structures such as vesicle and auxiliary cells – the latter are not always easily found – are decisive for the taxonomy even as high as at suborder level. The examination of the naturally colorless wall structures of broken spores such as composition of layers, thickness, pigmentation, ornamentation, histochemical reactions and germination structures allows (only) experts further characterization down to species level. MORTON et al. (1999) admit that "morphological characters have been a continual source of frustration for mycorrhizologists concerned with identification of experimental units".

1.5.2 Intraradical mycelium

The most commonly measured mycorrhizal feature to reflect the AM status of plants has been the percentage of colonized root length. However, direct correlation between colonization rate and nutrient uptake by AMF hyphae is not compulsive (SMITH & READ, 1997), especially not, when estimated late in the growth period or when soil water content was involved as an experimental factor. Generally, the colonization rate mirrors not only the formation of infection and the consequent interradical mycorrhizal development but also the root growth (ALLEN, 2001). The time course of the colonization rate follows usually a sigmoidal function. Rather than the final plateau value, the preceding *log* phase of rapid increase is decisive to evaluate the efficiency of AMF (SMITH & READ, 1997).

Unfortunately, some AMF colonization fails to be stained by standard methods such as trypan blue (AN et al., 1990). The quantification of colonization rates is a source of error and requires a high number of replications to restrict variation within tolerable dimensions (BIERMANN & LINDERMAN, 1981). Sensitivity to observer effects may be compensated by magnified intersection techniques but increase labor intensity (McGONIGLE et al., 1990). Qualifying the percentage rate of characteristics such as the colonization intensity of intercellular hyphae, their entry points, arbuscules, and vesicles may make the examination more profound. The staining of fungal chitin has the fundamental disadvantage that all sorts of structures including dead ones were stained. In contrast, functional and vital staining of

enzymes located in the fungal hyphae such as succinate dehydrogenises, alkaline phosphatase and hydrolases make it possible to determine the proportion of the fungus that is active (SMITH & READ, 1997).

1.5.3 Extraradical mycelium

The ecologically most significant part of the mycorrhizal symbiosis represents the least accessible one: the network of the external mycelium (READ, 1992). Extraction and quantification suffer from incomplete recoveries and especially the fine hyphal branches are very difficult to distinguish from those of soil saprophytes and of root pathogens (SMITH & READ, 1997). An efficient method for studying the impacts of the external mycelium are growth containers, firstly introduced by SCHÜEPP and co-workers (1987), separated by membranes, which are permeable only for hyphae, whereas other compartments are accessible both to roots and hyphae. Element fluxes can be monitored in such compartments by natural or radioactive isotopes as markers (JAKOBSEN, 1999). Split root systems are another approach (e.g. AUGÉ & DUAN, 1991). However, both systems implicate rather artificial and space limited conditions (MÄDER et al., 2000).

1.5.4 Interaction with other soil biota

Native soil microorganisms and mesofauna interact with AMF. Beside dispersal by ants and earthworms, mycorrhiza hyphae and spores may be consumed by nematodes (BRUNDRETT et al., 1996; INGHAM et al., 1986; ALLEN et al., 1987), collembola (THIMM & LARINK, 1995) and other soil microarthropods such as mites (KLIRONOMOS & KENDRICK, 1996; RILLIG & ALLEN, 1999). Consequently, a possible AMF response may be “grazed“ off under field conditions (FITTER, 1985a; McGONIGLE & FITTER, 1988; ALLEN, 1991; SMITH & READ, 1997).

Preliminary processes of an AMF colonization such as spore germination can be enhanced by microbial effects like the removal of toxins and germination inhibitors, production of specific stimulatory compounds and the maintenance of elevated CO_2 concentration (SMITH & READ, 1997). Conversely, the system of extraradical hyphae creates a mycorrhizosphere in the soil (AZCÓN-AGUILAR & BAREA, 1992) in which so called growth promoting bacteria find suitable sites on or nearby mycorrhizal structures promoted by exudates. However, the absorptive hyphae compete with other microorganism for nutrients and may reduce microbiological fixation of ammonia.

Because of interactions of a studied *Rhizobacterium* with AMF and biological N_2 fixation in peas, which had both positive (on soil aggregation) and negative (on AMF colonization and plant growth at mineral N supply) effects, BETHLENFALVAY and co-workers, (1997) suggest the term **a**gro**s**ystem-**a**ffecting **r**hizobacterium (ASAR) instead of the generally used "growth promoting bacteria".

The most prominent interaction of *Rhizobia* with AMF is largely plant mediated e.g. by competition for photosynthates (REINHARD et al., 1994; REINHARD, 1995) and by enhanced P supply via AMF for higher needs in N_2 fixing legumes (MARSCHNER, 1995), while general mycorrhizospheric effects may also exist.

1.5.5 Host plant water relation

The mechanisms of potentially enhanced drought resistance of mycorrhizal plants compared to non-mycorrhizal were firstly investigated by SAFIR and co-workers (1971). Interestingly, even after three decades of intensive research, there is no final elucidation (BRYLA & DUNIWAY, 1997a). The major disagreement concerns whether or not the cellular P status as a consequence of enhanced mycorrhizal P uptake is the principle reason (NELSON, 1987; SMITH & READ, 1997) or other effects of arbuscular mycorrhiza influence the water status of host plants significantly (VLEK et al., 1996). Very recently, this complex was excellently reviewed by AUGÉ (2001) although there is no ultimate conclusion. The evidence of direct water uptake by the extraradical fungal mycelium was demonstrated by FABER and co-workers (1991), however, rejected by GEORGE and co-workers (1992) with an experiment using a comparable hyphal compartment technique. Non-hydraulic (i.e. phytohormonal signals) may be induced by mycorrhizal roots in drying soils and improve stomata control irrespectively of the leaf water status (AUGÉ & DUAN, 1991; EBEL et al., 1996). Generally, phytohormones such as cytokinins, abscicic acid, and gibberelin-like substances (DIXON et al., 1988; DRÜGE & SCHÖNBECK, 1992; DANNEBERG et al., 1992) as well as auxins (MÜLLER, 1999) and ethylene (BESMER & KOIDE, 1999) may be involved in the plant-fungus relations.

The micro symbiont fungus represents a large sink of about up to 20 % for the plant's photosynthesis (SMITH & READ, 1997). Higher leaf contents of sugars, amino acids and solved proteins in a changed metabolism can decrease the osmotic potential of leaves of mycorrhizal plants (SUBRAMANIAN & CHAREST 1995; HAMPP & SCHAEFFER, 1999).

Indirectly, the mycorrhizal formation of aggregates and pores as mediated by the external mycelium (section 1.5.3; LINDERMAN, 1994; SMITH & READ, 1997) creates more suitable soil structures and may enhance plant water uptake under drought conditions (DAVIES et al., 1992), as well as increase both water infiltration and retention. Drying of soils enhances compactness of aggregates and reduces water conductivity (SCHULZE, 1993).

Generally, the importance of mycorrhizal nutrient uptake will be amplified when the nutrient flux through the soil solution is limited by diffusion speed at distances enlarged by the tortuosity of the water film around soil particles. Consequently, improved uptake of plant nutrients less mobile in dry soils such as ammonia is reported (TOBAR et al., 1994a; TOBAR et al., 1994b, SUBRAMANIAN & CHAREST, 1999). The same mechanism reduces even more the availability of phosphorus in dry soils (VIETZ, 1972; HINSINGER, 2001) and enforces mycorrhiza dependency (KOIDE, 1993). This may feed back on the regulation of water economy at high cellular P status.

1.6 Project background of the experimental work

The presented work was started in the Alentejo (Portugal) as part of the EU project Diversification of the cropping system and utilization of symbiotic associations to reduce production costs and environmental pollution under dryland farming in Mediterranean regions (CARVALHO, 1992). Economic limitations of the farming system are due to the restriction of the yield potential by both climatic and pedological conditions. Climate imposes multiple stress to crop growth. Production at high temperatures during the grain filling period are combined with drought stress from spring onwards. Conversely, an excess of water occurs during winter. Both forms of water stress strongly vary in length and intensity. The inter-annual variation of total annual precipitation is enormous, ranging from 300 to 1100 mm. The pedological limitations are mainly due to a high percentage of relatively infertile and shallow soils of low water holding capacity. The infertility is often caused by limited P availability for plants growing in acid soils of high P absorption capacity. Soil erosion is regarded as the major environmental problem of agricultural land use, beside water pollution deriving from nitrate leaching in context with low N fertilization efficiency. Additional water pollution is provoked by erosion when P is flushed with top soil into surface water (SHARPLEY et al., 2001). Rain fed agriculture in the Mediterranean areas is mainly based on autumn/winter sown crops. During the time of cultivation, precipitation largely exceeds evaporation. Moldboard ploughing is the practiced traditional soil tillage system in the region, even in the most

undulating landscape. These conditions associated with a weak structural stability in the majority of soils imply a great risk of soil loss by erosion (CARVALHO, 1997).

A diversification of the copping system, which is widely based on wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus* L.) production, was intended by introducing N_2 fixing grain legume crops such as fababean (*Vicia faba*), chickpea (*Cicer arietinum* L.), and forage peas (*Pisum sativum* L.) in order to reduce N fertilizer input. These legumes were integrated in experiments of crop rotation, soil tillage, weed control, and they were the target of breeding programs and of the research for efficient *Rhizobia* strains. Another aspect of the project was concerned with diversification by the introduction of "alternative" crops such as oilseeds for industrial purpose e.g. safflower (*Carthamus tinctorius* L.).

Altogether, cropping conditions and research objectives were worthwhile for the development of an efficient utilization of mycorrhizal association.

The working plan of the EU project section "mycorrhizal association" is based on following structure according to the initial project proposal.

AMF spores from air dried soil samples should be quantified after trapped with suitable bait plants in pot cultures (DIEDERICHS et al., 1996). Infectivity of soils also should be estimated in most probable number (MPN) tests by enumeration of infective propagules and extension of AMF colonization in host plants. The mycorrhizal response was intended to be measured by differences in biomass of host plants growing in pots filled with native soil or with soil heated at 110 °C (i.e. with vs. without native AMF propagules) (DIEDERICHS et al., 1996). Selected spores should be isolated and multiplied for taxonomic identification in pure cultures. Subsequently screened for efficiency by inoculation bioassays in the greenhouse, spores evaluated as efficient may serve for inoculum production. Those inocula should be tested again under field conditions in order to improve yields. The main objective of the project was to substitute expensive P fertilizer such as triple phosphate by means of a suitable AMF inoculum.

1.7 Methodological reflections

The working plan for the AMF section (CARVALHO, 1992) is derived from the concept of SIEVERDING (1991) and based on sequenced experimental steps as described above, referred to as the *sequential approach* later on in this work.

However, already the recovery of relevant AMF from the field may be incomplete in trap cultures. Some difficulties arise from the takeover of inadequate methods of related research

areas such as the MPN technique from general microbiology, as discussed later in section 3.1.3. Colonization rates describe only partly the mycorrhizal status of the plant and even less informative is the enumeration of AMF spores for the mycorrhizal status of the soil (see also section 3.1.3).

The major problem is created by the choice of the experimental conditions, which has fundamental impacts on what is considered to be the mycorrhizal effect. In particular, field conditions have important properties for mycorrhiza (KRUCKELMANN, 1975; STEFFAN, 1989; BODDINGTON & DODD, 2000). The production system with all interfering biotic and non biotic limiting factors is the field, not the pot. Any promising production component has finally to be evaluated in field trials as the target environment. A successful introduction of AMF or other manipulation of mycorrhiza in the production process will be related to the agronomic growing conditions such as soil tillage, rotation, fertilization, pests and their control, and optional irrigation. Residues from previous crops, natural vegetation and weeds (SCHREINER et al., 2001) affect the abundance of AMF propagules and development of colonization. The closer the test conditions match the production conditions, the more relevant will be the research. Pot experiments on isolated factors are easier to interpret and therefore generally preferred. However, the transferability is increasingly restricted by the higher degree of complexity under field conditions. The factor *mycorrhiza* is more than just inoculation, not to mention after soil sterilization, because it interacts strongly with its environment. The inclusion of the relevant test condition is therefore essential for the determination of the mycorrhizal effects.

The objective of the EU project was the substitution of mineral P fertilizer by a biofertilizer AMF inoculum. However, the maximization of the plant benefits by the mycorrhizal symbiosis demands not only the isolated study of the two symbiotic partners but also their interactions with all the components of a more or less anthropogenically manipulated agro-ecosystem. Low-input agriculture requires highly susceptible and responsive cultivars, of course in combination with efficient AMF, to improve nutrient efficiency. In contrast, non-responsive and non-susceptible crops are usually favored in combination with high fertilization (VLEK et al., 1996) as long as the lack of mycorrhiza does not decrease significantly soil structural stability or resistance to pathogens (SMITH & READ 1997).

1.7.1 Soil volume and plant density

Surprisingly little awareness is attributed to the fact that the soil volume in which the plants were tested must exert a great influence on the mycorrhizal response. As argued in section 1.2, quality and quantity of the potentially accessible soil volume may enhance the uptake of relatively immobile nutrients. The volume dependency gains more importance in the large soil volume of a field trial than in a comparably small pot, especially in Mediterranean dry land farming systems with relatively low plant densities. Hence, even a small improvement as effected by AM may be multiplied by such a volume factor and gain agronomic importance. The interaction between mycorrhizal response and soil volume was shown in pot trials for *Allium cepa* (BÅÅTH & HAYMAN, 1984). The indirect evidence as a response to plant density was demonstrated in pots for *Trifolium subterraneum* (FACELLI et al., 1999) and in field trials for *Hypericum perforatum* (MOORA & ZOBEL, 1998), for *Pisum sativum* (HAYMAN et al., 1982), for *Abutilon theophrasti* (KOIDE, 1994), and for two *Fabaceae* species (ALLSOPP & STOCK, 1992). However, the mycorrhizal benefit for larger field grown plants with a proportionately larger root system may decrease in comparison to small pot plants only grown for a short period, when intraspecific competition in high input cropping systems results in higher plant and root densities (BLOSS & PFEIFFER, 1984).

SMITH and READ (1997) conclude that (1) the potentially mycorrhizal plant species are unable to use soil resources effectively in the absence of colonization. (2) Colonized plants have a combined root and hyphal length density which permits effective use of a much greater volume of soil than non-colonized plants, with the consequence that interplant competition is potentially strong. (3) Responses to mycorrhizal colonization will be greatest at the lowest planting densities, both on an individual and on a population basis.

Above-ground space competition may create disadvantages for the mycorrhizal responsiveness of plants when their photosynthesis is reduced because of an insufficient light regime (TESTER et al., 1986; SMITH & READ, 1997; FACELLI et al., 1999). High plant densities were discussed for difficulties of transferring results on mycorrhizal response from pot to field experiments (FITTER, 1985b; McGONIGLE, 1988; FACELLI et al., 1999).

1.7.2 Pot experiments

Usually, the cultivation of plants in pots imposes a distribution of roots and water which is shifted to some extent in an unnatural way to the container walls and particularly to the bottom. Here, mycorrhizal hyphae cannot play the same functional role as in spatially

unrestricted soil systems without containment in which only root competition may restrict the space indirectly. Special attention must be paid to circular root growth soon after elongation (McGEE et al., 1999; TORRISI et al., 1999). Plant density in pot experiments is usually much higher as compared to field experiments and, consequently, the soil nutrient concentration, when the absolute nutrient supply per plant unit is set comparable to field conditions. In the case of phosphorus, the high concentration can imply a suppression of AM (SMITH & READ, 1997).

The evaluation of infectiousness in terms of the extension of the colonization in pot trials does not automatically give valuable information about the effectiveness of the investigated AMF (see section 1.5.2). The relevance of inoculation bioassays in sterile soils under the exclusion of other competing soil micro- and meso-organisms in small pots may be restricted. Besides, the creation of uniform AMF inocula is difficult (SMITH & READ, 1997).

Generally, larger pot volumes seem to promote the development of AM propagules (LOVATO, 1994). Field and pot cultivation differ also in other characteristics relevant for a potential mycorrhizal response, such as elevated (soil and air) temperature. Different temperature optima are known for the colonization process of different AMF (SCHREINER et al., 2001). Indirectly, growing speed both of plants and of fungal hyphae may be of importance. A lower maximum colonization rate was observed for plants with fast root growth such as cereals, in contrast to plants with slowly growing roots such as *Allium porrum* or *Trifolium subterraneum* (SMITH & READ, 1997). A fast and extensive colonization may be a criterion for efficiency. Low temperature can slow down or even suppress mycorrhiza development (SIEVERDING, 1980; WILSON & TRINICK, 1982; TOMMERUP, 1983b; SMITH & READ, 1997).

1.7.3 Soil sterilization and fumigation

All physical methods of soil sterilization such as autoclaving, steaming or dry heat have a positive or negative impact on soil chemistry, especially via mobilization of P (LANGE NESS, 1998) or toxic heavy metals such as Zn and Mn (AZCÓN-AGUILAR & BAREA, 1992). Available forms of N can be released from organic matter by γ irradiance (READ, 1992; KAHILUOTU et al., 2000). The chemical sterilization agents that are usually applied for soil fumigation are often highly toxic general biocides such as formaldehyde (SIEVERDING, 1991), methyl bromide (PLENCHETTE, 1983; SYLVIA et al., 1993), or methyl isothiocyanate (JAKOBSEN, 1986; SIEVERDING, 1991; WEBER 1992). The latter

two not only kill soil microorganism but also weeds, which has a strong impact on mycorrhizal abundance (SCHREINER et al., 2001). Likewise, the utilization of systemic soil fungicides such as BENOMYL® (MERRYWEATHER & FITTER, 1996; BODDINGTON & DODD, 2000; KAHILUOTU & VESTBERG, 2000) can have in the same way undesirable side effects (JAKOBSEN, 1994) such as modulation of the AMF community structure (SCHREINER & BETHLENFALVAY, 1996a) and phytotoxic effects. BENOMYL® (benzyladenine) is known to change senescence processes in plants (SKENE, 1972; VANSTADEN & NTINGANE 1996). There is also a risk of intoxication for researchers and technicians. Additionally, methyl bromide is a so called ozone killer.

The application of paper filtered soil suspension, often called leaching, to sterilized soils is inadequate to re-establish the soil biology simply due to the fact that pore sizes of about 4 µm ∅ let through only (some) bacteria and there is no guarantee that these reach the microsites they are used to occupying. Leaching of soil suspensions sieved at larger mesh sizes of e.g. 37 or 38 µm ∅ (JAKOBSON, 1987; KAHILOUTO et al., 2000) may contain infective propagules such as hyphae and very small AMF spores of fine glomalean endophytes.

1.7.4 Consequences and requirements for an innovative approach

Error susceptibility in combination with weak measures and high labor intensity, not to mention the lack of clear and feasible taxonomy, manageable only with the help of (conflicting) experts, complicate a successful implementation of arbuscular mycorrhiza in crop production. Many of the conceptual difficulties are linked to side effects of soil sterilization. A strategy of avoidance are inoculation bioassays in pots, which creates new serious restrictions concerning soil volume and other pot specific modification of growing conditions.

However, it is fundamental for modern mycorrhiza management to evaluate the native AMF and potential AMF inocula under relevant conditions instead of using a re-inoculated sterile system (VLEK et al., 1996). Only in extreme high input (greenhouse) horticulture with regular soil disinfections or for the restoration of mining spoils may such artificial conditions have practical importance.

More integrated approaches avoiding the multipartite evaluation process of the sequential approach (see section 1.6) conceptualized by SIEVERDING (1991) or DODD and

THOMSON (1994) may reduce the risk of multiplying too many possible mistakes by too many steps.

1.8 AMF resistant pea mutants as a new experimental tool

A simple but elegant way of providing non-mycorrhizal controls exists by the utilization of AMF resistant isogenetic mutants. Such isolines were detected particularly in peas deficient of symbiotic characteristics. Pea mutants are a traditional research tool for studies on genetics of the symbiosis with *Rhizobium* (POTSMA, 1990; DOWNIE & BONFANTE, 2000) but were also used as non-fixing controls for the determination of the proportion of biologically fixed N_2 (SAGAN et al., 1993). With the first report on AMF resistance of DUC et al. (1989), these mutants became a potential tool for mycorrhiza research. However, most studies so far were mainly aimed at the identification of the colonization process (GIANINAZZI-PEARSON et al., 1995; SMITH & READ, 1997; PETERSON & BRADBURY, 1999).

AMF resistant "*myc* $^{-}$" mutants were found nearly exclusively with legumes such as *Medicago sativa* L. (BRADBURY et al., 1991), *Medicago truncatula* Gaertn. (SAGAN et al., 1995), *Melilotus alba* Desr. (LaRUE & WEEDEN, 1994; PETERSON & BRADBURY, 1999; HIRSCH et al., 2000), *Phaseolus vulgaris* L. (SHIRTLIFFE & VESSEY, 1996), *Lotus japonicus* L. (WEGEL et al., 1998), *Vicia faba* L. (DUC et al., 1989) and *Pisum sativum* L.. In the latter, mutants derived from the forage pea varieties FRISSON (DUC et al., 1989) and FINALE (GIOVANNETTI & SBRANA, 1998), and from the garden pea variety SPARKLE (LaRUE & KNEEN, 1994). The related genes *sym* 8, *sym* 9 (*myc* $^{-}$), and *sym* 13 were used for the genetic transformation of the roots of var. LINCOLN (BALAJI et al., 1994) and var. SPARKLE (BALAJI et al., 1995).

Recently, as first non-legume hosts, mutants with highly reduced AMF colonization were reported for tomatoes (*Lycopersicon esculentum* L.) and for barley (*Hordeum vulgare* L.) (BARKER et al., 1998; SMITH & READ, 1997). While all other mutants were derived by mutagenesis with chemicals, mostly ethyl methane sulfonic acid, or γ irradiation, the above mentioned alfalfa lines were obtained by serial re-crossing for ineffective or non-nodulating character (BARNES et al., 1988).

Two types of mycorrhizal resistance were proposed (GIANINAZZI-PEARSON, 1996; GIOVANNETTI & SBRANA, 1998). First is the "early" *myc* $^{-1}$ type which forms an appressorium in first cells of cortical epidermis but does not allow further hyphal growth into roots. Second is the "late" *myc* $^{-2}$ type in which intraradical colonization is possible but shows

no differentiation in arbuscules. Very recently, *myc* $^{-}$ mutants were reviewed by MARSCH and SCHULTZE (2001). They classify the *myc* $^{-1}$ type as *pen* $^{-}$ according to no fungal penetration of root epidermis.

Non-mycorrhizal mutants can be a helpful tool as a control in the evaluation process under field conditions and are proposed by GIANINAZZI et al. (1995) especially for the reason that soil disinfections with all its implication becomes obsolete. Strikingly, at present only two relatively small field experiments with AMF resistant pea plants have been carried out to study the mycorrhizal response. The first was by LOVATO (1994) using FRISSON with its isogenetic *myc* $^{-1}$ mutant P2 at very high N fertilization. Very recently in the second, KAHILUOTU and co-workers (2000) tested mycorrhizal *nod* $^{-}$ *myc* $^{+}$ mutants of SPARKLE in comparison with its non-mycorrhizal *nod* $^{-}$ *myc* $^{-1}$ isomutant.

1.9 Objectives

The overall objective of the work presented was closely linked to the EU project "Utilization and management of symbiotic associations – selection and introduction of arbuscular mycorrhiza".

One proposed experimental technique for the selection procedure was the MPN test (MPN 93) in order to estimate the infective propagules in soils from experimental sites of the project. Two host plants were chosen, safflower and chickpea, creating cooperation with other participants of the project. The first was concerned with oilseed production and the second with *Rhizobia*-legumes symbiosis.

An improvement of the proposed bioassays comparing non-mycorrhizal plants growing in sterilized soil with mycorrhizal plants growing in non-sterilized soil was intended in order to create experimental conditions close to those in the field. Therefore, a bioassay in large soil monoliths under natural light and temperature regime was carried out (SAF 94). Beside mycorrhiza as an inoculum, drought, the most limiting factor in Mediterranean dry land farming, was exemined as an experimental factor. It should be proved whether safflower colonized by native AMF is also more productive under drought stress than non-mycorrhizal as compared to well watered plants. Leaf area related transpiration and water use efficiency for grain yield and biomass production were additionally chosen as evaluation variables.

Because higher P uptake is considered as the main beneficial AM effect, P fertilization was included as the third experimental factor in a subsequent similar experiment (SAF 95) to

prove whether the possible enhanced drought resistance of mycorrhizal plants is a direct or an indirect effect of improved P nutrition.

In order to overcome the fundamental restriction of an inoculation bioassay in sterilized soil, alternative appropriate mycorrhizal controls are sought in particular for field experiments. Parallel to safflower experiments, the two field pea isolines – the symbiotic FRISSON and the non-symbiotic mutant P2 – were examined in first minor trials under Mediterranean dry land farming condition (PF 94, PF 95, PF 95 a, PF 96 a) to determine whether they are suitable for a potential utilization as a screening and evaluation crop for mycorrhiza in field experiments. The possible benefit from N_2 fixation in FRISSON was intended to be suppressed by high mineral N fertilization.

A central objective is to prove whether arbuscular mycorrhiza can be reliably assayed by differences in field growth of those two isolines as a genetically defined experimental factor. For this purpose, an additional pot experiment (PP 95) was carried out. The performance of the two isolines was hypothesized as similar under symbiotic and non-symbiotic environmental conditions at high N and P supply. The experimental factor drought stress as the dominant growth limiting factor under the regional field conditions was included as before in safflower experiments to answer the question of whether mycorrhiza can directly enhance plant growth under water restriction independently of N and P limitations.

A fundamental idea of the work presented is using the set of the two pea isolines as a bioindicator crop to screen for effective native AMF directly under field conditions as an integrated approach. Therefore, a major field experiment (PF 96) was carried out at three sites. The experimental restriction by nitrogen should be controlled by the application of N fertilizer up to a level of no response. The yield differences between the two isolines are hypothesized as site specific and characterizing the efficiency of native AMF under given conditions.

However, together with the promising alternative approach, new methodological difficulties are emerging. In order to compensate for the conceptual problem of nitrogen fixation, N_2 fixation was to be suppressed by high ammonium nitrate fertilization (PF 96, PP 97d) and the utilization of non-fixing but mycorrhizal mutants in comparison with *nod*$^-$ *myc*$^{-1}$ mutants (PF 96 b).

Liming, factorial inoculation with mutualistic and phytopathogenic fungi, fungicide application, and both N and P response were studied in detail by additional pot experiments (PP 97a, b, c, d, e) in order to elucidate the reasons for strong mycorrhizal effects at one site.

The background of the objective was to contribute to the development of a simple but efficient and reproducible method. The assessment of arbuscular mycorrhiza in particular under field conditions is desirable in a way which avoids complicated, environmentally hazardous, expert-intensive, and expensive experimental techniques. The research efforts would be simply reduced to growing different lines of a crop that is common throughout the world. As a consequence, the mycorrhiza research for agriculture would be more accessible to others than only mycorrhizaists.

2 General methods

2.1 Geographical location of experimental sites, soils, and climate

The Alentejo in the South of Portugal is a traditional agriculture region. The Romans already produced olives, wine, and cereals in relative fertile Vertisols around Beja in the lower Alentejo. The investigated sites belong to the district of Évora, only Elvas belonging to the district of Portalegre, both situated in the province of the higher Alentejo. Farm sizes are large for historical reasons, but productivity is generally low (BASCH, 1998; see section 1.6).

Tab. 1: Sites and soils of field experiments

Site Specific Naming	**Évora** Fundação Eugénio de Almeida, San Manços	**Portel** Herdade de Vale Figueira de Baixo	**Mitra** Horta Experimental da Universidade de Évora	**Elvas** Estação National de Melhoramento de Plantas (ENMP)
Latitude North	38°30'	38°21'	38°32'	38°54'
Longitude West	07°46'	07°49'	08°01'	07°07'
Altitude [m]	200	230	230	200
Soil type (FAO)	Vertic Luvisol	Ferric Luvisol	Humic Cambisol	Luvisol
pH (in KCl)	5.3	4.6	6.0	7.2
Soil texture	Sandy loam	Loamy to silty sand	Sandy loam	Sandy loam
Skeletal phase [%]	2-3	50	1-2	1-2
Land use	Normal (typical farmland [1])	Extensive (Montado [2])	Intensive (horticulture)	Relatively intensive (experimental station)

[1] Plant species list see appendix A-Tab. 22
[2] for the Alentejo typical agro-silvo-pastoral land use system with cork oaks. Plant species list see A-Tab. 23

The most strikingly different soil of all sites was the Ferric Luvisol at site Portel, investigated in detail by STAUSS and KALLIS (1992) as documented in the appendix in A-Tab. 9 to A-Tab. 15. It differed in particular in higher acidity, free Fe and a fine soil content of only about 50 % in the top soil. The parent material is *rañas*. The Ferric Luvisol was originally described as a classical tropical Ferric Acrisol (STAUSS & KALLIS, 1992) with all typical properties of intense (relict) weathering. However, high contents of kaolinite (A-Tab. 14) enforced the renaming as Luvisol due to the FAO criterion (STAHR, per. comm., 1994).

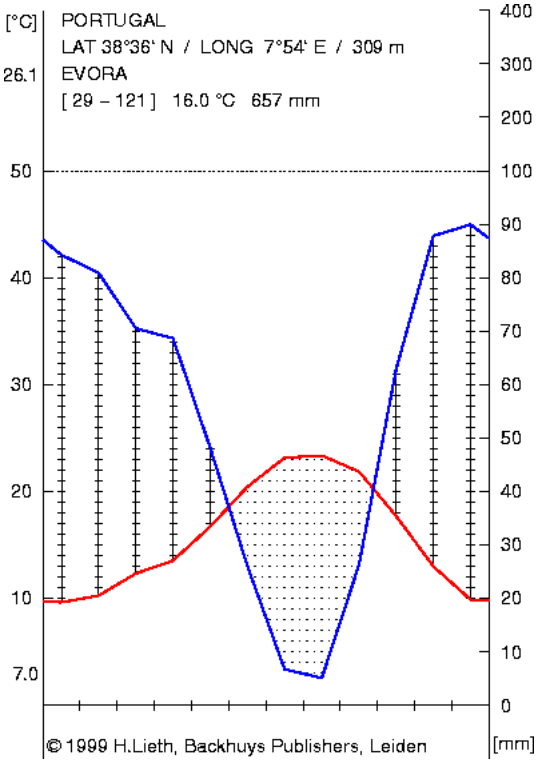


Fig. 1: Climate diagram of Évora, Portugal (LIETH et al., 1999)

Its development is characterized by poor humus accumulation and a very strong acidification. No base cations were accumulated, however, a very strong formation of oxides took place. The soil is highly loamed and, as a relict, rubified. Furthermore, the Ferric Luvisol is very lessive and shows a strong formation of aggregates and depletion of Si. It has been highly influenced by a perched water table in former times. The main rooting zone was about 55 cm and the mechanical profile depth was > 150 cm. The plant available field capacity was calculated as 72 mm in the main rooting zone and 108 mm for the first meter soil depth, for which total field capacity was 354 mm. The total organic N was determined in the Ap horizon at 0.04 (%) as very low. The total P soil content was high, however, plant available P extremely low as determined by CAL (calcium-acetate-lactate) extraction (A-Tab. 11).

Conversely, the Luvisol from Elvas had the highest pH and CEC due to its origin from slates. The Vertic Luvisol from Évora shows its Vertic properties in the subsoil (A-Tab. 17). The topsoil is formed by colluviums of different geological origins predominated by acid Grano-Diorits which is also the geological parental formation for the Humic Cambisol at Mitra. However, the soil has been exposed to a permanent anthropogenic influence since the 17th century in a cultivated cloister garden. Outside of the cloister walls, soil was sampled in a *Montado* for some pot experiments. The site was very heterogeneous, as were the soil types, which were characterized as Leptosol and Cambic Arenosol (HOLLAND, 1991). For more details on comparison of soil chemistry, see A-Tab. 18.

The climate is distinguished as Meso-Mediterranean due to an Atlantic influence, although frost can occur (Fig. 1). The factor water is still crucial for dry land farming in particular because of its irregularity. Therefore, smaller and larger water reservoirs were used to compensate for water deficits. The biggest artificial lake of western Europe *Alqueva* was

recently constructed to provide water for 110,000 ha of intensive irrigation in the Alentejo by damming up the river Guadiana from the beginning of 2002.

2.2 Climate chamber

For several pot experiments, a climate chamber *model* Fitoclima 700 EDTU (ARALAB S.A., Portugal) was used. The location was in an air conditioned building at the University of Évora, Núcleo da Mitra. It had a size of 2 (width) x 3 (length) x 3 (height) m. Air temperature was kept between 16 °C (night minimum) and 24 °C (day maximum). Daylight was programmed for 14 h at 350 µE $m^{-2}s^{-1}$ PAR including one hour transition phase in five steps to night darkness (Fig. 2). Relative humidity was maintained constantly at 55 % during safflower and at 70 % during pea experiments.

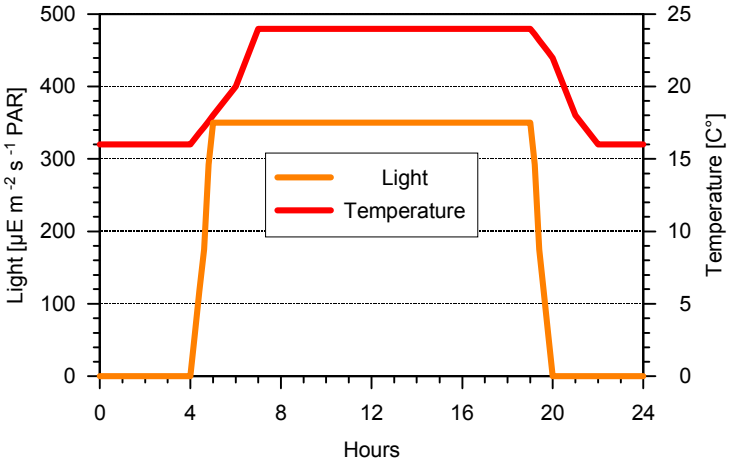


Fig. 2: Climate chamber programming

2.3 Chemical soil analysis

For N_{min} analysis, soil samples of about 5 g field fresh fine soil (< 2 mm) were filled in Erlenmeyer flasks. Soil water content was determined from samples of same size and source by drying overnight at 105 °C. Samples were shaken for 30 min in 50 ml 2 M KCl (*aqua bidest.*) to extract nitrate and ammonium (MAYNARD & KALRA, 1993). Extracts were filtered in N free filter papers (Whatman) and were stored frozen in plastic bottles. Melted solutions were diluted by factor 40. Subsequently, NO_3 and NH_4 concentrations were determined photometrically at UV spectrum by continuous flow measurement (CENCO, Germany).

For the determination of plant available inorganic soil P content, samples of 5 g dried soil were extracted according to BRAY I method by shaking for 5 min with 30 ml 0.03 M NH_4 + 0.025 M HCl at pH 2.60 (BRAY & KURTZ, 1945). Subsequently, P was measured

spectrophotometrically at 630 nm with an autoanalyser *model* Technicon II (Pulse Instrumentation Lts, Canada).

2.4 Plant material and element analysis

Plants were generally harvested when mature and separated into grains, straw and roots. Straw included all remaining parts of flowers and pods. Shoots were defined as straw plus grain. Straw was separated in some pot experiments into leaves and stem. When leaf area was estimated, only the green was taken into account.

Pots were soaked for several hours and roots were washed out on 2.5 and 1 mm sieves. For safflower, coarse roots > 2 mm were separated from fine roots, which were difficult to quantify.

Plant material was dried at 70 °C (safflower and chickpea) and 60 °C (peas) to constant weight for dry matter (dm) determination. However, grain samples of pea mutants and line SPARKLE were dried at 35 °C to enable further utilization as seed material. Difference of grain constancy weight between 35 and 60 °C was taken for FRISSON samples and used later on for P2 and all SPARKLE isoline grain yields. From field trials, the entire central plots were harvested. Yields were stated per plant and per square meter. The latter take the plant stand at harvest into account.

Subsamples of straw and grain were firstly ground in a steal mill and secondly in a CULLATI micro fine mill (IKA) on 2 mm for P analysis. For N and natural isotope analysis, samples were additionally pulverized for 10 minutes in a piston-action ball mill (RONDON & THOMAS, 1994). Nitrogen was analyzed as total N from subsamples of 0.5 mg by CARLO-ERBA autoanalyser NA 1500. For P analysis, dried ground subsamples of about 0.5 g were digested in 5 ml $HClO_4$ HNO_3 1:2 mixture (v/v) for 90 min plus 30 min, and after addition of 3 ml 6 N HCl, another 45 min plus 30 min at 160°C and 220°C, respectively. After cooling, the digestion solutions were transferred and filtered in 50 ml flasks, and filled up to the mark with *aqua dest.* (JUO, 1981). Total P was determined colorimetrically by the molybdenum-blue method in an autoanalyser *model* Technicon II (Pulse Instrumentation Lts, Canada). References of ground maize plants were included with every 40 samples to check digestion and analytic procedures.

Nitrogen and P uptakes of shoots are defined as added contents of grains and remaining shoots, respectively, calculated from separated determination of tissue concentration and dry matter.

Exemplarily, discrimination of natural isotopes ^{15}N and ^{13}C was analyzed mass spectrometrically (FINNIGAN MAT 251) in the Isotope Laboratory of the University of Göttingen (REINEKING et al., 1993) to assess biological nitrogen fixation by the natural abundance method (^{15}N) (RENNIE & RENNIE, 1982) and drought stress (^{13}C) (FARQUHAR et al., 1989). The enrichment $\delta^{15}N$ was calculated for the natural air standard in ($^{0}/_{00}$) as:

$$\delta^{15}N = \frac{^{15}N_{sample} - {}^{15}N_{standard}}{^{15}N_{standard}} \cdot 1000$$

The $\delta^{13}C$ discrimination was calculated in ($^{0}/_{00}$) for the PDB standard (CRAIG, 1957; LÜTTGER, 1996) as:

$$\delta^{13}C = \frac{^{13/12}C_{sample}}{^{13/12}C_{standard}} \cdot\text{-}\ 1000$$

2.5 Mycorrhiza

2.5.1 Characterization of native AMF population

The identification of the AMF was mainly carried out at IAT (University of Göttingen) by courtesy of HEINZEMANN (CARVALHO, 1995) according to MORTON and BENNY (1990), SCHENCK and PERÉZ (1990), and WALKER (1986) as listed in appendix A-Tab. 1. Own observations and preparations were additionally discussed with DODD (per. comm.).

At Évora (see section 2.1, Tab. 1,), spores of *Glomus mosseae* and a hyaline small sized (∅ 50-70 µm) *Glomus* sp. were very abundant. These two species were frequent also in all other examined soils. The latter was characterized as *Glomus aggregatum* by HEINZEMANN (CARVALHO, 1995). However, characterization was rejected by others (DODD, per. comm., 1994) and cannot be seriously achieved as long as no pure culture of an isolate is established. Furthermore, only *Acaulosspora* sp. Y150 was found in Évora.

In soil from the Montado site Mitra (see section 2.1), which was used later as AMF inoculum, beside *G. mosseae* and the *Glomus* sp., so far *Glomus* sp Y120, *Glomus* sp. B50, *Glomus* sp. B300, *Acaulosspora* sp. Y150, *Acaulosspora* sp. HY90, *Scutellospora* sp. H130, and *Scutellospora* sp. HY300 have been found and characterized by HEINZEMANN (A-Tab. 1). Furthermore, few *Glomus fragilistratum* spores and a red-brown, thick-walled *Acaulospora* spec., disregarded in the EU-report (CARVALHO, 1995), were additionally identified by courtesy of DODD. Colonization seemed to be dominated by *Glomus* species detectable by spore formation inside of the root (SCHENCK & PERÉZ, 1990), and “fine glomalean endophytes” which also had been discussed as *Glomus tenue* (ROSENDAHL et al.,

1994). In the Ferric Luvisol from Portel, a large *Scutellospora* frequently seemed to colonize pea roots. Vesicles were relatively seldom observed in stained roots, especially in pot experiments. Spiny auxiliary cells, characteristic of that genus, were detected at extraradical hyphae. Unfortunately, also this fungus could not be isolated as a pure culture up to now which is obligatory for serious taxonomic determination (see section 1.5.1). However, the scutellum as the genera decisive morphological feature was clearly visible in some preparations. The spores seemed to be similar to those described by HEINZEMANN as *Scutellospora* sp. HY300. Furthermore, (often dead) spores of *Glomus* cf. *invermaium* were found which were named *Glomus* sp. B50 by HEINZEMANN.

2.5.2 Extraction of AMF spores

After stirring the soil sample in a bucket, the sandy soil particles were allowed to sediment for 1 minute. The supernatant was sieved through a combination of sieves (BRUNDRETT et al., 1996). The widest of 630 µm mesh width retained coarser organic material, while the finest of conventionally 45 µm retained the spores. An intermediate mesh width of 125 µm was used for a pre-separation of a spore sample by size. The filtrate was transferred to 100-ml centrifugation tubes. In a first step, the organic material was floated at 1500 rpm for about 5 minutes while spores and mineral soil of higher density formed the pellet. In a second step, the soil-spore mixture of the pellet was homogenized in an aqueous sugar solution of 70 % (w/w) in which the spores were floated while mineral soil formed the new pellet. Now, the supernatant contained the spores and was washed quickly onto a 45 µm sieve in order to prevent osmotic damage. BRUNDRETT and co-workers (1996) recommended this classical two step centrifugation in particular for samples rich in organic material. An alternative procedure is sugar gradient centrifugation (DANIELS & SKIPPER, 1982) which combines the two steps described above. Using this method, the centrifugation tube was half filled with sugar solution. Then, the sample was stirred with a glass rod and topped up carefully with water. After centrifugation, spores at the boarder layer of the two phases were extracted with a 100 ml syringe and washed onto a 45 µm sieve.

2.5.3 Typification and counting of AMF spores

Spores from 50 g field fresh soil from Portel, which was screened on 4 mm, were cleaned from attached organic material under a dissection microscope (WILD M5) at 50x magnification with fine tweezers. The spore samples were separated by typifying morphological features such as size, color, hyphal connection and spore surface to different groups and determined as

far as possible. Subsequently, they were counted in a nematode plastic dish of 15 cm diameter with 10 concentric indentations. In order to confirm the division and attempt taxonomic determination, reference samples, so called vouchers, of those groups were prepared in PVLG and PVLG/Melzer´s solution according to the recommendation of the IBG (BEG, 2002) and were observed under a stereo-microscope at 40 to 1000x magnification.

2.5.4 Staining of AM structures and estimation of colonization rate in fine roots

Mycorrhizal chitin was stained with lactic trypan blue according to PHILIPS and HAYMAN, (1970), KOSKE and GEMMA (1989), WEBER (per. comm., 1993) and own modification (Tab. 2).

Tab. 2: Procedure to stain AM chitin in fine roots by trypan blue

Step	Reagent	Time	Temperature
1. Dispigmentation	KOH 10 %	60 min	65 °C
2. Washing	Tap water	(well)	Ambient
3. Acidification	HCl 2 M	20 min	Ambient
4. Staining	Trypan blue 0.1 % in Lactic acid 90 %	12 min (chickpea) 18 min (safflower, pea)	65 °C
5. Destaining	Lactic acid 90 %	30 min (2-3 d)	65 °C (ambient)

Pieces of stained roots were placed without overlapping in petri-dishes marked with cross lines. Mycorrhizal colonization rate was determined by the grid line method (100 cross lines) under a dissection microscope (WILD M5) at 50 x magnification and strong through light of a fiber cold light (Schott KL 1500). The percentage of root length colonized by AM fungi based on arbuscules, vesicles, external and internal hyphae connected to arbuscules. The frequency of arbuscules and vesicles at each observed cross section was classified, similar to TROUVELOT et al. (1986) in three levels (low: 1-33 %, medium: 33-66 %, high: 66-100 %).

Non-specific fungal alkaline phosphatase was stained according to TISSERANT and co-workers (1993). In contrast to chitin staining with trypan blue, this simple staining method visualizes an important functional feature of mycorrhizal hyphae and structures: the capability to hydrolyze monophosphate esters. Enzyme staining provides a powerful marker for the evaluation of efficiency of arbuscular mycorrhizal colonization (TISSERANT et al., 1993).

Samples of washed fine roots of peas were digested for 2 h at room temperature with 150 units of cellulase extract of *Aspergillus niger* (Sigma C2415) and 150 units of pectinase (Sigma P5146) dissolved in 10 ml 0.05 M Tris/citric acid buffer (pH 9.2) with 0.5 g *D*-sorbitol (Sigma S1876) as substrate. After rinsing with *aqua bidest*., root pieces were processed for 16 h at room temperature in a reaction medium containing 10 mg Fast Blue RR salt (Sigma F0500) and 10 mg α-naphthyl acid phosphate as substrate. As activation, 5 mg Mg Cl_2 and 5 mg $MnCl_2 \cdot 4\ H_2O$ in 10 ml of 0.05 M Tris/citric acid buffer (pH 9.2) were used. Roots were washed thoroughly in tap water and the reaction was stopped by a sodium hypochlorite solution of 1 % active Cl. Stained roots were cut into 1-cm pieces and a subsample of about 30 root pieces was mounted in glycerol on slides. Under a stereo-microscope (LEITZ, Germany) at 40-400x magnification, colonization rate and development of arbuscules were classified according to TROUVELOT et al. (1986) by five levels: (none: 0 %, very low: 1-25 %, low to medium: 25-50 %, medium to high: 50-75 %, very high: 75-100 %).

2.6 Statistics

Results were tested with STATISTICA (1998) by analysis of variance after verifying normality (Kolmogorov-Smirnov, Sharpiro Wilk) and homogeneity of variances (Levene's). When necessary, values were ln transformed as indicated. However, according to LINDMAN (1974), small derivations from these distributional assumption were not seen as fatal as long as no correlation between means and variation and no strong violations were observed. Contrast analyses were performed in individual cases. Tukey's honest significant differences (HSD) was applied as post hoc analysis at $p<0.05$, because of the intermediate strength between Scheffé and Newman-Keul post hoc tests. In one indicated case, the least significant difference (LSD) test was applied to reveal tendencies. The combined data of different sites were analyzed as a nested design. Comparison of two groups was done by t-test or non parametric U-test at $p<0.05$. Data in percentages such as harvest index, colonization rates, ratios etc. were arcsinus-root-transformed. Significances for all tests including F-test are stated as * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, and n.s. when not significant at $p>0.05$ or by exact p-values in some tables. In various tables and figures, different letters are used to state significant differences of (arithmetic) means according to Tukey's HSD at $p<0.05$. Variations were stated as standard variation (SD) for *populations* in pot trials and for *samples* in field trials, indicated by error bars in graphs.

3 Safflower experiments

3.1 Quantification of mycorrhizal propagules by MPN tests with safflower and chickpea as host plants (MPN 93)

3.1.1 Introduction

The information of how infective is a soil concerning AM symbiosis may be crucial for further investigation on the improvement of mycorrhizal functioning in agro ecosystems.

The most probable number (MPN) test is used to determine the infective units of mycorrhizal propagules in soils, such as spores, extraradical hyphae, infected live or dead root pieces. It was one of the first steps on the selection of effective native AMF of soils from experimental sites of the EU project working plan. AMF spores isolated from soils with high propagule concentrations would be the object of subsequent bioassays in pot and field experiments.

The principle of the most probable number (MPN) test consists of dilution series with a definite degree of replication. This classical microbiological method goes back as far as 1915 to the concept of McCRADY. It is based on the probability of whether or not an infection is established in serially diluted media. The most probable number *d* of organisms can be calculated by the formula

$$d = -\frac{1}{v}\ln\left(\frac{s}{n}\right)$$

when *s* of *n* samples of a volume *v* are left sterile (COCHRAN, 1950). The method was adapted from bacteriology (ALEXANDER, 1965) for mycorrhiza research by PORTER (1979). SIEVERDING (1991) recommended fourfold dilution series with 5 replications as a standard technique to evaluate the infectiousness of arbuscular mycorrhiza. He derived his formula from FISHER and YATES (1970):

$$d = 10^{\log\,(x \cdot \log a - k)}$$

where *x* is the sum of infected pots per replication, *a* the factor of dilution, and *k* a constant from table VIII2 in which, however, an error must be corrected (per. comm. DODD, 1994).

Another way of calculation follows the equation of COCHRAN (1950) in which *e* is *Euler's* number:

$$\frac{(n-s_1)\cdot v_1\cdot e^{-(v_1\cdot d)}}{1-e^{-(v_a\cdot d)}}+\frac{(n-s_2)\cdot v_2\cdot e^{-(v_2\cdot d)}}{1-e^{-(v_2\cdot d)}}+\frac{(n-s_3)\cdot v_3\cdot e^{-(v_3\cdot d)}}{1-e^{-(v_3\cdot d)}}=s_1\cdot v_1+s_2\cdot v_2+s_3\cdot v_3$$

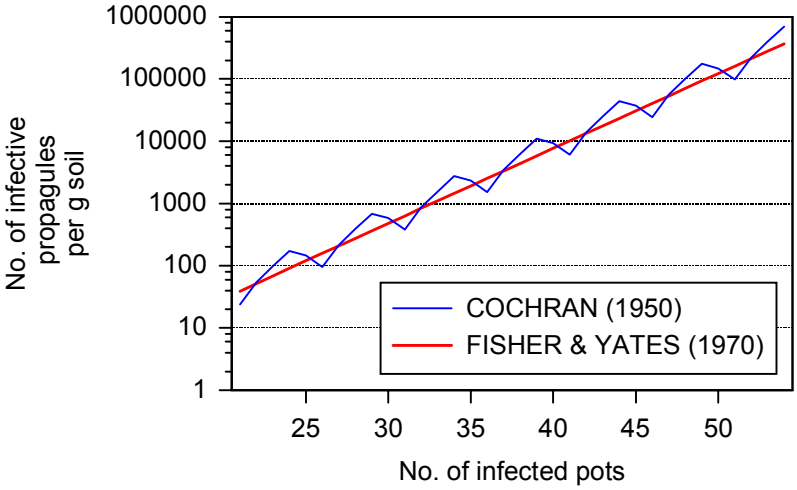


Fig. 3: Comparison of two MPN calculation methods
Modeling of fourfold dilution series (5 replications) with continuously growing infection in most diluted replication and without skips in lower diluted pots, respectively.

Both methods are available as BASIC-programs (INRA Dijon, France; CISRO Perth, Australia) but can also be adapted easily for spread sheets as exemplarily demonstrated in A-Tab. 2 and A-Tab. 3 (see Fig. 3 for model calculation). Non-infective controls are included in the MPN test. The comparison of the plant growth in undiluted parent soil to heated soil should function as a simple bioassay for the assessment of a potential plant growth response to mycorrhiza. Two different host plants were chosen, safflower and chickpea, creating cooperation with other participants of the project. The first was concerned with oilseed production and the second with *Rhizobia*-legumes symbiosis. The MPN test may be host plant related.

The experiment should answer the following question: How many infective mycorrhizal propagules can be found in the arable soils of the Alentejo? Can soils with high mycorrhizal potential be identified in order to investigate the AMF of those for further inoculum production? Are there differences in MPNs of practical importance resulting from the calculation method and from the host plant used? Does the comparison of host plant growth in heated and undiluted parent soil allow an evaluation of the mycorrhiza effect? Is the MPN test a suitable method for mycorrhizal research?

3.1.2 Materials and methods

Four different soils of agricultural sites from Alentejo were tested: (1) Vertisol from Beja, (2) Luvisol from Elvas (project site on-farm near Vila Boim: soil, derived from slates, which is similar to that from on-station ENM, but less fertile), (3) Vertic Luvisol from Évora and (4) Leptosol / Cambic Arenosol from the *Montado* site in Mitra (see section 2.1), all screened on 4 mm and air dried. About 21 kg of soil of each site were heated for 24 h at 100 °C in an oven.

Two different host plants were chosen (see section 1.9). Surface sterilized seeds of chickpea (*Cicer arietinum* L. var. ELVAR) and safflower (*Carthamus tinctorius* L. var. WARAMIN) were pre-germinated for 48 h at 36 °C on filter paper. Plants were cultivated in 250 ml cone pots and watered with 50 ml *aqua dest.* every 2 days. Pots were collocated in a support at 10 cm distance to allow free drainage and were protected against contamination with 20 cm high transparent plastic sheets around the pots. The supports were put on benches in a climate chamber (see section 2.2). No fertilizers were applied. Chemical soil properties are listed in A-Tab. 18. The experiment was arranged as 1 factorial complete randomized block (CRB) design with 5 replicates, however, separated for the two plant species. Blocks were orientated into the direction of the air stream of the climate chamber. The experimental factor was the concentration of infectious soil mixed by fourfold dilutions from 4^0 to 4^{-6} of heated with non-heated soil plus an additional non-infective control. The experiment was carried out stepwise with the two host plants in a series of 80 pots separated for each soil, respectively.

Plants were harvested at 60 DAS and shoot dry matter was determined. Fine roots were stained with lactic trypan blue (section 2.5.4) and AMF colonization was determined qualitatively in all samples under a dissection microscope at 50x magnification. When doubts occurred, slides were prepared for an additional control under the stereo-microscope at 100 to 400 x magnification. Additionally, the colonization rate in the parent soil was quantitatively determined under a stereo microscope at 100x magnification in a subsample of 100 root pieces. If any AM structure was found in a root sample, the respective pot was counted as "infected". The number of infective propagules was calculated according to FISHER and YATES (1970) or COCHRAN (1950). Formulas were written in a MS® EXCEL spread sheet (see A-Tab. 2 and A-Tab. 3) as recommended by JARSTFER and SYLVIA (1997).

3.1.3 Results and discussion

3.1.3.1 Mycorrhizal propagules and colonization

Unfortunately, a slight contamination with AMF in controls occurred in 4 of the 8 MPN tests with soils from Évora and Mitra. The resistance to heat stress of AMF can vary (SCHREINER et al., 2001) and was apparently higher than 100 °C in those soils. In consequence, infective propagules could not be calculated here.

The native AMF were very low in infectivity in the two remaining soils. As the host plant, safflower was more susceptible to AMF than chickpea (Tab. 3), in the Luvisol within the 95 % confidence limit. Additionally, the mycorrhizal colonization rate was always higher. In soil from Mitra, safflower even reached an AMF colonization of 83 % (data not shown).

Tab. 3: Mycorrhizal propagules and colonization in two soils from the Alentejo (MPN 93)

Soil (site)	Vertisol (Beja)		Luvisol (Elvas V.B.)	
Host plant	Safflower	Chickpea	Safflower	Chickpea
Infective propagules per g dry soil *(lower and upper confidence limit 95 %)*				
FISHER & YATES (1970)	**0.15** *(0.07-0.32)*	**0.07** *(0.03-0.14)*	**1.08** *(0.17-0.74)*	**0.03** *(0.01-0.00)*
COCHRAN (1950)	**0.28** *(0.06-1.20)*	**0.09** *(0.04-0.21)*	**1.61** *(0.67-3.88)*	**0.04** *(0.01-0.13)*
AMF colonization rate (%) in undiluted soil (**mean** ± SD)				
	65 ± 26	**13** ± 27	**53** ± 9	**16** ± 20

The MPN test for the Vertic Luvisol from Évora was repeated (DIEDERICHS, unpublished data) with safflower as the host plant in a climate chamber at IAT. The fourfold soil dilution (heated to non-heated) followed the same schedule as described in section 3.1.2, but was extended to a higher dilution rate of 4^{-12}. The number of infective propagules (Tab. 4) was higher by magnitudes than in any other MPN test before with soils from the region. Soil sampling took place at Évora in the same year, but after harvest at the end of June. The air drying process may affect the viability of propagules especially in hot climates (SIEVERDING, 1991). In contrast to MPN series at Évora, irradiance reached in the IAT climate chamber 800 $\mu E\ m^{-2}\ s^{-1}$ PAR.

Tab. 4: Mycorrhizal propagules in Vertic Luvisol (MPN 94)
Estimation by MPN test in climate chamber at IAT with safflower as the host plant (calculation from raw data of DIEDERICHS, unpublished)

Calculation method:	FISHER & YATES (1970)	COCHRAN (1950)
Infective propagules per g dry soil	**7193**	**7427**
(lower and upper confidence limit on 95 %)	(2279-9633)	(3082-17899)

3.1.3.2 Effects on host plant growth

The growth effect of soil sterilization on host plant chickpea was negligible (Tab. 5). Only in soil from Évora could a small significantly negative response to plant growth be observed. The negative mycorrhizal effect may be related to ineffective AMF or to indirect undesirable sterilization effects, like elimination of phytopathogens and P mobilization via sterilization (compare A-Tab. 18). Nematodes were frequently found in all soils (CARVALHO, 1993). Rooting may be space limited in the small pots and therefore the advantage of better soil exploitation by fungal hyphae without significance. Nutrient supply was poor due to small quantities of relatively infertile soils (A-Tab. 18) far from the optimum soil P concentration for AM effects, which is in the magnitude of about 30 µg g^{-1} plant available P (BRUNDRETT et al., 1996). Light restriction cannot be excluded. The irradiance in the climate chamber did not reach the recommended level of at least 500 µE $m^{-2}s^{-1}$ PAR (JARSTFER & SYLVIA, 1997).

Tab. 5: Shoot production of safflower and chickpea in preliminary bioassays (MPN 93)
Treatments are mycorrhizal (**M1**) and non-mycorrhizal (**M0**) in four different soils. In soils marked [1], a small AMF contamination in non-mycorrhizal treatment occurred. Letters show significant differences between shoot dry matter (dm) for mycorrhiza treatment within plant species and soils (t-test at $p<0.05$). Bold values are **means** ± standard deviation (SD).

Treatment	Soil (Site)	Vertisol (Beja)	Luvisol (Elvas V.B.)	Vertic Luvisol [1] (Évora)	Lepto-/Arenosol [1] (Mitra)
M1	safflower dm [g]	**0.94** ± 0.19 b	**0.30** ± 0.05 b	**1.17** ± 0.34 b	**1.35** ± 0.17 b
M0	safflower dm [g]	**1.67** ± 0.14 a	**0.84** ± 0.11 a	**1.79** ± 0.10 a	**1.81** ± 0.08 a
M1	chickpea dm [g]	**0.93** ± 0.10 a	**0.60** ± 0.02 a	**0.63** ± 0.08 a	**0.84** ± 0.14 a
M0	chickpea dm [g]	**1.11** ± 0.15 a	**0.61** ± 0.08 a	**0.52** ± 0.06 b	**0.82** ± 0.11 a

3.1.3.3 Methodological reflections

The exact MPN values can be calculated by computer programs and, consequently, approximation with tables or even graphs are no longer reliable, although still in practice (JARSTFER & SYLVIA, 1997).

However, the following statistical and biological assumptions are critical for applying MPN tests in mycorrhiza research. Soil microorganisms are usually not *normally distributed,*

which is one of two principal assumptions for the MPN estimation (COCHRAN, 1950). Soil cannot be perfectly homogenized (STRIBLEY, 1987) like *real* microbiological media, i.e. agar. Nevertheless, the concentration of propagules affects AMF infection (WILSON & TOMMERUP, 1992) and may be crucial for the mycorrhizal colonization process under field conditions in soils of different degrees of perturbation. In this context, the other principal assumption demanded by COCHRAN (1950) may be violated: The growth of the microorganism – or in application for AM – the development of intraradical fungal hyphae has to be initiated by not more than one single propagule. Another violation derives from possible interaction of the culture medium *soil* (ADELMAN & MORTON, 1985) with the infection process. Independently of the number of contained propagules, high phosphorus soil concentration can suppress mycorrhization. However, the growth medium itself is not objective of the MPN test. Optimum conditions such as (highly mycotrophic) host plants, temperature, irrigation and duration of the assay are demanded. Nevertheless, not everything optimal for the fungus, which prefers moderately low P availability and moderate water content in soils, is inevitably optimal for the host plant, too. When the host plant is regarded as part of the *growing medium* for the tested microorganism AMF, some conceptual confusion arises from what the optimal conditions are and how to determine them. For example, when root growth is limited by an AM promoting factor, the probability of an infection event may be restricted.

PORTER (1979) was sensitive in some respect to the methodological problems and admitted that the fulfilling of the necessary assumption could not be stated from his own experiment. However, convincing proof of the accuracy of the MPN procedure has yet to be presented, although it is regularly applied and recommended as a standard method. It would be easily done at least for viable and infective spores mixed as a known quantity into the soil from which the MPN could be estimated. Besides, such an accuracy check would be worthwhile for the recovery rate of spore extraction, too. “Inasmuch as the probability of inhibitions cannot easily be predicted...” ALEXANDER (1982) already recommended “...that preliminary counts be made to determine the recovery of known population added to soil in varying population densities”.

Generally, it is much more difficult (and time consuming) to detect an AMF infection even in a subsample of roots, than a bacterial colonization in a petri dish where *yes* or *no* is unambiguous. However, the “transformation of the medium or the microorganism itself, after it has undergone multiplication, must be easily recognizable in the substrate” (ALEXANDER,

1982). Theoretically, all roots must be examined (JARSTFER & SYLVIA, 1997) till at least one AM structure can be verified or not. That implies another, rather optimistic technical assumption that all living fine roots are recovered quantitatively and were stained properly.

An experimental mistake we made in this context was to use the entire pot volume as a calculation reference, although the upper parts were not rooted. It is better to fill an inoculation layer in the center of the pot. Consequently, the results obtained imply an unknown underestimation. Another methodological problem was that fine roots of chickpea tended to form a darkly stained central cylinder, complicating the detection of mycorrhizal structures which were often formed in the adjacent parenchyma (own observations; SMITH & READ, 1997).

The MPN test for mycorrhiza unfortunately combines qualitative errors with quantitative results. There is a high risk of underestimation caused by the *human factor* of missing colonized root pieces, additionally amplified by the fact that some mycorrhiza may not been stained (AN et al., 1990; MORTON, 1985). The application of the MPN test for individual species as done by AN and co-workers (1990) could be even more error prone by combining the disadvantages of both methods.

Biologically, assumptions for the MPN test were impaired by disregarding species in dormancy (TOMMERUP, 1983a). In addition, growing conditions of the host plant such as duration in particular and, to a lesser extent, temperature influence the number of estimated infective propagules (WILSON & TRINICK, 1982). The observed differences between safflower and chickpea with respect to AMF infection confirm the earlier observation of host specificity in AMF (ADELMAN & MORTON, 1986). Caused by thoroughly mixing, only *robust* infective propagules were estimated, consequently disregarding the infection potential of the extraradical mycelium in less disturbed soils.

The bioassay may be of limited value in such small pots. However, it is indispensable to control contamination or insufficient (soil) sterilization. Papers on MPN often do not refer to the calculation method, either to COCHRAN (1950) or to FISHER and YATES (1970). However, means can differ enormously as shown in the model calculation Fig. 3 (section 3.1.1) and, consequently, so do confidence limits.

Unfortunately, there is no agreement about what a *high* or a *low* number of propagules is, which conflicts with the intended idea of creating comparable units. For example, SIEVERDING (1991) defined a high density as in excess of 20 infective propagules per g dry soil, whereas 37 was a low (control) level compared to a 1000-fold value assumed as high by

HARINIKUMAR and BAGYARAJ (1996). LOVATO (1994) indicated a good infectiousness for a natural AMF potential at 0.44 and a highly infective inoculum at 6.60 infective propagules per g dry soil. Generally unacceptably high are the errors represented as a confidence level of 95 %.

3.1.3.4 Conclusion

In evaluation of all those circumstances, the MPN test cannot be recommended for the investigation of arbuscular mycorrhiza, although leading mycorrhizologists such as SMITH and READ (1997) or JARSTFER and SYLVIA (1997) do so. Researchers of INRA Dijon (per. comm. TROUVELOT, 1994) use *Trifolium repens* (three plants per 50 ml pot) which may be a little more reliable at least for a rough estimation of mycorrhizal infectivity than our own experimental approach. However, the quality of results which is determined by the conventional 95 % confidence limit is not proportional to the quantity of experimental input: the MPN test is particularly characterized by its labor-intensity (AN et al., 1990).

Mycorrhiza papers on MPN tend to suppress confidence limits of most probable numbers obtained (e.g. AN et al., 1990; HARINIKUMAR & BAGYARAJ, 1996; MOHAMMAD et al., 2000) and treat them incorrectly as means in ANOVA post-hoc tests. Error propagation has a strong impact on such probability calculations, but is not considered. Consequently, such MPN results may be better called “EPN” (error propagated numbers). The only way to treat MPN by ANOVA is to process the respective raw data of infected and not infected pots.

It is very useful to include sterile controls in the test set up (PORTER, 1979; WILSON & TRINICK, 1982; JARSTFER & SYLVIA, 1997). The reduction of the very high errors may be possible by using fewer steps of dilution and, conversely, more replication (ALEXANDER, 1982; RIDOUT, 1994; JARSTFER & SYLVIA, 1997). But better, MPN tests should be simply abandoned for mycorrhiza research and alternative standardized bioassays developed to replace them.

3.2 Mycorrhizal response of safflower in an open-air soil monolith trial at continuous water supply and temporal drought stress (SAF 94)

3.2.1 Introduction

An improvement of bioassays comparing non-mycorrhizal plants growing in heated soil with mycorrhizal plants growing in non-heated soil was needed. Resembling field conditions, a bioassay in large soil monoliths under natural light and temperature regime was carried out. The inoculum of previously tested local AMF was investigated on well watered and drought stressed plants compared to a heated inoculum as a control. The irrigation was interrupted 60 days after emergence (DAE) to impose water deficit during generative growth. The treatment was chosen to simulate the most limiting factor of Mediterranean dryland farming. In contrast, well watered plants were irrigated up to grain-filling.

The aim of this experiment was to examine the impact of arbuscular mycorrhiza on growth and water relations of safflower as affected by drought stress. Mycorrhiza may improve yields and drought stress resistance (see section 1.5.5 and 1.9).

3.2.2 Materials and methods

The experimental layout was arranged as a 2-factorial complete randomized block design with five replicates. The experimental factors were (1) AMF inoculation and (2) exposure to temporal drought stress in contrast to optimal water supply. The trial was carried out open-air (Tab. 6) at the "Herdade Experimental da Mitra", University of Évora. The site was protected by 1.2 m high walls. Rainfall was excluded by a shelter roof placed 80 cm above ground in order to allow optimal air circulation. PVC tubes (*model* ECO-PLAS PVC -10- ∅ 315 0.4 MPa, SANIDIN, Portugal), 35 cm in height with 30 cm inner diameter which retained 20 l soil monoliths from the field site Évora were used as containers. The lower edges of the tubes were sharpened to improve soil penetration. These cylinders

Tab. 6: Climate conditions during experiment SAF 94
Data as daily average from station MITRA (courtesy Dept. of Geophysics, University of Évora) based on the first 90 DAE of growing period; PAR photosynthetically active radiation)

Air temperature [°C]			Relative humidity	Light
mean	max	min	[%]	[E m^{-2} d^{-1} PAR]
18.7	26.7	11.6	60.8	46

were placed on 7 cm supports above a 40 cm ∅ dish to allow unrestricted soil water percolation. A nylon net was tightened at the bottom in order to fix the monolith in the tube.

The soil substrate was treated as follows: Monoliths from site Évora were collected randomly two weeks after soil tillage by chisel in September, 1993. The soil, a Vertic Luvisol (details see in section 2.1, A-Tab. 9 and A-Tab. 18), was heated for 43 h at 100 °C. At least 80 °C were reached for 24 h in the inner soil core and AMF exclusion was successfully obtained, as a previous test had shown. After heating, first 13 cm of top soil were replaced by a 3 cm inoculation layer with soil from Mitra *Montado* site (see section 2.1) which was covered with 5 cm of the above mentioned homogenized heated top soil of the soil monoliths. Non-mycorrhizal (nm) control treatments received the same inoculation soil, but heated for 24 h at 100 °C.

The soil was carefully water saturated with demineralized water for 3 days from both cylinder openings. Subsequently, cylinders were placed on supports and the soil water was allowed to drain for 24 h. The soil moisture of those monoliths was estimated at **30** ± 3.5 % (v/v). For further calculation this mean was defined as water holding capacity (WHC). The field capacity (pF 1.8) of this soil had been estimated at about 28 % (v/v) (courtesy SOMMER).

The soil inoculum was free from nematodes and showed comparably high mycorrhizal infection in first bioassays. For estimation of infectious propagules, an MPN test with chickpea and safflower as host plants was carried out in a climate chamber (see section 2.2). Soil microorganism were reestablished in all cylinders with filtered soil suspension which was obtained from a vacuum paper filtration (Whatman No. 4) at 4 µm. The experiment was originally carried out with *Cicer arietinum* L. (chickpea winter form, var. ELVAR). However, the first experiment failed: an AMF contamination was detected after 65 days in 3 of 10 controls. Consequently, that experiment was stopped and shoots were harvested. Shoot dry matter was still small (mycorrhizal **0.68** ± 0.29, non-mycorrhizal **0.73** ± 0.15 g, N=10) and not affected by inoculation treatment. It is suspected that the removal of infective propagules from the inoculum soil (ca. 15 liters of air dried soil) by heating for 24 h at 100 °C was incomplete: Similarly looking fine glomalean endophytes were found also in contaminated control pots with heated soil of the MPN experiment (see section 3.1.3). The monoliths of the non-mycorrhizal control were heated again for 43 h and the experiment was repeated with the *Compositae* safflower (*Carthamus tinctorius* L. var. WARAMIN) as a spring crop. The

variety WARAMIN from Iran is almost spineless and performed an intermediate production in previous field trials of EGER (CARVALHO, 1996).

In the repeated experiment, the infective propagules of the mycorrhizal treatment derived not only from the inoculation soil layer as in the original experimental set up. Additionally, colonization may have been spread out from previous infected chickpea roots, AMF hyphae, and possibly newly formed AMF spores. All cylinders were again reestablished for soil micro-flora (400 ml paper filtered - ∅ 4 µm - suspension each of ca. 20 liters of untreated soil). This procedure may have implied some (unknown) fertilization. The soil monoliths were carefully wetted again for several days from both openings.

Safflower was sown (8 seeds per cylinder) on 7 April. On 27 April or 12 days after emergence (DAE), respectively, seedlings were thinned to three. Root material was selected from each cylinder and stained for AMF infection. At 43 DAE, this procedure was repeated and one plant was left. Leaf area, length and width was determined with a CI-202 AREA METER (CID Inc., USA), and biomass and plant height of harvested plants were measured.

During the first 20 days, cylinders were covered with transparent plastic sheets to avoid AM contamination from aerosols, to reduce soil evaporation and to accelerate emergence. Subsequently, the experiment was protected by the above mentioned transportable shelter roof until the time of transpiration measurement.

From sowing to transpiration measurement, each cylinder was irrigated in total with 5 l demineralized water. Irrigation was applied every 2-4 days, depending on weather conditions.

The soil water content was maintained at 24 % (v/v) corresponding to 80 % of WHC during irrigation, which was about 86 % of field capacity. After verifying the absence of AM structures in non-mycorrhizal and, conversely, the establishment of colonization in mycorrhizal treatment, all cylinders were closed to block soil evaporation. For this purpose, tops were covered with 1 cm polystyrene and additionally sealed with paraffin. Only small holes for the shoot and an irrigation tube were left. The outside surface and underside of the cylinders were wrapped in white PVC sheets and taped tightly. They were put upon 3 cm thick polystyrene sheets for heat insulation and mechanical protection. Flexible PVC tubes (80 cm length, 0.5 cm ∅) were perforated with a hot needle (100 holes) and implanted as an irrigation system in a sand layer around the plant stem at 1-2 cm depth. The end was soldered together and the top closed with a cork stopper.

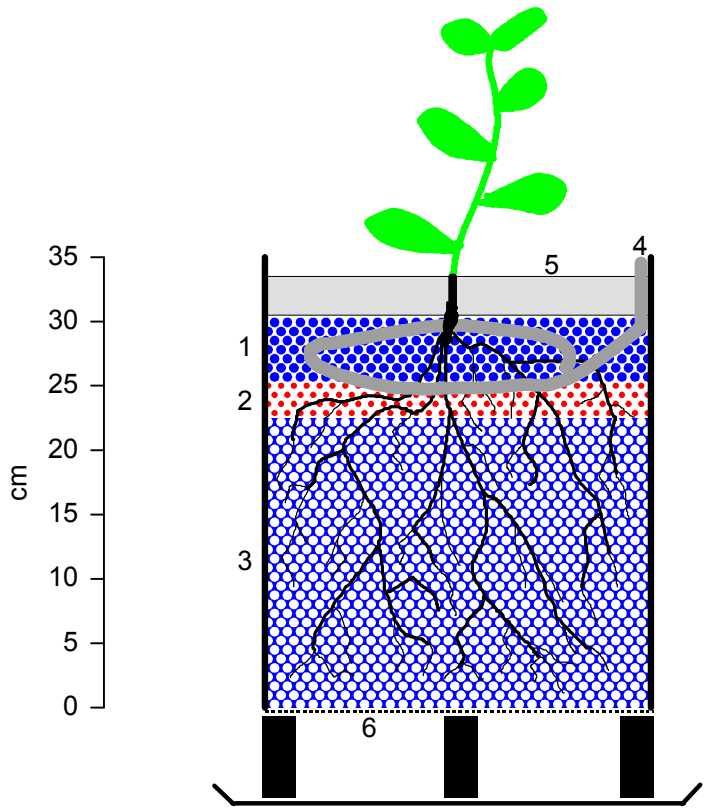


Fig. 4: Constitution of the soil monolith container system in trial SAF 94 (lateral cut)
(1) tilled and homogenized top soil, (2) inoculation soil, (3) soil monolith, (4) irrigation tube, (5) polystyrene sheet, (6) nylon net. Before transpiration measurement (4) and (5) were added and supports with dish were removed.

Irrigation was stopped to induce drought stress at 60 DAE with the exception of a single application of 500 ml at 75 DAE. The soil water content during the drought treatment started at **80** ± 10 % (w/w) WHC and was lowered to **35** ± 5 % (w/w) WHC at harvest. In well watered monoliths **50** ± 7 % (w/w) WHC was determined at maturity two weeks after last irrigation.

Transpiration loss of well watered plants was replenished up to 105 DAE when plants had already flowered for two weeks. All cylinders were first weighed daily at 8 h local time one by one on a digital balance (OHAUS I 10, USA) with an accuracy of ± 2 g. Then, around two hours later, well watered treatments were irrigated with demineralized water at the previously determined rate. Weighing and irrigation routine began each day with another randomly chosen plant.

Evaporation water loss of the irrigated container system was estimated after shoot harvest on a hot day in August. The cut shoot stem was sealed with Parafilm®. A negligible mean loss of 1.7 % of the applied amount of water was determined. At this day, soil temperature was also measured in one monolith, exemplarily. A maximum of 33 °C was reached in the most inner part.

Leaf transpiration respectively conductivity of leaves were measured by a porometer *model* AP4 (DELTA T DEVICES, England) 77 DAE at 6 h local time as an alternative approach. Results (n = 3 leaves by 3 measurement replications, data not shown) had the same range as the weighing method, however, at a much higher variation. Consequently,

measurement of transpiration was continued only by weighing. Stomatal resistance was analyzed by a ^{13}C discrimination technique with plant straw material of final harvest (see section 2.4).

Phenological state such as plant height, total shoot length (TSL) and number of leaves were noted 12 and 21 DAE. Additionally to these measurements, width and length of all leaves were taken on 43, 51, 55, 61, 66 and 81 DAE with a ruler and leaf area was calculated according to the correlation of previously scanned leaves from first harvest (parameters see Fig. 7: in the following section). Numbers of flowers were observed daily during the blooming period.

Plant fresh weight was calculated using two regressions. The first was between leaf area and biomass at 43 DAE (parameters see Fig. 7 in the following section). Additionally, a correlation was obtained between total shoot length (TSL) and fresh weight (FW) of safflower plants harvested on 22 June from a project field trial (Vertic Luvisol, Évora) at blooming:
FW (g) = 1.127 + 0.2121 TSL (cm); (n=10, r = 0.96)

Because the increment of plant fresh weight during the running experiment could not be determined destructively, a non-destructive estimation was calculated using the equation above for plants higher in FW than in the regression equation of Fig. 7. Transpiration loss calculation was corrected daily accordingly those plant fresh weights estimations by linear interpolation.

Shoots of mature plants were harvested at least 112 DAE and separated for leaves, grains, petals, remaining flower and remaining shoot.

It was necessary to asses herbivore loss because of damage caused by a weevil (*Curculionidae*). The assessment for each flower followed three criteria: (1) Total loss in two cases when the entire flower was cut off. (2) Partly grain loss was estimated on a scale from 1 to 4. (3) Grain damage of the single grains, which caused lower thousand grain weight, were assessed on a three-point scale. The correction was always based on the average yield of unaffected flowers from the respective treatment.

Soil monoliths were soaked overnight and subsequently main root systems were pulled out carefully. The original intention of washing the remaining roots on 2.5 and 1 mm sieves was rejected by recognizing in the first sample that quantitative fine root washing was not reliable. The advanced senescence in combination with the large soil volume and relatively high amounts of organic matter did not justify the enormous effort to determine this error

prone, although important feature. Furthermore, large differences in shoot growth suggested similar differences for root length.

The entire soil from monoliths was caught in large plastic tubs and air dried for 10 d, then weighed and homogenized. Triplicate sub-samples of each monolith were dried to constant weight at 105°C. The mass of dry soil and bulk density were calculated under consideration of residual soil water content. Bulk density of dry soil was determined with **1.6** ± 0.05 g cm^{-3}. Climate data (Fig. 5) were obtained from the Dept. of Geophysics UE for station Mitra which was only a few hundred meters from the experimental site.

The saturation deficit (Δ e) was calculated from relative air humidity (h) and air temperature. By computing saturation water vapor (e_s) of Smithsonian Meteorological Table No. 94 (LIST, 1968) against temperature, the following equation was obtained:
$e_s = a + e^{(b+c\ T)}$, where e_s is expressed in (hPa) and T in (°C). The constants a, b and c are -7.15998468, 2.46870586 and 0.04808163, respectively. The average error between calculated and tabulated values from 14 to 41°C in intervals of 0.1 is less than 0.92 %. As relative humidity (h) is defined as the ratio of actual water vapor pressure in the atmosphere (e_a) to saturation water vapor (e_s), it follows that: $e_a = e_s \cdot h$ and saturation deficit $\Delta\ e = e_s - e_a$.

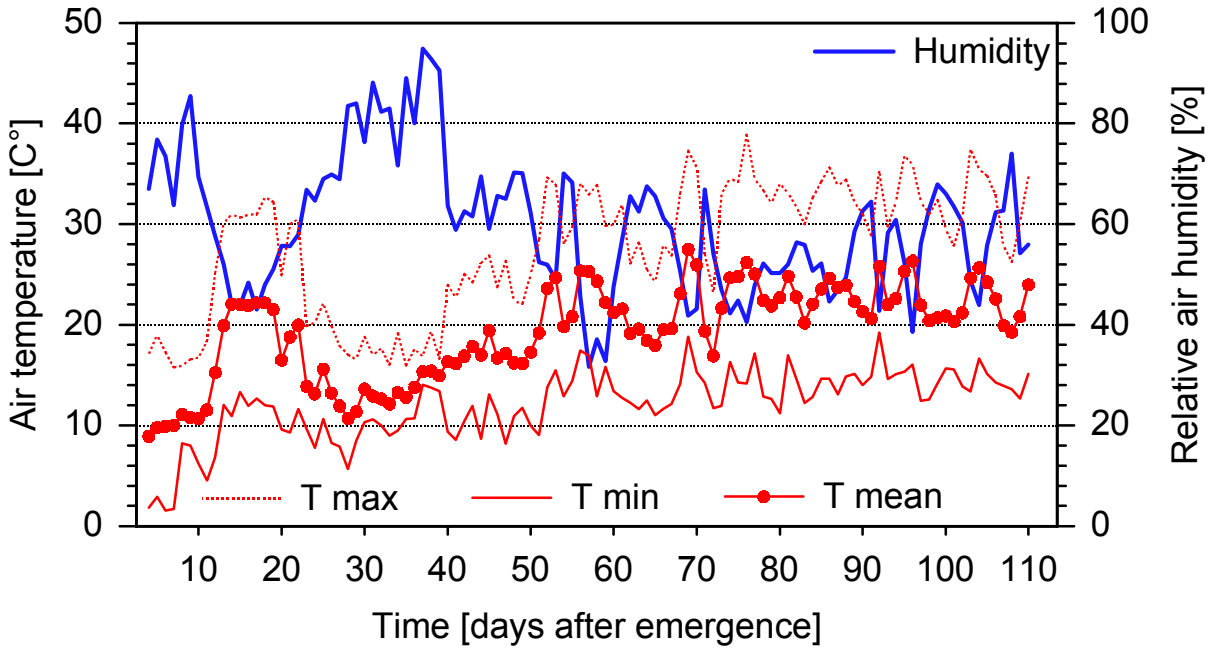


Fig. 5: Climate conditions in the time-course of safflower monolith trial SAF 94

3.2.3 Results

3.2.3.1 First harvest

Fresh weight increment of plants in the course of the experiment was needed to correct the net weight of planted pots in the calculation of transpiration. Therefore, correlation between leaf properties and fresh weight from plants of first harvest at 43 DAE were used. Fig. 6 and Fig. 7: show good correlation for the non destructive estimation of the leaf area and fresh weight.

The biomass of plants did not differ when transpiration measurements were started (Fig. 6). However, thinning led to high variation in 1st harvest because (mostly upper) extremes were taken to gain maximal equality. In contrast to biomass, shoot height was significantly higher without mycorrhiza until 43 DAE.

3.2.3.2 Dynamic of shooting and leaf growth

Mycorrhiza improved leaf growth significantly (Tab. 7 and Fig. 8b) in contrast to plant height (Fig. 8a) up to 66 DAE, only the irrigated mycorrhizal plants were significantly taller than all others at maturity.

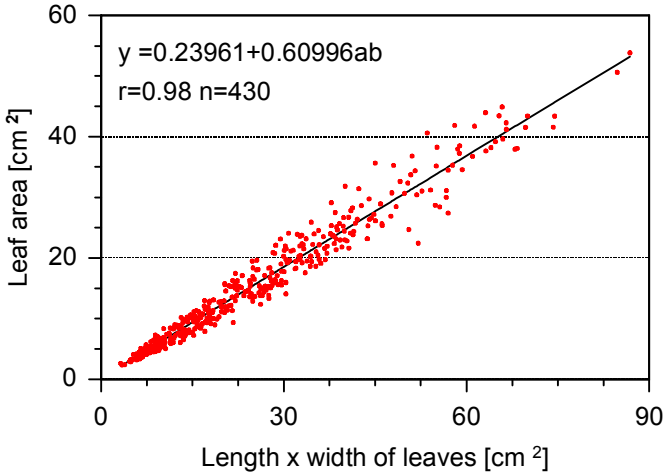


Fig. 6: Non-destructive leaf area estimation of safflower from length **a** and width **b** of leaves by linear regression

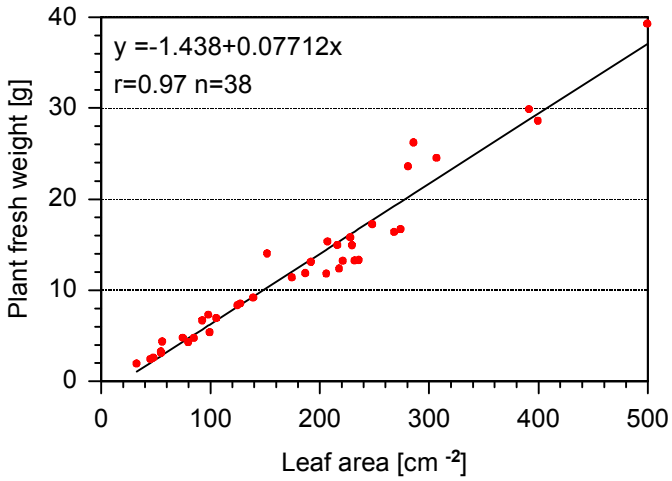


Fig. 7: Non-destructive biomass estimation of saf flower from leaf area by linear regression

Tab. 7: Growth of safflower plants at 43 DAE in monolith trial SAF 94

Treatments are mycorrhizal (**M1**) and non-mycorrhizal (**M0**) Letters indicate significant differences of **means** ± SD according to U-test (n = 19).

Treatments	Shoot dm [g]	Leaf area [cm²]	Height [cm]
M1	**1.05** ± 0.62 **a**	**205** ± 102 **a**	**17.2**± 5.9 **b**
M0	**1.06** ± 0.90 **a**	**142** ± 79 **b**	**24.8**± 12.8 **a**

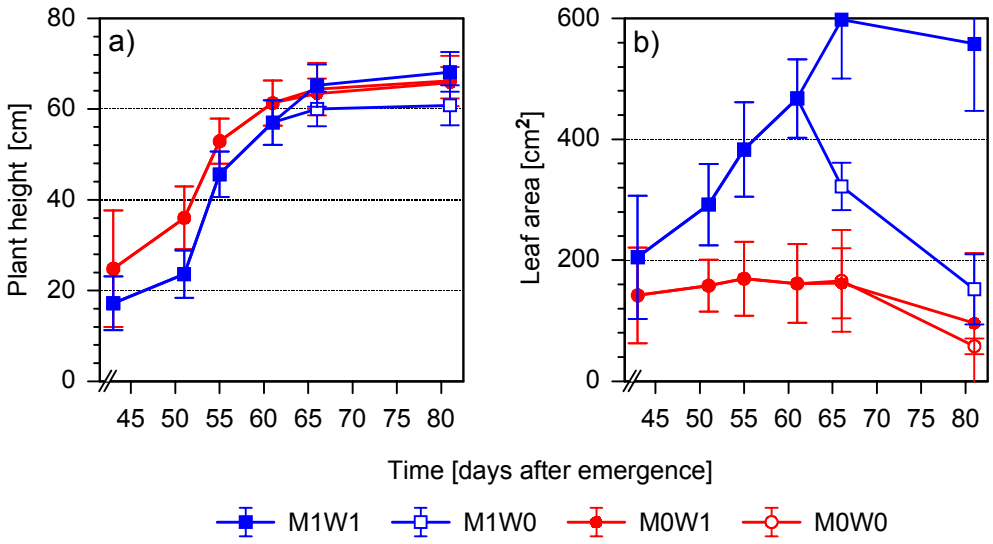


Fig. 8 Height and leaf growth of safflower plants in monolith trial SAF 94
Treatments are: **M1** with AMF, **M0** without AMF, **W1** well watered, **W0** irrigation stop at 60 DAE. Means of 5 replicates with SD indicated by error bars.

3.2.3.3 Yields, growth variables, water consumption and flowering

The plant response to AMF was expressed in a three- to fourfold increase of leaf area with irrigation. Exposed to drought stress during late vegetative growth, however, non-mycorrhizal safflower maintained constant leaf area as compared to well irrigated controls with AMF. In contrast, stressed mycorrhizal plants dramatically reduced the area of green leaves after 61 DAE. Irrigated, they still increased leaf area significantly up to 66 DAE (Fig. 8b).

Safflower growth was more improved by irrigation than by mycorrhization as indicated by higher F-values (Tab. 8). However, shoot dry matter of irrigated safflower was more than three times higher and had double the grain yield of those affected by AMF. Although grain yield and harvest index were reduced by weevils (*Curculionidae*), the harvest index was lowest in the M1W0 treatment.

Yield benefit by mycorrhiza was enlarged when irrigated in contrast to stressed plants. However, thousand grain weight was lower in the mycorrhizal treatment. Water supply during generative growth had a strong impact on production caused by a greater number of seeds. Pallet dry weight (see A-Tab. 4 in appendix) has an economic importance according to coloring properties, beside the oil content of seeds (REHM, 1989). Assimilating leaves were almost dried up at harvest. Therefore, leaf area from Fig. 8b better mirrors the assimilating conditions than leaf dry matter in Tab. 8. The double value of leaf-to-shoot ratio in M1W0

treatment (A-Tab. 4) indicates a kind of "unbalanced" amount of leaves for that growing condition. The total cumulative shoot length of plants under water stress was significantly longer when mycorrhizal.

Irrigated non-mycorrhizal plants mostly transpired more strongly especially in peaks of high saturation deficits than mycorrhizal (Fig. 9). The average transpiration over all treatments and measuring days was **55** ± 27 mmol cm^{-2} d^{-1} at about 15 h day light with an average irradiance of 949 µE m^{-2} s^{-1} PAR between 6 and 21 h (GMT) or about 10 mmol m^{-2} s^{-1}.
Transpiration rate in M1W0 treatment was decreasing with time (Fig. 9). The extraordinary irrigation of 500 ml did not change transpiration in contrast to the M0W0 treatment. Drought stressed mycorrhizal plants produced significantly more effective shoot biomass per unit of water. Drought stress corresponded to a significantly higher stomatal resistance measured with the ^{13}C discrimination technique as higher $\delta^{13}C$ in straw tissue (see A-Tab. 5 in appendix). However, no significant treatment effect were found for water use efficiency based on grain yield.

Tab. 8: Growth and water consumption of safflower plants in monolith trial SAF 94
Experimental factors are **M1** with AMF, **M0** without AMF, **W1** well watered, **W0** irrigation stopped at 60 DAE. All dry matter (dm), length and transpiration data are per plant. TGW is thousand grain weight. Different letters indicate significant differences (Tukey's HSD). Grain yield related data were corrected by assessment for herbivore loss. Significance of 2-way ANOVA F-values is indicated by * (see section 2.6). For more detailed data, see A-Tab. 4.

Treat-ment	Shoot dm [g]	Grain dm [g]	TGW [g]	Harvest Index [%]	Leaf dm [g]	Cumulative shoot length [cm]	Transpi-ration [l]	Water use efficiency Shoot [g l^{-1}]	Grain [g l^{-1}]
M1W1	55 a	13.3 a	28 b	25 ab	7.1 a	345 a	23.9 a	2.2 bc	0.57 a
M0W1	17 b	6.5 b	44 a	35 a	1.9 c	122 bc	8.5 b	1.9 c	0.78 a
M1W0	15 b	2.8 c	25 b	17 b	4.0 b	192 b	4.0 c	3.1 a	0.70 a
M0W0	10 b	2.6 c	37 ab	26 ab	1.4 c	111 c	3.5 c	2.5 b	0.74 a
F-values of isolated and combined effects (degree of freedom: 1 effect, 16 error)									
M	59***	23***	16**	9*	79***	67***	52***	14**	n.s.
W	74***	91***	n.s.	7*	17**	20***	127***	37***	n.s.
M x W	35***	21***	n.s.	n.s.	9*	15**	46***	n.s.	n.s.

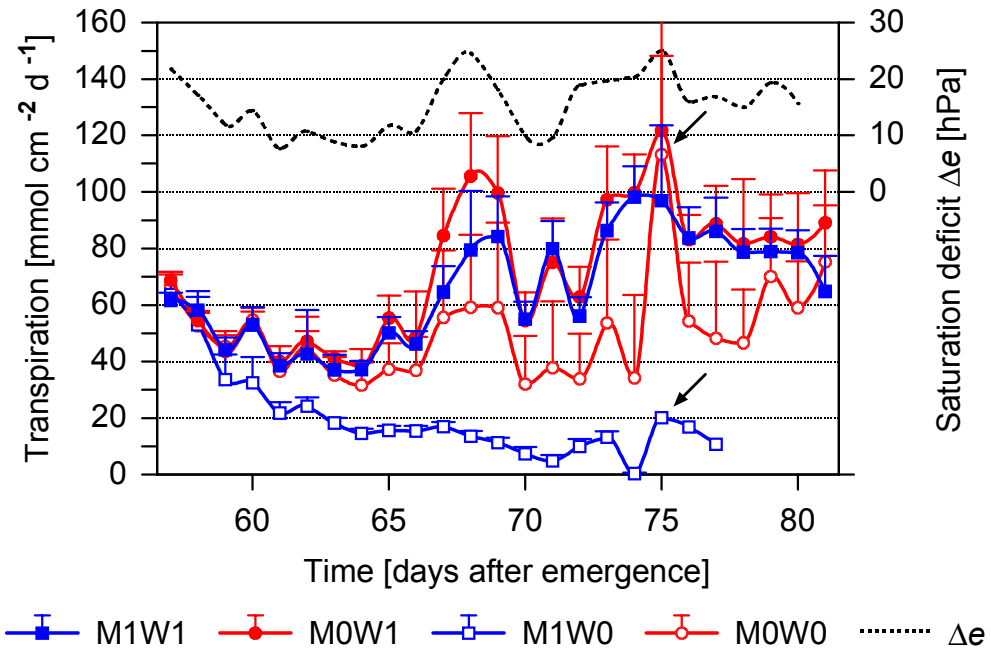


Fig. 9: Daily transpiration of safflower plants in monolith trial SAF 94
Treatments are: **M1** with AMF, **M0** without AMF,**W1** well watered,**W0** irrigation stop at 60 DAE. Arrows indicate extraordinary irrigation at 75 DAE for **W0** treatment. Means of 5 replicates with SD indicated by error bars

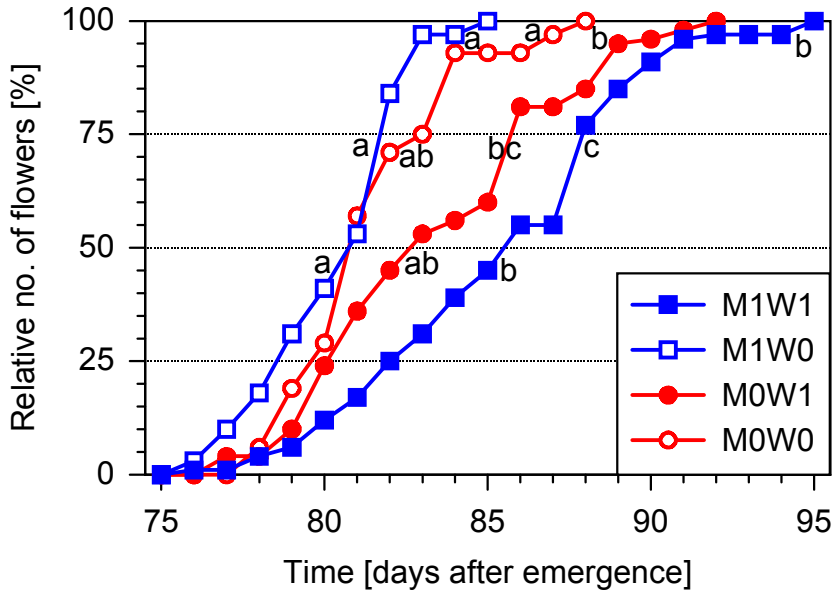


Fig. 10: Flowering of safflower plants in monolith trial SAF 94
Treatments are **M1** with AMF, **M0** without AMF, **W1** well watered, **W0** irrigation stop at 60 DAE. Different Letters show significant differences among treatments in days (Tukey's HSD) reaching 50, 75 and 100 % of total blooming.

Flowering was not influenced by AM but significantly retarded in the well watered treatment after half of all flowers were blooming. Before, differences were not significant. The absolute amount of flowers was by far the highest in the M1W1 treatment (see A-Tab. 4)

3.2.3.4 Mycorrhizal colonization

No AM was found at any of the sampling dates (43, 51, 112 DAE) in the non-mycorrhizal treatment. The inoculation treatment already established an AMF colonization at 12 DAE which was well developed at 43 DAE (Tab. 9). However, no growth differences between plants of the two AMF treatments were apparent at that time.

Tab. 9: AMF colonization of safflower (SAF 94) Trypan blue staining of a mixed root sample at 12 DAE and of 10 plants (**mean** ± SD) at 43 DAE. Frequencies of arbuscules (arb.) and vesicles (ves.) were classified to three levels.

	Sample day			
AMF colonization [%]	**12 DAE**		**43 DAE**	
	31		**72** ± 15	
Frequency [%] of	arb.	ves.	arb.	ves.
classified: low	**11**	**7**	**7**± 3	**4**± 2
medium	**12**	**7**	**24**± 6	**15**± 8
high	**7**	**2**	**34**±16	**19**±13

Safflower from MPN test (section 3.1, Tab. 3), growing in small cone pots filled with the soil, which was used as inoculum, was colonized by AMF at a rate of **84** ± 6 % at 54 DAE. At this time plants were flowering, a period in which often the plateau maximum of colonization is reached (SMITH & READ, 1997). Colonizing AMF seemed to be dominated by *Glomus* spec. and "fine glomalean endophytes".

3.2.3.5 Uptake and concentration of phosphorus

Compared to non-mycorrhizal plants, the P uptake of mycorrhizal plants was more than five times higher after thorough watering, and three times higher after drought stress (Fig. 11c). Also the P concentrations in straw tissue were significantly higher. (Fig. 11a). However, the P concentration was strikingly higher in the drought stressed mycorrhizal plants as compared to the other treatments (Fig. 11 b).

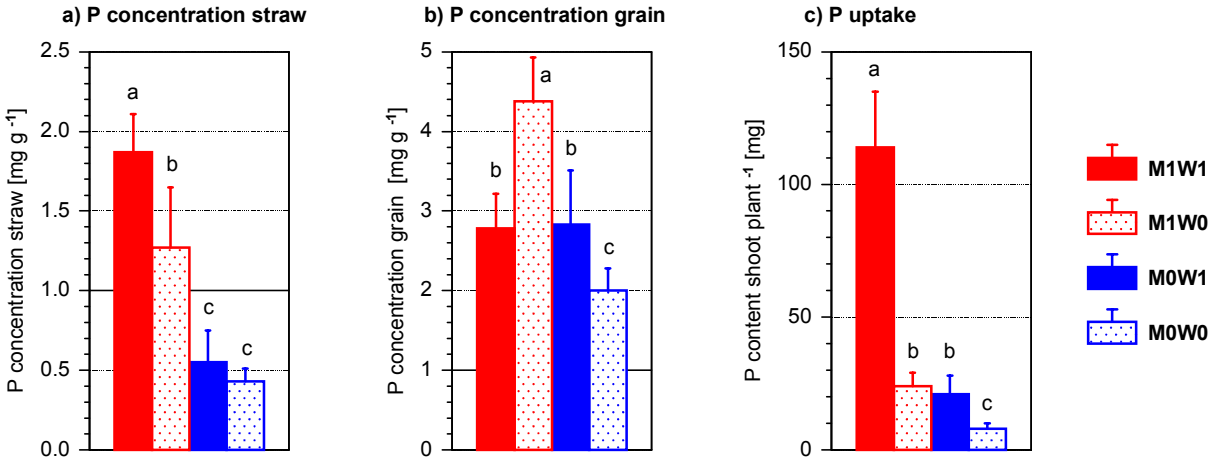


Fig. 11: P concentrations and uptake in safflower monolith trial (SAF 94)
Treatments are **M1** with AMF, **M0** without AMF, **W1** well watered, **W0** irrigation stop at 60 DAE. Different letters show significant differences among treatments. Means of 5 replicates with SD indicated by error bars (more details in A-Tab. 5)

3.2.4 Discussion

3.2.4.1 Growth, water relation, and P nutrition

The better vegetative growth of mycorrhizal plants did not result in better grain yield under water limited conditions. AM promoted plants with greater leaf area obviously consume more of the soil stored water. Thus, the mycorrhizal benefit must have been counterproductive at a certain threshold of water supply.

The water use efficiency (WUE) for biomass production was improved by AMF in the experiment presented. The values of water use efficiencies obtained were in the normal range for C3 plants as reported with 1.05 to 2.22 g dm l^{-1} by REHM (1989) whereas SIEVERDING (1980) found much higher water consumption from 0.08 to 1.25 g dm l^{-1} in greenhouse pot experiments .

Under the assumption that the most important AMF benefit is the enhanced P uptake (BOLAN, 1991), higher absolute transpiration should be considered as being caused by higher C demands from both symbiotic partners at a higher plant leaf area. The P benefit on positive water regulation for plants which is generally discussed as an important AMF effect (see section 1.5.5) will be over compensated when water becomes the absolute limiting factor on plant growth.

However, grain yield related WUE was not effected by AM. In contrast, the harvest index which is considered as the most important production variable under water restricted

conditions (PASSIOURA et al., 1993), was significantly reduced by AMF. Analyzing alleged "drought resistance" promoting components, PASSIOURA and co-workers emphasize the counterproductivity of too vigorous vegetative growth on harvest-index and call it "haying off" in the extreme case. Something similar happened in the experiment presented. WEBER (1992) draws a similar conclusion on chickpea dryland cropping in Syria for very infectious and, tested in pot bioassays, "effective" AMF. VETTERLEIN (1993) reported proportionality between growth response and water consumption on P fertilization in pearl millet. The faster shooting of mycorrhizal safflower may be induced by P deficiency as a kind of emergency maturity.

Probably, the balance between leaf area and available water supply during the growth period has some influence on effectiveness of AMF on crop yields in the Mediterranean regions which are strongly characterized or even defined by the highly varying water condition (see section 1.6; CARVALHO & BASCH, 1996). The chosen treatments approximately corresponded with 170 and 700 mm plant available precipitation minus soil evaporation for the first 77 days. The higher water demand of mycorrhizal safflower apparently did not cause grain yield depressionunder such extreme seasonal and absolute water conditions: the haying off syndrome was no more dramatic than the growth depression of the non-mycorrhizal plants. Independently of this, an enhanced drought resistance may have occured in mycorrhizal plants. Although theoretically possible, it could not be verified here, because such an effect would have been covered by the haying off syndrome.

3.2.4.2 Methods

Soil monolith gross weights of about 40 kg are at the limit of possible practical maintenance without technical support by a crane. An alternative could be to use logger systems (TDR, micro tensiometer) in microplot systems or lysimeters instead of manpower and weighing method. Finally, it might be useful to come back to classical pot experiments, regardless of all the restrictions discussed (see section 1.7.2).

A further problem is that reliable root quantification and mycorrhiza staining cannot be made from mature plants (see chapter 3.2.2). More replicates for an additional harvest would be necessary or the bioassay would have to be limited on vegetative growth, thereby disregarding the agronomicaly important aspect of final grain production. However, the mycorrhizal response may be delayed by parasitic properties of certain AMF in the early stage of the symbiosis (SMITH & READ, 1997).

Soil monoliths simulate field conditions in terms of undisturbed soil structure, but do not make it possible to overcome the problem of soil heterogeneity. Variation in bulk density and water content can still easily disturb the experiment. Soil temperature in cylinders was certainly higher than in field sites, despite some measures for insulation. It would be preferable to build the container system within the field or garden soil, as is done in micro plot experiments. Sealing the soil monolith may have some negative effects on root and AM respiration or development. However, AM was already developed well before the relatively short transpiration measurement period. Also root concentration was relatively small due to low plant density.

3.3 Short-time bioassay for AM with safflower in a climate chamber (SAF 95 a)

3.3.1 Introduction

In the preceding safflower experiment of 1994, an enhancement of leaf growth by AMF in the first weeks was noted. In contrast, shooting of non-mycorrhizal plants was faster (section 3.1.3.2, Tab. 7, Fig. 8). This developing pattern was studied in pots which provided sufficient rooting space for a relatively short growing period. However, the smaller containers made it possible to carry out the experiment in a climate chamber under better controlled conditions which also allowed the quantification of the root mass. The aim was to confirm that AM improves early leaf growth and retards shooting of safflower. Additionally, the root relations were to be investigated.

3.3.2 Materials and methods

Pre-sterilized soil, the Vertic Luvisol from Évora of safflower monolith experiment 1994 was autoclaved for 60 minutes at 121°C. Mycorrhizal treatment received 300 g of inoculum soil (see section 3.4.2) and 700 g sterilized soil, while non-mycorrhizal treatment received only 1 kg of the latter. A filtrate (∅ 4 µm) of 50 ml from about 125 g untreated soil was added to all pots. Climatic conditions were as reported in section 2.2. The four replicates of the two treatments were randomized, rotated and irrigated with demineralized water every two days. At 28 DAS or 21 DAE, the entire plants were carefully pulled out of the soil and separated for leaves, remaining shoot stem, and roots. Few remaining fine roots were quantitatively picked up by tweezers and all roots were washed from attached soil. The single leaves were scanned for leaf area measurement (CI-202 AREA METER; CID inc., USA). Dry matter of plant

components was determined at 70 °C. A small subsample of fine roots was stained with trypan blue and state of AMF colonization was proved (section 2.5.4).

3.3.3 Results

In the early growth stage, mycorrhiza induced leaf growth instead of stem growth in non-mycorrhizal safflower plants. Differences in leaf area and dry matter were not significant at $p<0.05$, but they were at $p<0.10$. More clearly, the significantly different ratio between stem and leaf biomass mirrored the favored leaf development in mycorrhizal plants. Shoot-root-ratio was significantly higher with mycorrhiza at almost equal total plant biomass (Tab. 10). AM was well developed in mycorrhizal roots (data not shown) and a small contamination was found in one isolated root from controls.

Tab. 10: Results from bioassay in climate chamber with safflower (SAF 95 a)
Treatment **M1** = mycorrhizal **M0** = non-mycorrhizal. P-level of t-test is **significant** (s.) when $p<0.05$ and not significant (n.s.) when $p>0.05$.

Means (n = 4)	**M1**	**M0**	P-level	**M1** vs. **M0**
Leaf area [cm²]	135	94	0.075	> n.s.
Stem length [cm]	2	11	**0.014**	< s.
Plant height [cm]	9	11	0.196	< n.s.
Shoot dm [g]	0.56	0.46	0.207	> n.s.
Root dm [g]	0.42	0.55	0.268	< n.s.
Shoot-to-root ratio	1.43	0.86	**0.038**	> s.
Plant dm [g]	0.98	1.01	0.873	≅ n.s.
Leaf dm [g]	0.48	0.32	0.086	> n.s.
Stem dm [g]	0.08	0.14	**0.043**	< s.
Leaf-to-stem ratio	6.7	2.7	**0.041**	> s.
Specific leaf area [cm² mg^{-1}]	0.298 *n=38*	0.313 *n=45*	0.131	< n.s.

3.4 Mycorrhizal response of safflower in an open air pot trial as affected by water supply and P fertilization (SAF 95)

3.4.1 Introduction

Higher P uptake is considered as the major beneficial AMF effect and was significantly enhanced in mycorrhizal safflower of the preceding experiment SAF 94 as compared to non-mycorrhizal. The benefit may consist not only of a growth response but also of an enhanced drought resistance at limited water supply. In order to separate the mycorrhizal effect from a nutritional effect, P fertilization was added as the third experimental factor. This subsequent experiment was carried out with large pots under a natural light and temperature regime. In

contrast to the experiment of 1994, the soil water contents were held constantly on a well watered and on a permanent drought stress level to allow drought acclimatization.

The purpose of the trial was to examine whether local AMF can improve the growth of safflower under water limited conditions independently of P benefits, under consideration of transpiration rates and water use efficiency (compare sections 1.5.5 and 1.9).

3.4.2 Materials and methods

The experiment was arranged in a three factorial complete randomized block design with four replicates. The treatments at two levels for each factor were:

(1) Mycorrhiza - **M1** with AMF inoculum, **M0** without AMF

(2) Phosphorus - **P1** with 50 µg g^{-1} P fertilization, **P0** no P fertilization

(3) Water - **W1** well watered at a soil water content of about 80 % water holding capacity
W0 drought stressed at a soil water content of about 40 % water holding capacity

Pots were located in the experimental garden "Mitra" of the University of Évora as in the experiment of the year before, with a natural light and temperature regime (Tab. 11). Rainfall was excluded when necessary by a transparent plastic roof which could be rolled up.

Pots of 7.3 l volume were filled with 8.3 kg soil from Évora (Vertic Luvisol). The unfertilized soil was sieved on a 4 mm screen, air dried and finally sterilized twice for 24 h at 120 °C in an oven with a 24 h cooling interval. Soil temperature reached 90 °C in the inner part of the (cleaned) oil barrel which was used as a soil container during heating.

An air dried AMF inoculum of about 17 % (w/w) of the pot content was added to the M1 treatment, which had been multiplied in the same soil substrate with maize plants. Hence, the AMF inoculum was of the same source (Mitra *Montado* site) as in 1994.

Tab. 11: Climate conditions during experiment SAF 95
Data as daily average from station MITRA (courtesy of the Dept. of Geophysics, University of Évora) based on the first 90 days of growing period; PAR photosynthetically active radiation.

Air temperature [°C]			Relative humidity	Light
mean	max	min	[%]	[E m^{-2} d^{-1} PAR]
20.5	29.1	13.3	57.5	38

The P1 treatments received 50 µg g^{-1} P as $NaH_2PO_4 \cdot H_2O$. The soil was mixed in an aseptic concrete-mixer. Soil microorganisms without AMF were reestablished in all pots with 50 ml paper filtered (∅ 4 µm, Whatman N° 4) soil suspension from the intercrop soil.

Surface sterilized seeds of safflower (*Carthamus tinctorius* L.) var. WARAMIN were selected for equal weight and pre-germinated at 30 °C on agar in petri dishes. In each pot four seedlings were planted on 27 April 1995.

At 18 days after planting (DAP), plant stands were thinned to one plant per pot. The roots of the harvested plants were stained with lactic trypan blue (Tab. 2, section 2.5.4) and plant dry matter was determined.

The leaf area of the remaining plants was estimated non-destructively (see section 3.2.2 and Fig. 7:) and the irrigation treatment was started. Pots were weighed, irrigated with demineralized water (error ± 1 ml) and rotated one position forward within blocks each day in the morning hours. The water holding capacity (WHC) was defined as the WHC of soil monoliths in experiment SAF 94 (see section 3.2.2). Accordingly, treatment W1 was moistened to a level of about 24 % and W0 to 12 % v/v soil water content, respectively. On hot days during generative growth, the well developed plants of the W1P1 treatments were additionally irrigated in the afternoon. Transpiration was usually determined in 24 hour intervals und was correlated with the air saturation deficit Δe. In a few cases, measurements differed from 24 hour interval and daily transpiration, and Δe was corrected accordingly under consideration of day length. Evaporation was reduced by covering the soil with 1 kg acid washed sterile sand (∅ 1-2 mm). The pots were wrapped in aluminum foil and placed on 3 cm thick polystyrene sheets for heat insulation.

Leaf area was estimated at 27, 33, 39, 50 DAP, when also plant height and the cumulative length of main axis and lateral branches were measured. The latter was repeated after 66 days. The occurrence of flowering was noted daily. Shortly before maturity was reached at 84 DAP, on average about two weeks after last flowering, plants were harvested in order to obtain intact fine roots. The soil was soaked overnight in tap water and roots were washed out. Fine roots were stored in an aqueous solution of (v/v) 5 % acetic acid and 5 % ethanol for subsequent mycorrhizal staining with lactic trypan blue.

Plants were separated into leaves, flowers, grains, stems plus branches, and coarse roots and were dried to constant weight at 70 °C. Final leaf area was measured before drying with a LiCor Li 3000 planimeter (LiCor, USA). The area of degenerated leaves was taken from the last non-destructive leaf area estimation and the values were corrected accordingly.

Plant transpiration in (mmol cm^{-2} d^{-1}) was calculated from water loss minus soil evaporation divided by linearly interpolated leaf area. Soil evaporation was estimated from bare control pots without plants. During the running experiment, an additional error in soil

evaporation measurement was detected. The delayed infiltration of 500 to 700 ml water which was applied in W1P1 treatments resulted in some direct evaporation immediately after irrigation. To estimate this effect, dishes of pot diameter were filled with 1 kg sand and were placed on the control pots. The dishes were irrigated with 500, 400, 300, 200 or 100 ml and water loss was monitored for 1 h during a hot day. The corresponding loss for W1P1 treatments was estimated at about 20 ml for the morning hours and at 80 ml for the afternoon. Transpiration values were corrected accordingly. Gross weight of pots was also corrected constantly during the experiment for linearly interpolated fresh weight increments (see section 3.2.2).

3.4.3 Results

3.4.3.1 First harvest

Thinning out 3 of 4 plants at 18 DAP was oriented towards obtaining equally developed single plants per pot (Tab. 12). However, the M1P1 treatment already was effective and therefore discriminated negatively while the M0P1 treatment was discriminated positively as indicated by the index in Tab. 12. Although the P0 treatment was positively discriminated, the unfertilized non-mycorrhizal plants were already significantly less developed when the irrigation treatment was started. However, differences in P content were not significant.

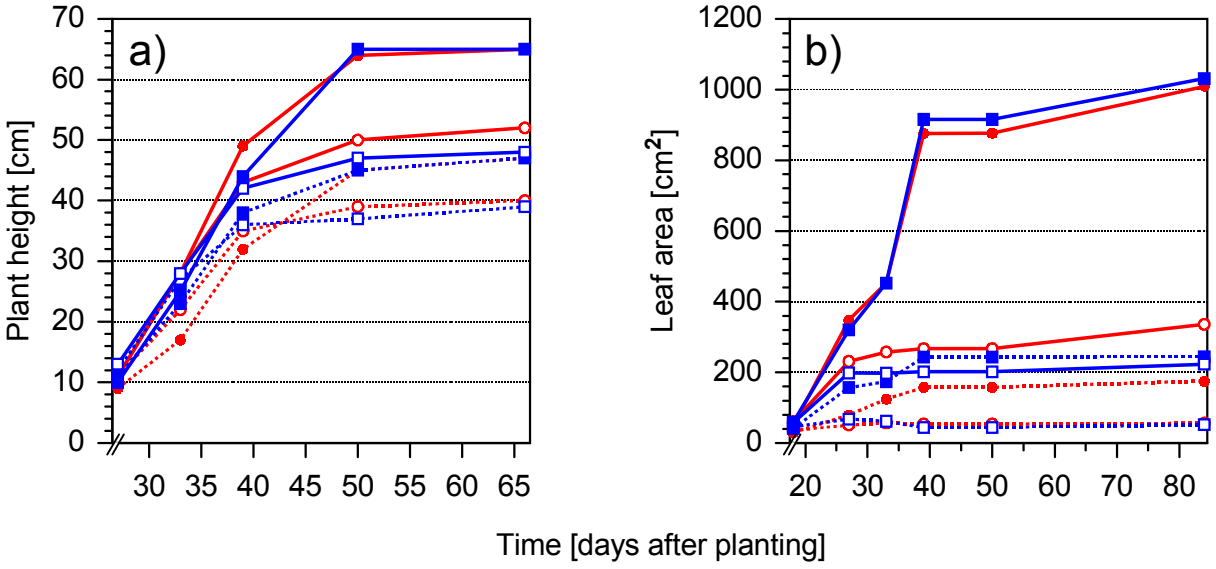
Tab. 12: Biomass, P uptake, and leaf area of safflower plant at 18 DAP (SAF 95)
Treatments are **M1** with AMF, **M0** without AMF, **P1** 50 µg g^{-1} P fertilization, **P0** no P fertilization. Biomass of harvested plants (n=24) was determined without fine roots. P concentrations were measured in mixed samples. Leaf area was estimated from plants (n=8) which carried on in the experiment. Different letters indicate significant differences (Tukey's HSD) of **means** ± SD. The index 100 represents the **M1P1** treatment.

Treatment	Plant dm [mg]	Index	P conc. [mg g-1]	P content [mg]	Leaf area [cm²]	Index
M1P1	**311** ± 108 **a**	100	**1.91**	**0.595**	**58** ± 14 **a**	100
M1P0	**154** ± 56 **b**	49	**3.74**	**0.576**	**43** ± 6 **ab**	74
M0P1	**162** ± 75 **b**	52	**2.09**	**0.339**	**54** ± 15 **a**	93
M0P0	**106** ± 25 **b**	34	**4.29**	**0.455**	**32** ± 5 **b**	55

3.4.3.2 Dynamic of shooting and leaf growth

	27d	33d	39d	50d	66d
M1P1W1	a	a	ab	a	a
M1P1W0	a	a	ab	bc	bc
M1P0W1	a	a	ab	bc	bc
M1P0W0	a	a	ab	c	c
M0P1W1	a	a	a	a	a
M0P1W0	a	a	ab	b	b
M0P0W1	a	a	b	bc	bc
M0P0W0	a	a	ab	bc	c

	18d	27d	33d	39d	50d	84d
M1P1W1	a	a	ab	a	a	a
M1P1W0	a	a	ab	bc	b	bc
M1P0W1	ab	ab	bc	bc	b	bc
M1P0W0	ab	a	d	d	c	d
M0P1W1	a	ab	a	a	a	a
M0P1W0	a	ab	ab	ab	b	b
M0P0W1	b	b	cd	cd	b	c
M0P0W0	b	ab	d	d	c	d



F-values of isolated and combined effects (degree of freedom: 1 effect, 24 error)

Factor	**Plant height** [cm]				**Leaf area** [cm²] (ln transformed)					
	33d	39d	50d	66d	18d	27d	33d	39d	50d	84d
M	n.s.	n.s.	n.s.	n.s.	n.s.	5*		n.s.	n.s.	n.s.
P	5*	16***	81***	73***	22***	25***	121***	136***	253***	331***
W	n.s.	n.s.	49***	45***	-	n.s.	23***	52***	189***	214***
M x W	n.s.	n.s.	7*	5*	-	n.s.	n.s.	n.s.	5*	5*

Fig. 12: Height and leaf growth of safflower plants in pot trial SAF 95
Treatments as in Tab. 14. Graphs are means of 4 replicates. Different letters beside the legend indicate significant differences (Tukey's HSD) of plant height (a) and leaf area (b, ln transformed) for respective days (d) after planting. Significant p-values of corresponding ANOVA F-values in the table were indicated by * ($p<0.05$) ** ($p<0.01$) *** ($p<0.001$) or n.s. when not significant. Other interactions and treatment effects were not significant.

Plant height was strongly enhanced by P fertilization. From 50 DAP onwards, height growth was also enhanced with water supply as indicated by high F-values. At this date, a slight interaction with the AMF inoculation was detected (Fig. 12 a).

The appearance of leaf development was similar. However, differences between the P1W1 and the other treatments were much more prominent. In contrast to stem growth, there was a slight positive impact by mycorrhiza on leaf growth at 27 DAP and the effect of water supply was much stronger as indicated by high F-values (Fig. 12 b).

3.4.3.3 Yields, growth variables, water consumption and flowering

At harvest, mycorrhiza had no effect on yield variables of plants under differential irrigation and P fertilization. The most productive treatments were with 60 g shoot dry matter the P1W1. In contrast, the P0W0 treatments with only 3 g dm achieved the lowest biomass production. The values were strikingly similar for both mycorrhizal and non-mycorrhizal plants (Tab. 13). However, the plants depended strongly on P and water supply. Phosphorus fertilization increased in particular yield variables such as harvest index, whereas irrigation especially increased leaf variables, shoot production and transpiration as indicated by larger F-values. Only a small interaction of mycorrhiza with irrigation can be detected. Mycorrhiza increased safflower growth after thorough watering without P, but showed a tendency to decrease drought stressed growth with P fertilization. The last differences were not significant due to high standard variation (A-Tab. 6). Shoot contents of P were especially high in **P1W1** treatments with high biomass production (A-Tab. 6).

An interaction between P fertilization and irrigation was determined for leaf dry matter, harvest index and water use efficiency.

The only variable with a significant mycorrhizal main effect was WUE for shoot production. Across all other treatments, mycorrhizal safflower produced 0.17 g dry matter per liter transpired water or 8.4 % more biomass than non-mycorrhizal. The pair wise comparison for mycorrhizal treatments in combination with both other experimental factors did not show a significantly higher transpiration of non-mycorrhizal safflower. (Tab. 13).

Tab. 13: Growth and water consumption of safflower plants in pot trial SAF 95
Treatments as in Tab. 14. Different letters indicate significant differences (Tukey's HSD). F-values were indicated as in Fig. 12. Variables marked ^{ln} were ln transformed. For quotient *grain WUE*, transformation was applied for both the nominator *grain dm* and denominator *transpiration*. For shoot related WUE, one replication in treatment **M1P1W0** with an extremely high value (4.04 g l^{-1}) was deleted and Tukey's HSD for unequal n (*Spjotvoll/Stoline*) performed. Shoot dry matter was corrected for WUE calculation by initial value (4.4 % of harvest weight in average) when transpiration measuring started at 18 DAP. More details including P uptakes see A-Tab. 6.

Treatment	Shoot dm	Grain dm	Leaf dm	Cumulative	Harvest	Transpi-	Water use efficiency	
	plant^{-1}	plant^{-1}	plant^{-1}	shoot length	Index	ration	Shoot	Grain
	[g] ^{ln}	[g] ^{ln}	[g]	[cm] ^{ln}	[%]	[l] ^{ln}	[g l^{-1}]	[g $^{l-1}$] $^{ln/ln}$
M1P1W1	59.8 **a**	23.7 **a**	8.85 **a**	393 **a**	40 **a**	27.5 **a**	2.16 **ab**	0.86 **a**
M1P1W0	12.3 **b**	3.4 **b**	2.49 **bc**	77 **b**	28 **b**	4.5 **b**	2.27 **a**	0.77 **b**
M1P0W1	15.8 **b**	6.5 **b**	2.70 **bc**	87 **b**	41 **a**	8.8 **b**	1.75 **cd**	0.73 **b**
M1P0W0	3.0 **c**	0.6 **c**	0.78 **d**	41 **c**	18 **c**	1.4 **c**	1.99 **abc**	0.38 **c**
M0P1W1	58.8 **a**	23.7 **a**	9.05 **a**	378 **a**	40 **a**	28.3 **a**	2.07 **abc**	0.84 **a**
M0P1W0	17.4 **b**	5.9 **b**	3.45 **b**	120 **b**	34 **ab**	9.1 **b**	1.87 **bcd**	0.65 **b**
M0P0W1	10.5 **b**	3.7 **b**	1.67 **bcd**	87 **b**	34 **ab**	6.5 **b**	1.60 **d**	0.55 **b**
M0P0W0	3.3 **c**	0.6 **c**	0.90 **cd**	46 **c**	17 **c**	1.7 **c**	1.95 **abc**	0.34 **c**
F-values of isolated and combined effects (degree of freedom: 1 effect, 24 error)								
M	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	11***	n.s.
P	213***	173***	250***	223***	25***	175***	29***	143***
W	163***	174***	170***	170***	77***	200***	6***	138***
P x W	n.s.	n.s.	68***	n.s.	13***	n.s.	11***	11***
M x W	n.s.	4***	n.s.	22***	5***	7***	n.s.	n.s.
M x W x P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	5***	n.s.

Transpiration rates, calculated per day and leaf area, followed in all treatments the saturation deficit (Fig. 13). Drought stress in W0 treatments did not result in lower transpiration rates but in faster leaf senescence in 3 of 4 treatments as shown by missing values in graphs after 74 DAE.

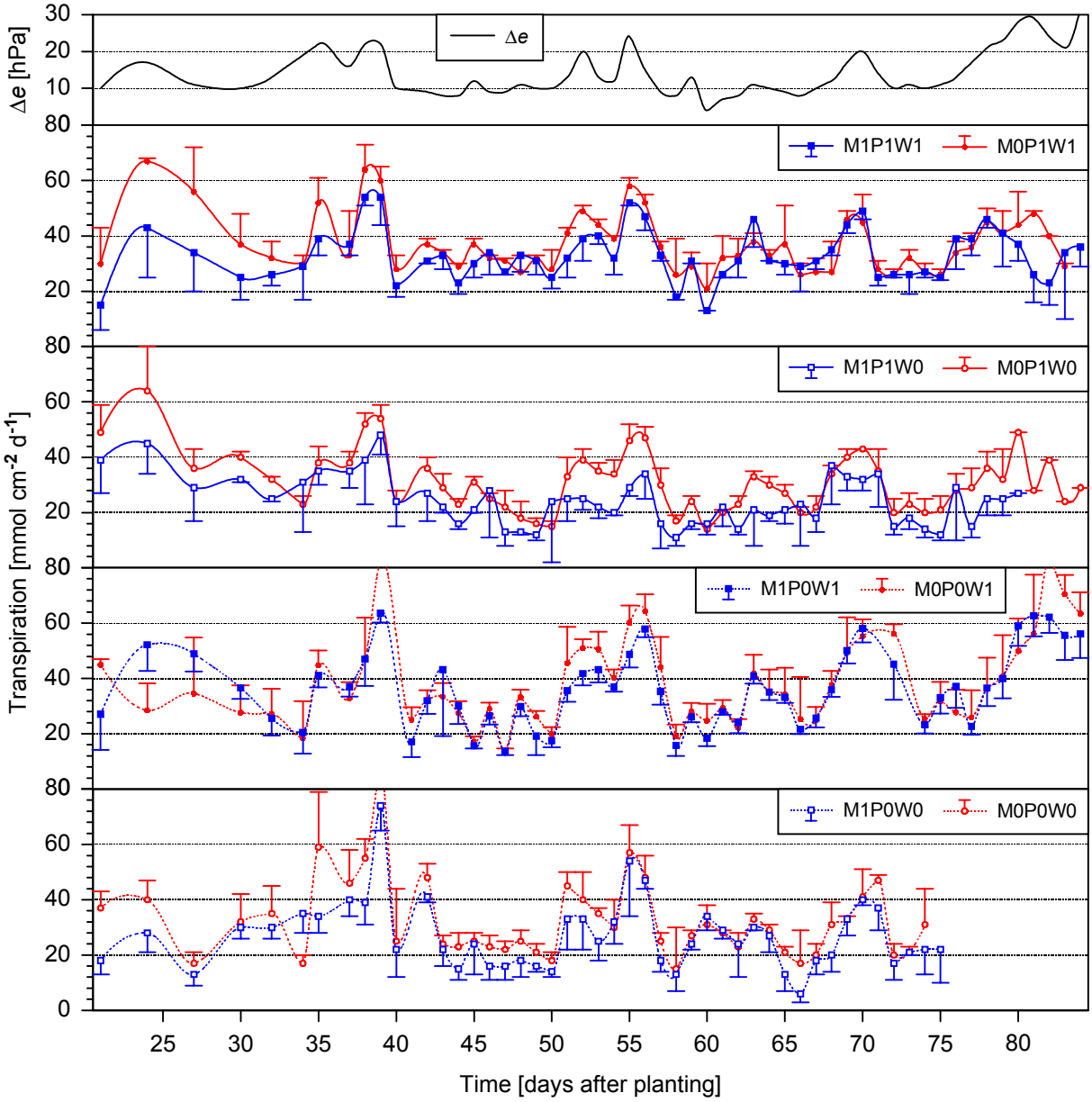


Fig. 13: Daily transpiration rates of safflower plants in pot trial SAF 95
Treatments are **M1** with AMF, **M0** without AMF, **P1** 50 µg g^{-1} P fertilization, **P0** no P fertilization, **W1** soil water content about 80 % of WHC, and **W0** soil water content about 40 % of WHC. Bars indicate standard deviation (down **M1**, up **M0** treatments)

The average transpiration over all treatments and measuring period was 32.5 ± 14.5 mmol cm^{-2} d^{-1} at about 15 h daylight with an average irradiance of 703 µE m^{-2} s^{-1} between 6 and 21 h (GMT) or about 6 mmol m^{-2} s^{-1}.

Flowering was earlier in all drought stressed treatments, whereas mycorrhiza had no significant effect (Fig. 14).

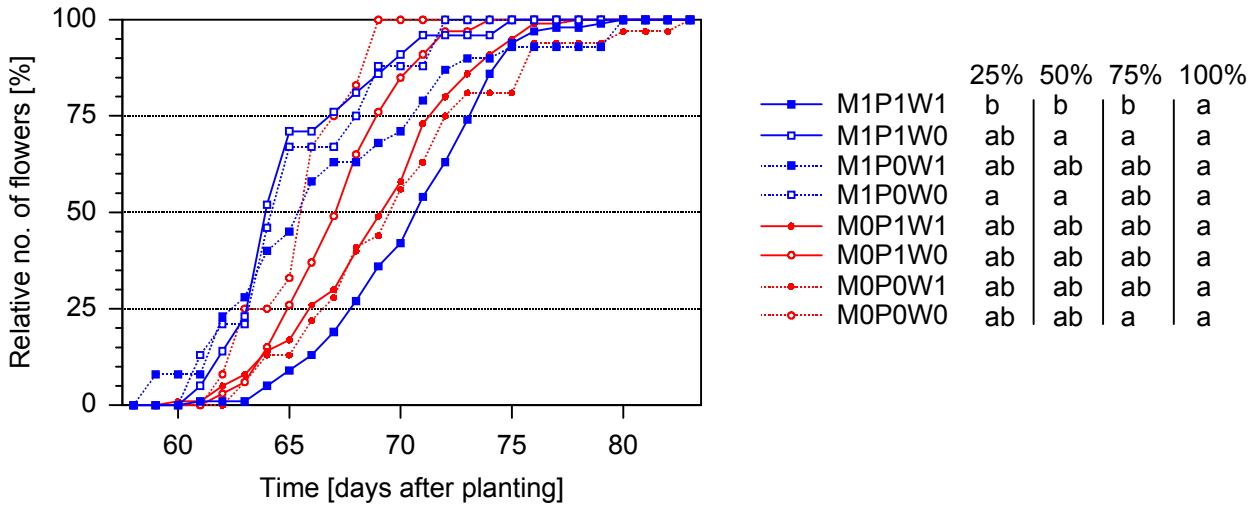


Fig. 14: Flowering in safflower pot trial SAF 95
Different letters show significant differences (Tukey's HSD) among treatments in days reaching 25, 50, 75, and 100 % of total blooming. Treatments as in Fig. 13. For absolute numbers of flowers, see A-Tab. 6

3.4.3.4 Mycorrhizal colonization

No AMF colonization was found in the M0 treatment at 18 and 84 DAP, only a single isolated infection was detected in one fine root subsample at final harvest. At 18 DAP, the AMF colonization of P fertilized plants was clearly less (Tab. 14) than of non-fertilized plants.

At 84 DAP, the P fertilization enhanced significantly the colonization in well watered plants whereas drought stressed plants tended to be less infected. Drought strongly suppressed AM as indicated by a high F-value. (Tab. 14).

Tab. 14: AMF colonization in safflower pot trial SAF 95
Treatments as in Fig. 13. **Means** ± SD before irrigation treatment (18 DAP) are of n=8 and at harvest (84 DAP) of n =4. Different letters indicate significant differences according to U-test (18 DAP) and Tukey's HSD (84 DAP). The corresponding significant F-values ($p<0.001$) of 2-way ANOVA (84 DAP) are 42 (**W**) and 14 (**P** x **W**).

Treatment	**AMF colonization** [%]	
	18 DAP	**84** DAP
M1P1	**6** ± 4 **b**	
M1P1W1		**81** ± 5 **a**
M1P1W0		**21** ± 11 **c**
M1P0	**36** ± 11 **a**	
M1P0W1		**55** ± 14 **b**
M1P0W0		**38** ± 15 **cb**

3.4.4 Discussion

3.4.4.1 P fertilization

The soil used was unambiguously highly P deficient as demonstrated by the enormous P effect. It cannot be excluded that some irregularities may have occurred in the fertilization procedure. The P was mixed as powdered

and dried solid $NaH_2PO_4 \cdot H_2O$ into the total amount of soil. Although a concrete-mixer was used for 15 minutes for thispurpose, the blending of 30 g fertilizer with 130 kg soil may not have been as successful as necessary. A refined method was used by BRYLA and DUNIWAY (1997a), carefully spraying nutrient solution into the soil while the concrete-mixer was operating

Biomass and inevitably roots were more developed in the P fertilized treatments. Enhanced absolute root growth (of taller plants) can also influence colonization rate due to a dilution effect (see section 1.5.2; ALLEN, 2001). Consequently, the prominent P effect on plant growth in combination with lower mycorrhizal colonization at 18 DAP and particularly in the well watered treatment at 84 DAP does not necessarily express a mycorrhizal suppression by P fertilization in the experiment. Unfortunately, no root length could be measured to elucidate this question. However, the preceding experiment SAF 95 which is comparable for the early growth stage, revealed a higher proportion of roots in mycorrhizal as compared to non-mycorrhizal safflower (section 3.3.3, Tab. 10). Most plants react to AMF in such a way (SMITH & READ, 1997).

3.4.4.2 Growth response to AMF

Independently of P fertilization, an extraordinarily high variation occurred in treatment M1P0W1 (A-Tab. 6). Here, different AMF colonization in quantitative and qualitative terms may have played a role, but could not be estimated: the use of a mixed inoculum, a so called *cocktail* (AUGÉ, 2001), may involve the risk of plants in the different pot replicates being colonized by different AMF species, depending on the (casual) first contact. Nevertheless, the species with the highest competition strength will be dominant. Generally, species may differ in efficiency for P uptake (SMITH & READ, 1997), causing higher variations within the same treatment. However, the inoculation with the AMF *cocktail* was closer to field conditions than with a pure culture.

3.5 General discussion of safflower experiments

3.5.1 Comparison of open air safflower trials SAF 95 and SAF 94

The experimental conditions between 1994 and 1995 differed concerning water stress treatment, soil preparation and soil containment. No pest damage occurred in 1995. In 1994, the inoculum procedure may be more effective due to a “nurse effect” (SMITH & READ, 1997) from the mycorrhizal chickpea plants previously grown (see section 3.2.2).

Another difference may be important: While in 1994 leaching (400 ml) was taken from ca. 30 kg untreated soil, soil in 1995 was cultivated twice (safflower and maize). Quantity and quality in terms of possible nutrient contents was consequently less in trial SAF 95 but this has not been further analyzed.

The much higher grain yield with P fertilization independent of mycorrhization in 1995 had the range of the yield increase by AMF inoculation the year before. The contradictory result concerning the mycorrhizal effect between the two years cannot explained by climatic differences. The growing period in 1995 was 1.8 °C warmer and 3.3 % relative humidity drier (Tab. 6, section 3.2.1 and Tab. 11, section 3.4.1), mainly a consequence of planting 12 days later (Tab. 15). Irradiance during the experiments was higher in 1994 but still optimal in 1995. The comparison of the PAR irradiance data shows inconsistencies: The spring of 1995 cannot have been 20 % darker than the spring of 1994. However, raw data of hourly means were intensively proved and double checked with the responsible scientists of the Dep. of Geophysics, UE. The year 1994 was the start of their measuring campaign.

Tab. 15: Timing of safflower experiments SAF 94 vs. SAF 95

Time schedule of agronomic procedures and measures	**Exp. SAF 94**		**Exp. SAF 95**	
	(Date)	(DAE)	(Date)	(DAP)
Sowing	07 April		-	-
Planting	-	-	27 April	0
Emergence	15 April	0	27 April	0
Thinning out	27 April	12	18 May	21
Begin of transpiration measurement	11 June	57	19 May	22
Harvest	1 August	108	20 July	84
Time of transpiration measurement	51 days		62 days	

Although drought stress treatments were different, well watered treatments M1(0)W1 are comparable with M1(0)W1P0 treatments (Tab. 16). The almost threefold soil volume and fourfold soil weight, respectively, are supposed to be main reasons explaining a 5 times stronger mycorrhizal response for biomass and grain yield. However, the difference was not significant in the second experiment.

The drought stress treatment of experiment SAF 94 may have been more effective since it was exerted on the entire soil volume of the monolith. In contrast, a strong gradient of the soil moisture in the stress treatment may have occurred in the pot experiment SAF 95: The limited irrigation did not allow a complete rewetting of the lower parts of the pot soil. Consequently, the soil volume from which water was extracted by the plant was smaller.

Tab. 16: Comparison of mycorrhizal effects on well watered safflower plants in experiment SAF 95 vs. SAF 94

Difference ΔM is the difference between mycorrhizal treatments. The percentage response or relative effect of M is calculated as (M1-M0)·100/M0. Difference Δa is the difference between experiments in year 1994 and 1995 for each mycorrhizal treatment. The relative effect a is calculated as (SAF94-SAF95)·100/SAF95. Plant dry matter (dm) before the onset of the transpiration measurement was derived non-destructively from leaf area.

	Exp. SAF 95				**Exp. SAF 94**				**Comparison 95 to 94**			
	Treatments				Treatments				Δa	Effect	Δa	Effect
Variables	M1 W1	M0 W1	ΔM	Effect M [%]	M1 W1	M0 W1	ΔM	Effect M [%]	M1 W1	a [%]	M0 W1	a [%]
Shoot dm plant^{-1} [g]	15.8	10.5	5.3	50	55.0	15.0	40.0	267	39.2	248	4.5	43
Grain dm plant^{-1} [g]	6.5	3.7	2.8	76	13.0	2.6	10.4	400	6.5	100	-1.1	-30
WUE shoot [g l^{-1}]	2.0	1.8	0.1	6	2.2	1.9	0.3	16	0.3	13	0.1	4
WUE grain [g l^{-1}]	0.7	0.6	0.2	33	0.6	0.8	-0.2	-27	-0.2	-22	0.2	42
Transpiration [l]	8.8	6.5	2.3	35	23.9	8.5	15.4	181	15.1	171	2.0	30
Start plant dm [g]	0.3	0.2	0.1	41	2.2	0.9	1.3	141	1.8	569	0.7	293

3.5.2 Comparison with other experiments

In field trials at the Évora site (see section 2.1, Tab. 1) of EGER with safflower as spring crop, seed yields up to 80 (1992/93) and 61 g m^{-2} (1993/94) could be reached at NPK fertilization of 75/100/85 kg ha^{-1} (CARVALHO, 1996). Yields were similar to the average in the EU of 63 g m^{-2} (FAO, 1995). However, the var. WARAMIN sown at 11 March 1994 performed intermediately with a yield of only 35 g m^{-2}. Shoot biomass reached 9.8 g dry matter per plant at a plant density of 36.5 m^{-2}. Regarding yield potentials of up to 400 - 480 g m^{-2} (WORKER et al. 1965; WEISS, 1983), the as best tested var. of safflower could realize at the Évora site about 15 %, and the var. WARAMIN only 8 % of the yield potential under Mediterranean dry land farming conditions.

Low yields were explained by EGER (CARVALHO, 1995) with severe damage by different insects, mainly a seed beetle which was apparently the same weevil as in the 1994 monolith trial presented. The biomass production was in the range of drought stressed safflower plants in the experiment presented. However, irrigation could increase yields by multiples.

Nevertheless, in the view of EGER (CARVALHO, 1997), safflower showed a good vegetative growth and flower production in field trials. Flowers were not damaged by insects, offering the possibility of growing safflower for dye production.

Safflower was recently studied for mycorrhiza, water relation and P nutrition. BRYLA and DUNNYWAY (1997a) confirmed that the enhanced P uptake by AMF particularly affects the variable leaf area of safflower which enable better growth by larger assimilation. Generally, this makes safflower interesting for studies on water relations in interaction with P supply. Leaf area estimation of large safflower plants with up to 250 leaves, including crown leaves of flowers, was not very sophisticated but error sensitive, and large leaved sunflower (KOIDE, 1985a & 1985b) is in this context certainly the better model plant. The advantage of carrying out the experiments until maturity is counteracted, unfortunately, by the disadvantage of an unreliable root determination. The short-time bioassay, however, revealed higher shoot-to-root ratios for mycorrhizal safflower at a plant biomass equivalent to that for non-mycorrhizal plants (section 3.3).

BRYLA and DUNYWAY (1997a & 1997b) conducted impressive work on AMF, P nutrition, and water relation in safflower. They presented more detailed variables, such as stomatal conductance, relative leaf water contents (RWC), osmotic leaf water potentials, and root length and their AMF colonization at 2 cm depth increments in 50 cm long container tubes. Furthermore, soil water conditions were precisely monitored. The non-mycorrhizal safflower was fertilized with 34 mg P kg^{-1} as K_2PO_4 at 5 µg g^{-1} plant available P soil content, accepting an enhanced P uptake as a side effect, because they intended to create both root length and leaf area similar to that of mycorrhizal plants. Drought stress of up to -0.8 MPa was applied by withholding irrigation beyond 22 DAE. However, the result remains basically the same as in the experiments presented here. No alteration of transpiration by colonization of arbuscular mycorrhizal fungi under drought stress condition was verifiable in safflower. Conversely, BRYLA and DUNYWAY (1997a) determined higher drought resistance and osmotic adjustment in non-mycorrhizal drought stressed safflower compared to mycorrhizal by higher RWC and greater (i.e. more negative) leaf water potential Ψ_l and greater osmotic

potential at full turgor Ψ_π^{100}, respectively. In contrast, DAVIES et al. (1992) had found higher leaf water potential and transpiration in repeatedly drought stressed mycorrhizal pepper plants independently of plant size and P content. However, in a repeated experiment by BRYLA and DUNYWAY (1998), this could not be confirmed for drought adapted safflower and wheat.

Merely a higher water use efficiency was found in our own safflower experiments for (different) shoot biomass production under both forms of stress, temporal drought and continuously low water supply. Apparently, this was more an indirect effect of different plant size than of mycorrhization. The same phenomenon was already found by SIEVERDING (1980) in pot experiments with *Sorgum bicolor*, *Guizotia abyssinica*, *Eupatorium oderatum*, and *Trigonella foenum-graecum*.

An important difference between our own safflower experiments and those of BRYLA and DUNYWAY (1997a & b; 1998) must be stressed. The growing conditions, in particular nutrient supply, under which safflower was cultivated, were much better and the cultivar S555 itself may be more productive than WARAMIN. The latter, with mycorrhiza in the growth chamber experiment (SAF 95 a) at 21 DAE, achieved a leaf area of only 135 cm^2 compared to 400 cm^2 of S555 in the cited experiment. Under open air conditions in pot trial SAF 95, leaf area was in the best case only about 20 % of the respective surface of S555 after 18 d. Furthermore, the other cultivar grew faster and developed already floral components at 27 DAE. However, the open air experiments presented were carried out till maturity and the results have therefore a qualitatively different value of information. The magnitudes of transpiration rates in mmol m^{-2} s^{-1} of about 10 (exp. SAF 94) and 6 (exp. SAF 95) were comparable with those of BRYLA and DUNYWAY (1997a) between 4 and 6. Well watered conditions corresponded in the latter study with water holding capacity of containers. Interestingly, mycorrhizal safflower had a significantly higher transpiration rate than non-mycorrhizal. Conversely, in the presented experiments, transpiration of non-mycorrhizal plants tended to be increased in both well watered and drought stressed treatments. Although the experiments were carried out with less technical support than those of BRYLA and DUNYWAY, the experimental set up was much closer to field conditions. The results obtained may help to improve the Mediterranean dry land farming with safflower in consideration of mycorrhiza symbiosis.

4 Experiments with *myc* $^{-}$ and *myc* $^{+}$ peas

4.1 Growth response of symbiotic and non-symbiotic peas on inoculation with micro-symbionts and water supply (PP 95)

4.1.1 Introduction

There is a general dilemma of all conventional bioassays in mycorrhizal research: it is not possible to determine the mycorrhizal response on native AMF under natural growing conditions. The experimental factor mycorrhiza is – as demonstrated in safflower experiments – either an inoculation or the exclusion of mycorrhiza is achieved by soil sterilization or fumigation.

In an effort to overcome such fundamental restriction, the AMF resistant and non-nodulating (*myc* $^{-1}$ and *nod* $^{-}$) pea mutant P2 (compare section 1.8) was tested in a first pot experiment against the symbiotic parental isoline FRISSON. Drought stress as the most important production determining factor in Mediterranean dry land farming may be crucial for their further utilization as an AMF screening and evaluation crop in field experiments here. Mycorrhization may increase drought resistance in plants by several mechanisms (AUGÉ, 2001; section 1.5.5). Therefore, the two pea isolines were examined with different water supplies under both sterile and symbiotic conditions at high fertilizer input in order to prove the following two hypotheses:

(1) Under a good and restricted water supply, pea mutant P2 of var. FRISSON does not have any production relevant genetic defects other than the lack of symbiosis.

(2) AM symbiosis increases pea growth under drought stress and high P supply.

4.1.2 Materials and methods

The experimental conditions in the climate chamber used were set as described in section 2.2 at a relative humidity of 70 %, as required by forage peas. The soil (Vertic Luvisol, further description see A-Tab. 16, A-Tab. 17, and A-Tab. 18) was heated in an oven twice for 24 h at 120 °C with one day cooling interval and was fertilized with N/P/K 1.8/0.26/0.33 g per plant or 346/50/63 mg per kg soil, respectively. Nitrogen was applied as NH_4NO_3 half as salt before and half as nutrient solution (100 ml of 0.1 % N) weekly during the experiment. Phosphorus and K were mixed as KH_2PO_4 into the soil of each pot filled with 5.2 kg oven dried soil. The P application corresponded with a field fertilization as high as 240 (105) kg ha^{-1} P_2O_5 (P)

when calculated per plant at a density of 40 per m². The experiment was arranged as a randomized block design with 5 replication split for factor inoculation in order to avoid contamination with *Rhizobium*. Plants were irrigated daily with sterilized water on a balance and transpiration was estimated accordingly in consideration of soil evaporation from bare pots. Soil water content was maintained at 14 % w/w (24 % v/v) or 80 % of water holding capacity (WHC) in well watered (W1) and 7 % w/w (12 % v/v) or 40 % of WHC in drought stress (W0) treatment, respectively. Soil moisture had a maximal oscillation of ± 1.75 % (w/w) or 10 % of WHC. Corresponding soil evaporation of bare control pots was determined at 50 ± 9 ml (W1) and 25 ± 6 ml (W0) per day, respectively. The AMF inoculum, 50 g of a peat-soil-root mixture containing *Glomus manihot* (IAT 78-1 orig. SIEVERDING C-1-1) which was produced with maize in the same (heat) sterilized soil (courtesy DIEDERICHS), was thoroughly mixed in each pot. Radiculae of pre-germinated peas were inoculated with 1 ml medium (ca. $10^{8)}$ of *Rhizobium leguminosarum* strain *PS10* (courtesy ALHO) and two were sown in each pot. After two weeks, 1 of 2 seedlings was taken and fine roots were checked for AMF colonization. Because no mycorrhiza were observed in some inoculated plants of FRISSON, the inoculation was repeated, but in a modified way. Instead of mixing into the soil, 5 g of AMF inoculum were concentrated into the sowing mound. Now, AMF colonization could be confirmed in all FRISSON peas when checked again after two weeks. However, the inoculated treatments (IN1) emerged 18 d later than those not inoculated (IN0). For technical reason, IN1 treatments had to be moved for the last two weeks of the experiment into another climate chamber and were harvested 4 d earlier. The second climate chamber was the same model and had the same programming, however, was additionally occupied by other plants. The experimental factors were:

(1) Isoline: Symbiosis as **i**so**l**ines **FRI**SSON (*myc* $^{+}$*, nod* $^{+}$) and mutant **P2** (*myc* $^{-1}$*, nod* $^{-}$)
(2) Water: Irrigation at 80 % (**W1**) and 40 % (**W0**) of water holding capacity
(3) Inoculum: Symbiosis as **in**oculum with (**IN1)** and without (**IN0**) AMF and *Rhizobia*

4.1.3 Results and discussion

Shoot dry matter from thinned seedlings at 14 DAP was homogenous in non-inoculated treatments. However, when the inoculated part of the trial was repeated, the differentiated irrigation, which was started in the first trial set up only after 10 d, already influenced early growth (Tab. 17).

Under sterile conditions, FRISSON and mutant P2 showed no growth differences at final harvest, only root weight of drought stressed FRISSON was higher and consequently shoot-to-root ratio lower.

In a pre-test (A-Tab. 19) under well watered conditions and high N fertilization, however, no significant differences between FRISSON and mutant P2 were obtained in heated (100°C) soil without P fertilization. The impact of *G. manihot* on drought stressed plants could not be tested due to a very small AMF colonization of only 3 % in treatment Fri W0 IN1. Consequently, hypothesis (2) could not be confirmed and must be tested in a different experimental setup.

Tab. 17: Shoot dry matter perplant at 14 DAP (PP 95)
Treatments are **IL** (**iso**lines **FRISSON** and mutant **P2**), soil water content **W** 80 % (**W1**) and 40 % (**W0**) WHC, inoculum **IN** with (**IN1**) and without (**IN0**) AMF and *Rhizobia*, respectively. Bold values are **means** ± SD. Different letters indicate significant differences (Tukey's HSD).

Treatments IL W IN	Shoot dm plant^{-1} [mg]	
Fri W1 IN1	**175** ± 26	**bc**
Fri W0 IN1	**131** ± 26	**c**
P2 W1 IN1	**210** ± 21	**ab**
P2 W0 IN1	**134** ± 32	**c**
Fri W1 IN0	**267** ± 16	**a**
Fri W0 IN0	**244** ± 37	**ab**
P2 W1 IN0	**251** ± 41	**a**
P2 W0 IN0	**230** ± 34	**ab**

However, JENSEN (1985) also found a strong decrease of AMF colonization in 10 pea cultivars grown in a loamy soil when soil water content was reduced continuously to 50 % field capacity as compared to constant hold field capacity. For the forage peas of vars. BODIL and FINALE, colonization by natural AMF dropped from 21 to 2 and 22 to 4 %, respectively.

When well watered, the AMF colonization was reasonable at 55 % but the inoculation reduced plant weight as well as water use efficiency for grain yield significantly as compared to non-inoculated treatments. Additionally, this resulted in an interaction of the experimental factors isoline, water supply and/or inoculation. Drought stress decreased harvest index of FRISSON, not of mutant P2 (A-Tab. 18).

One replication of treatment P2 W1 IN0 showed further mutation and was treated as a missing value. That plant was growing well, but its flowers remained greenish and infertile, while all other plants had reached maturity. The same phenomon was observed in two other single plants of P2 at grain multiplication and in field experiment PF 96.

Nodulation was well developed in Fri IN1 treatments and not observed in sterile controls. Thus, the high N fertilization could not suppress nodule forming.

Tab. 18: Yield components of pea isolines in pot experiment PP 95
Treatments are **IL** (**i**so**l**ines **FRI**SSON and mutant **P2**), soil water content **W** about 80 % (**W1**) and 40 % (**W0**) water holding capacity, inoculum **IN** with (**IN1**), and without (**IN0**) AMF and Rhizobia, respectively. Bold values are **means** ± standard deviation. Different letters indicate significant differences (Tukey's HSD for unequal N (Spjotvoll/Stoine) with N=5 except treatment **P2 W1 IN0** with N=4). Dry matters (dm) and ratios indexed ^{ln} are ln, ^{ar} are arcsin root transformed for 3-way ANOVA. F-values are significant with * at p<0.01, ** at p<0.05, and *** at p<0.01) or not significant (n.s.).

Treatments	Plant dm [g] ^{ln}	Grain dm [g] ^{ln}	Harvest Index ^{ar}	Root dm [g]	Shoot / Root ^{ln}
Fri W1 IN1	**10.0** ± 2.9 b	**5.9** ±1.8 bc	**58** ± 1 a	**0.50** ±0.15 ab	**19** ± 3 ab
Fri W0 IN1	**7.5** ± 0.8 b	**3.8** ±0.4 c	**50** ± 5 bc	**0.44** ±0.18 b	**18** ± 5 bc
P2 W1 IN1	**28.9** ± 8.5 a	**13.1** ±2.9 a	**47** ± 5 c	**1.11** ±0.55 a	**27** ± 7 ab
P2 W0 IN1	**7.0** ± 1.4 b	**3.5** ±0.7 c	**50** ± 2 bc	**0.44** ±0.08 b	**15** ± 3 bc
Fri W1 IN0	**25.6** ± 4.3 a	**13.1** ±4.6 a	**59** ± 2 a	**0.88** ±0.32 ab	**30** ± 8 ab
Fri W0 IN0	**10.6** ± 1.3 b	**5.1** ±0.6 c	**48** ± 1 c	**1.10** ±0.34 a	**10** ± 3 c
P2 W1 IN0	**23.1** ±11.4 a	**12.8** ±7.1 ab	**54** ± 3 abc	**0.55** ±0.19 ab	**43** ±17 a
P2 W0 IN0	**8.7** ± 2.9 b	**4.8** ±2.2 c	**53** ± 5 abc	**0.43** ±0.13 b	**22** ±10 ab
F-values of isolated and combined effects (degree of freedom: 1 effect, 31 error)					
Isoline (**IL**)	n.s.	n.s.	5*	n.s.	8**
Water (**W**)	72***	57***	11**	n.s.	33***
Inoculum (**IN**)	10**	7**	n.s.	n.s.	n.s.
IL x **W**	9**	5*	11***	5*	n.s.
IL x **IN**	12**	n.s.	7**	15***	4*
W x **IN**	n.s.	n.s.	n.s.	n.s.	7*
IL x **W** x **IN**	8**	n.s.	n.s.	n.s.	4*

No main effect of isoline (IL) on any water consumption or efficiency variable was detected (Tab. 19). Irrigation treatment (W) had, as expected, the strongest influence as indicated by high F-values. The double amount of water supply was followed by a 2- to 3-fold transpiration per plant with only one exception: the inoculated FRISSON developed so poorly even when well watered that the water consumption had the range of drought stress treatments. As a consequence, there was a significant interaction of treatment factors IL x W on water use efficiency per plant biomass.

Tab. 19: Water consumption of pea isolines in pot experiment PP 95
Treatments and abbreviations as in Tab. 18. WUE is water use efficiency for plant or grain dry matter related to transpiration

Treatments	Transpiration [ml]	WUE plant [g dm l^{-1}]	WUE grain [g dm l^{-1}]
Fri W1 IN1	**1527** ± 515 bc	**6.61** ±0.65 ab	**0.32** ±0.08 b
Fri W0 IN1	**1838** ± 63 bc	**4.10** ±0.47 c	**0.55** ±0.01 a
P2 W1 IN1	**4083** ± 1051 a	**6.99** ±0.50 ab	**0.56** ±0.07 a
P2 W0 IN1	**1721** ± 165 bc	**4.04** ±0.57 c	**0.53** ±0.02 a
Fri W1 IN0	**4976** ± 1888 a	**5.60** ±1.24 b	**0.55** ±0.09 a
Fri W0 IN0	**1484** ± 190 bc	**7.12** ±0.24 a	**0.48** ±0.03 ab
P2 W1 IN0	**3463** ± 1835 ab	**6.79** ±0.26 ab	**0.48** ±0.11 ab
P2 W0 IN0	**1455** ± 659 c	**6.25** ±0.63 ab	**0.46** ±0.09 ab
F-values of isolated and combined effects (degree of freedom: 1 effect, 31 error)			
Isoline (**IL**)	n.s.	n.s.	n.s.
Water (**W**)	35***	23***	n.s.
Inoculum (**IN**)	n.s.	19***	n.s.
IL x **W**	n.s.	7***	n.s.
IL x **IN**	11***	n.s.	11***
W x **IN**	10***	48***	10***
IL x **W** x **IN**	11***	n.s.	11***

In the inoculated part of the experiment, the water use efficiency concerning the entire plant dry matter resulted for both isolines in a significant increase of about 40 % at the higher water supply. In the non-inoculated part, that pattern could not be observed indicated by a strong interaction of IN x W. FRISSON reacted here in the opposite way due to a very high consumption whereas mutant P2 showed no significant differences (Tab. 19). Water use efficiency calculated for grain yield mirrored the bad performance of well watered inoculated FRISSON which resulted in a small interaction of all factors. Water use efficiency related to transpiration may be more relevant as related to evapotranspiration because, apart from shadow effects, soil evaporation is not dependent on plant growth. Such shadowing by plants could not be considered for the calculation of transpiration with bare control pots in the experimental set up and may have caused an underestimation by lowering soil evaporation.

Generally, drought stress led to higher N concentrations (Tab. 20). Hence, the lack of water may have directly reduced photosynthesis and consequently growth. An indirect limitation of growth via reduced N uptake is less plausible although absolute N content in shoots of well watered plants was twice as high as in drought stressed plants.

Exposed to the better water supply, the P uptake increased threefold (Tab. 20). Phosphorus concentration was analyzed from a mixed sample of five replications because there was too little plant material in some treatments.

Growing conditions offered a *luxurious* supply of N and a *good* supply of P. Therefore, P uptake was improved more strongly than N uptake by better water supply, as indicated by the higher F-value (Tab. 20).

Tab. 20: Shoot uptake and straw concentration of N and P in pea pot experiment PP 95
Treatments and abbreviations as in Tab. 18.

Treatments	NITROGEN			PHOSPHORUS		
	Concentration		Content	Concentration		Content
	Grain [%]	Straw [%]	shoot plant^{-1} ^{ln} [mg]	Grain [mg g^{-1}]	Straw [mg g^{-1}]	shoot plant^{-1} ^{ln} [mg]
Fri W1 IN1	**4.71** ±0.14 **bc**	**2.20** ±0.40 **d**	**351** ± 93 **c**	**3.07**	**0.58**	**20.1** ± 6.1 **b**
Fri W0 IN1	**5.11** ±0.24 **abc**	**2.74** ±0.16 **cd**	**308** ± 34 **c**	**2.95**	**0.77**	**13.7** ± 1.3 **b**
P2 W1 IN1	**4.64** ±0.30 **c**	**3.41** ±0.16 **abc**	**1006** ±268 **a**	**2.88**	**0.95**	**51.7** ±13.2 **a**
P2 W0 IN1	**4.96** ±0.21 **abc**	**3.88** ±0.15 **ab**	**277** ± 54 **c**	**2.86**	**0.95**	**12.9** ± 2.7 **b**
Fri W1 IN0	**4.63** ±0.27 **c**	**2.39** ±0.46 **d**	**872** ±215 **a**	**3.36**	**0.76**	**52.8** ±13.8 **a**
Fri W0 IN0	**5.43** ±0.18 **a**	**2.90** ±0.26 **bcd**	**456** ± 61 **bc**	**2.70**	**0.68**	**16.7** ± 2.0 **b**
P2 W1 IN0	**4.92** ±0.34 **abc**	**3.49** ±0.85 **abc**	**856** ±300 **ab**	**3.66**	**0.96**	**56.2** ±29.9 **a**
P2 W0 IN0	**5.25** ±0.15 **ab**	**4.10** ±0.57 **a**	**383** ±112 **c**	**2.72**	**0.72**	**15.6** ± 6.4 **b**
F-values of isolated and combined effects (degree of freedom: 1 effect, 31 error)						
Isoline (**IL**)	30***	57***	n.s.			n.s.
Water (**W**)	6***	5*	57***			89***
Inoculum (**IN**)	n.s.	5*	16***			10***
IL x **W**	n.s.	n.s.	14***			7***
IL x **IN**	n.s.	n.s.	10***			6***
W x **IN**	n.s.	n.s.	8***			n.s.

The AMF inoculum of *G. manihot* was not effective in enhancing plant growth as reported by other studies (SIEVERDING, 1991; HEINZEMANN, 1994; LANGE NESS, 1998). A contamination of the inoculum with pathogens (KASIAMDARI et al., 2002) was not probable because inoculated mutants developed well and also the poorly colonized drought

stressed peas of FRISSON showed no significant differences in comparison to drought stressed pea plants without inoculum (Tab. 18). The ineffectiveness of the AMF may be related to the high NP fertilizer input in the experiment. It cannot be excluded that irradiance at 350 µE $m^{-2}s^{-1}$ PAR was insufficient. JARSTFER and SYLVIA (1997) recommend an illumination superior to 500 µE $m^{-2}s^{-1}$ PAR for AM bioassays. REINHARD (1995) found a significant response of pea plants grown under light regimes in climate chambers between 250 and 500 µE m^{-2} s^{-1} PAR in the context of AMF colonization and N_2 fixation. An irradiance as high as 900 µE m^{-2} s^{-1} PAR was consequently chosen to exclude light effects in a corresponding experiment with peas in tripartite symbiosis (REINHARD et al.,1994). However, light was necessary to increase continuously from low levels to prevent phytotoxic effects observed already at 750 µE m^{-2} s^{-1} PAR when supplied from the beginning.

The genetic defect of infertility observed in one of the mutants was annoying, but still tolerable for the presented data with four remaining replications. It should be borne in mind for further experiments with mutant P2 that this very rare defect can be detected easily but only relatively late during inflorescence.

In contrast to safflower experiments with more mature plants, the relatively coarse white colored and less senescent pea roots were much easier to process for root washing and consequently allowed a more reliable determination of root dry matter.

4.2 First field trial of var. FRISSON and *myc* $^{-1}$ mutant P2 at site Évora (PF 94)

4.2.1 Introduction

The evaluation of arbuscular mycorrhiza in field experiments is more meaningful than by bioassays in pots. The preceding experiment revealed that the *myc* $^{-1}$ mutant P2 has the same growth potential as the wild type FRISSON under growth chamber conditions even under restricted water supply. The forage pea line FRISSON is well adapted to growing conditions at high input in the Bourgogne (SAGAN et al., 1993) and Ile-de-France where the plant breeder is situated. However in the Alentejo, the soil and climate conditions are much less favorable for field peas. Therefore, one prerequisite for further field experiments must be proved:

Does this variety also perform reasonably under Mediterranean dry land farming conditions? Inasmuch as these conditions are yield limiting, the more interesting they are for the management of arbuscular mycorrhiza (see section 1.6). The central question arises:

Can the set of *myc* $^{-}$ and *myc* $^{+}$ peas function as indicators of a "field bioassay" in which the variable "yield difference" between the two isolines evaluates the mycorrhiza effect?

Possible advantages by N_2 fixation in FRISSON were expected to be negligible at high N fertilization. Although germ plasm multiplication was needed, no P fertilization was applied in order to allow maximal AMF colonization in the wild form FRISSON. Due to a limited amount of seeds, only a small first experiment could be started.

4.2.2 Materials and methods

The soil was tilled by heavy disc harrow on 18 October 1993 followed by single-axle rotary hoal before sowing. The previous crop was wheat (*Triticum aestivum* L.). A fertilization of 100 kg ha^{-1} N as NH_4NO_3 was given at sowing in order to suppress N_2 fixation benefits. An additional 50 kg of N ha^{-1} were applied during flowering on 15 March 1994. No P or K was fertilized. The experiment had a 1-factorial randomized block design with two replications.

Forage peas of the symbiotic isoline FRISSON as mycorrhizal treatment and the non-symbiotic isoline mutant P2 as non-mycorrhizal treatment by genetic means were sown on 22 November 1993. Plot size was 2 m x 1 m with 25 seeds per m² at a row distance of 20 cm.

The phenological development (plant height, live leaf nodes and flowers) of the central six plants of each plot was noted monthly. From 26 January to 18 February, the trial was protected by a net against birds which already had caused some damage. Weed control was

done by hand. On 14 May, mature central plants were harvested. All remaining seeds and plants were sampled in order to avoid the spread of germ plasm.

Samples of fine roots (4 internal plot replications) were taken by means of a cork auger (10 cm length, 75 ml volume) on 26 January close to pea plants of marginal rows and stained with trypan blue to confirm AM colonization. However, root samples were too small for a quantification of the colonization rate.

Soil samples of top soil were taken with a spade after harvest from each plot. The soil P concentration was determined by the Bray I method (see section 2.3). For further soil and climate data, see section 2.1, A-Tab. 8 (precipitation) , A-Tab. 17, and A-Tab. 18 (soil).

4.2.3 Results and discussion

On 18 December 1993, all pea plants had emerged, except for 3 which followed a few days later. Flowering and pod building of both isolines were compared around mid March. The plants of mutant P2 were generally much smaller from March onwards (Tab. 21).

The AMF resistance of mutant P2 demonstrated for greenhouse experiments (DUC et al., 1989) was confirmed under field condition. Arbuscular mycorrhiza developed very well in FRISSON (no quantification), whereas AMF entered only superficially into epidermal cells of P2 fine roots. Although appressoria were formed here, neither arbuscules nor internal hyphae were found inside of the root cortex.

Tab. 21: Agronomic data of two pea isolines in field experiment PF 94 (**means** ± SD are of 2 x 6 sub samples; n.d. = not determined).

Days after emergence		**39**	**63**	**87**	**119**
Plant height	**Frisson**	**6** ± 0.6	**11** ± 0.9	**22** ± 4.2	**29** ± 3.7
[cm]	**P2**	**6** ± 0.2	**10** ± 0.1	**13** ± 2.4	**19** ± 1.3
Leaf nods	**Frisson**	**10** ± 1.3	**18** ± 4.0	**40** ± 9.4	n.d.
plant^{-1} [no.]	**P2**	**10** ± 0.5	**15** ± 0.6	**23** ± 4.6	n.d.
Flowers	**Frisson**	0	0	**6** ± 2.2	0
plant^{-1} [no.]	**P2**	0	0	**3** ± 0.6	0

This morphological pattern was analyzed by GIANINAZZI-PEARSON (1996) in detail.

FRISSON performed 3- to 4-fold better than P2 in biomass, pod, and grain production (Tab. 22). The hundred grain weight and harvest index of FRISSON were slightly higher but grains per pod were not significantly different compared to mutant P2.

Tab. 22: Yields and yield components of two pea isolines in field experiment PF 94
Means ± SD are of 2 x 6 sub samples. Results of t-test with n=12 were indicated as p-level (n.s. = not significant).

Isoline	Grains plant^{-1} [no.]	Grains pod^{-1} [no.]	Pods plant^{-1} [no.]	Grain dm plant^{-1} [g]	Shoot dm plant^{-1} [g]	100 grain weight [g]	Grain yield dm [g m^{-2}]	Harvest Index [%]
Frisson	**42** ± 10	**3.8** ± 0.04	**11** ± 2.6	**6.0** ± 1.4	**10** ± 2.4	**15** ± 1.2	**216** ± 49	**58** ± 0.3
P2	**12** ± 3	**3.1** ± 0.39	**4** ± 1.3	**1.4** ± 0.4	**3** ± 0.9	**12** ± 0.3	**52** ± 16	**41** ± 0.8
t-test	< 0.0001	n.s. 0.1294	< 0.0001	< 0.0001	< 0.0001	0.0419	< 0.0001	0.0025

In a field trial in France, a relative growth difference of 28 % between the two isolines was determined (LOVATO, 1994; TROUVELOT et al., 1996). The application of 44 kg ha^{-1} P as triple phosphate did not completely compensate the differences between the two isolines although they were no longer significant. In contrast, a yield difference by more than a factor of three was found in the experiment presented, although fertilized with only 150 instead of 500 kg N ha^{-1} as in the French field experiment. There is no evidence to explain the 10-fold stronger effect with the smaller amount of applied N fertilization, but it can not be ruled out that winter rainfall had leached some nitrate. Phosphorus uptake was restricted by very low soil concentrations of **0.4** ± 0.1 µg g^{-1} P (Bray I) in a relative dry spring. The P uptake of FRISSON in comparison to mutant P2 was increased by a factor of 4.6, whereas a factor of 2.7 was calculated for N uptake (A-Tab. 27). Fertility of the French soil was much higher, particularly in P availability by a factor of 10 (compare section 4.5.3.7).

The exclusion of the undesirable site effect N_2 *fixation* in order to isolate the isoline effect onto the experimental factor *mycorrhiza* as a genetic treatment plays a key role in the validation of the proposed experimental tool.

Generally, the determination of the N_2 fixation rate is possible by ^{15}N enrichment or the natural abundance technique (ZAPATA, 1990). However, it is difficult to integrate such expensive and relatively complex experiments into studies which are primarily focused on AMF resistance. It failed in this experiment due to differences in lab replication up to factor four. The analysis for naturally enriched ^{15}N should exemplarily estimate the amount of biologically fixed N_2 in peas (HANDLEY & RAVEN, 1992).

Another approach to ensure that high amounts of N fertilizer suppress the symbiotic advantage of BNF in FRISSON might be a step wise fertilization up to a level of N saturation. This was estimated by SAGAN and co-workers (1993) in France for the site of the experiment of LOVATO (1994) at a level as high as 500 kg N ha^{-1}, but under irrigation.

The control of possible N effects by genetic means may be the use of existing *nod* $^{-}$ *myc* $^{+}$ mutants or mutants with ineffective nodulation (*fix* $^{-}$ *nod* $^{+}$ *myc* $^{+}$) although all other putative genetic defects beside the symbiotic must be thoroughly observed (KAHILUOTU et al., 2000).

It may also be of interest to inoculate with ineffective *Rhizobia* which could *shield* symbiotic peas from BNF and manifest the same high dependency on N supply as known for non-symbiotic AMF resistant peas. In contrast to AMF, the symbiosis with *Rhizobium* is restricted to a specific strain which is, once the infection is established, incompatible for other strains (ALBRECHT et al., 1999). Generally, strains differ in terms of efficiency concerning N_2 fixation and infectiousness with regard to the level of nodulation and competitive strength against other (native) strains (SCUPHAM et al., 2000).

4.3 Second field trial of variety FRISSON and *myc* $^{-1}$ mutant P2 at site Évora (PF 95)

4.3.1 Introduction

The first test trial was promising. However, the amount of plants was very low. Further multiplication of germ plasm in a climate chamber allowed a repetition of the trial at a larger scale with the same objectives as described in section 4.2.1.

4.3.2 Materials and methods

The field trial was situated close to the one of the year before. The soil (Vertic Luvisol) was tilled by single-axle rotary hoal directly before sowing. Previous crop was oats (*Avena sativa* L.). A fertilization of 250 kg ha^{-1} nitrogen as NH_4NO_3 (26 %) was given in order to exclude N_2 fixation benefits of the wild type compared to mutant P2. No other fertilizers were applied and soil was very low in P concentration (7 µg g^{-1} P Bray I) (CARVALHO, 1997). The experimental design was a one factorial randomized balanced block with four replications and the two pea isolines FRISSON and mutant P2 as treatment. Sowing was done by hand on 4 February 1995 in plots of 1.8 m x 4 m with 25 seeds per m² at a row distance of 20 cm.

From 27 February to 11 March, the trial was protected by a net against birds. Weed control was done selectively with 1 % TRIBUNIL® by hand sprayer on 13 March and subsequently by hand.

Root samples for estimation of AMF colonization (section 2.5.4) were taken 35, 72 and 90 days after sowing (DAS). On 18 April (72 DAS), three plants from each replication of FRISSON were used to assess N_2 fixation by acetylene reduction (HARDY et al., 1968). On 29 May first mature pods and plants were harvested. This was repeated on 2 June to avoid harvest losses caused by extraordinary drought. The remaining plants were harvested on 20 June. The central plots of 3.6 m², separated to next plot by two rows and 50 cm, respectively, were used for yield determination.

After harvest, soil samples were taken with a 2 cm ∅ soil auger and separated for 0 to 20 and 20 to 40 cm depth. Two samples of each of the 8 plots were mixed and P soil concentration was analyzed by the Bray I method (section 2.3).

4.3.3 Phenological observations

Emergence occurred on 27 February. Flowering (50 %) was observed for both isolines at 12 April 1995 (67 DAS). First differences between isolines occurred with pod forming one week later. The mutant P2 had fewer green leaves and showed faster senescence by this time. Simplified, three flowering periods induced by drought could be observed. Grain filling of the first suffered from freezing (a minimum air temperature of 0.0 °C was recorded at 3 m altitude on 21 April 1995 at Mitra) and of the latter two from severe drought (see A-Tab. 8). Most of the P2 plants did not flower a third time and consequently reached maturity faster. It was assumed that those plants had shallower root systems. However, root depth could be estimated only from peas with green parts, implying strong soil perturbation. Consequently, only few roots were dug out exemplarily with a spade and a fine screw driver a few days before the final harvest. These peas, mostly FRISSON, apparently reached between 40 and 70 cm rooting depth with still some soil moisture.

4.3.4 Results and discussion

In contrast to the year before, emergence was not complete (FRISSON **80** ± 12 %, mutant P2 **83** ± 7 %; at 31 DAS) but not significantly different between the two isolines. Plant density did not change till harvest.

No nitrogenase activity could be detected (courtesy ALHO, data not shown) in sampled plants and only small white nodules were found. Therefore, no evidence of N_2 fixation in FRISSON was found at 72 DAS.

AMF colonization in FRISSON, estimated from mixed root samples, started at a rate of 5 % at 35 (DAS), reached 39 % at flowering (72 DAS) and was 75 % at grain filling (90 DAS). No AM was detected in fine roots of P2 plants.

The symbiotic isoline FRISSON had an almost five times higher grain yield in comparison to mutant P2 (Tab. 23). The yield of both isolines was more than three times less than in 1994 (section 4.2.3, Tab. 22). This difference seemed to be related to the much drier year of 1995 (A-Tab. 8). Additionally, drought stress on plant growth was enhanced because sowing had been done two months later than the year before. The delayed sowing which was, however, still in the range of the indicated period for forage peas in the region (BASCH, per. comm.) was imposed by previous multiplication of P2 seed material in a climate chamber.

Tab. 23: Yield and yield components of two pea isolines in field experiment PF 95
Means ± SD, n = 4. All differences were significant according to Tukey's HSD of 1-way ANOVA as indicated by p of F-value.

Isoline	Grain dm plant-1 [g]	Shoot dm plant-1 [g]	100 grain weight dm [g]	Grain yield dm [g m^{-2}]	Straw yield dm [g m^{-2}]	Harvest Index [%]
Frisson	**3.3** ± 0.2	**7.9** ± 0.2	**12.3** ± 0.8	**67** ± 11	**93** ± 10	**42** ± 2
P2	**0.7** ± 0.2	**2.4** ± 0.4	**11.2** ± 0.4	**14** ± 3	**36** ± 6	**28** ± 4
P of F-value	0.0002	0.0002	0.0149	0.0003	0.0003	0.0016

In an NPK fertilizer trial at the same site (CARVALHO, unpublished data), the grain yield of winter sown forage peas (var. BALLET) sown in November reached only 30 g m^{-2} (average of all treatments) in 1994/95 and did not respond to different levels either of N (0 and 30 kg ha^{-1} N as NH_4NO_3), or of P (0, 22, and 44 kg ha^{-1} P as triple phosphate), or of K (0, 25, and 50 kg ha^{-1} K as K_2SO_4). Yield depression was probably more affected by freezing in late April due to more advanced pot building of winter peas at this time. In contrast, grain yields were 93 g m^{-2} in 1993/94, a normal year in terms of precipitation, and even 104 g m^{-2} in

1992/93, a relatively dry year (A-Tab. 8), but never showed a significant response to any fertilizer treatment. Plant densities were much higher in these trials, with about 63 plants m^{-2}.

After harvest, soil P (µg g^{-1} Bray I, **means** ± SD) was almost by factor 20 higher than in the field experiment in 1993/94, at **7** ± 3 ∅ 0-40 cm depth, **6** ± 5 at 0-20 cm and **8** ± 5 at 20-40 cm depth, but was still low. The same soil P concentration was found in a chickpea trial nearby before sowing in March (CARVALHO, 1997).

If other factors, particularly N nutrition, can be excluded for the large isoline response, it is speculated that P nutrition mainly enhanced root depth growth which was apparently responsible for the general plant growth under extreme drought conditions (SÁNCHEZ-DIAZ & HONRUBIA, 1994). Only long and deep reaching roots could still access some soil water, whereas smaller plants of P2 with a proportionally smaller root system could not. It must be considered in this context that larger plants simply consume more water (see section 3.2.4.1). Therefore, the (unknown) plasticity of plants to allocate more resources in shoot or in root growth has importance in whether the mycorrhizal effect is plant beneficial, as in the case of a deeper root system (e.g. as assumed in experiment PF 95), or counterproductive, as in the case (e.g. nearly in experiment SAF 94) of *haying-off* with too large a leaf area being disproportional to the limited water supply. In the pea pot experiment PP 95 (section 4.1), mutant P2 performed equally or better under well fertilized conditions under good and limited water supply. However under field conditions, nutrient uptake is affected by many factors such as nutrient and soil water distribution in gradients which are more or less reached by the (mycorrhizal) root system (VLEK et al., 1996). The access to these resources is, in particular under conditions of Mediterranean dry land farming, much more important than the absolute amount itself.

Fertilization trials up to a level of no response may be suitable to check mutant P2 for possible N deficits. The role of P in this experimental system is ambivalent because of suppression of AMF colonization at high levels of P fertilization on the one hand and interaction of the P status in the plant with water efficiency and root-depth-growth on the other hand. Finally, the P effect is known the most important beneficial contribution of AMF in P limited cropping systems (BOLAN, 1991).

The comparison of N and P shoot uptakes in pea plants from both years (A-Tab. 27) indicates a higher isoline response for N in 1994 (factor 4.6) than in 1995 (factor 2.7). Conversely, P was less strongly affected (factor 3.1) in 1994, whereas the P uptake of FRISSON was over five times higher than in mutant P2 in 1995. Straw N concentrations in both isolines

were extraordinarily low in 1994, at 0.8 % P straw concentrations were low in both years, at 0.05 % (A-Tab. 27). Hence in 1994, N supply may to some extent have limited the yield of the non-symbiotic mutant P2. However, in 1995 under enhanced drought stress, the lack of mycorrhiza was apparently the reason for a radically reduced P uptake in pea mutants. Conversely, a significantly higher straw N concentration of P2 plants may indicate that N was not the growth limiting factor.

4.4 Evaluation trials with different forage pea lines at site Elvas (PF 95 a & PF 96 a)

4.4.1 Introduction

The integration of the presented work into the EU-Project offered the possibility of carrying out two small sampling experiments in the seasons 1994/95 and 1995/96 at the national plant breeding station at Elvas. Beside the opportunity to sensitize plant breeders for the variable "mycorrhiza" (JENSEN, 1985; VLEK et al., 1996), the cooperation provided the infrastructure for the comparison of the two isolines FRISSON and mutant P2 with 120 Portuguese associations of forage peas as a winter crop on-station (section 2.1, Tab. 1). The soil of this site is a Luvisol which is more fertile than the one in Évora particularly in plant available P content (A-Tab. 16 and A-Tab. 18). Therefore, no yield differences between the two isolines are to be expected under sufficient N supply for the mutant P2 as long as other factors do not limit growth. The following questions should be answered:

Can the mutant P2 achieve the same yield level as FRISSON at high mineral N input under field conditions and a good P supply? How can the growth of var. FRISSON be classified within pea varieties adapted to regional cropping conditions?

4.4.2 Materials and methods

On 23 November 1994, 40 seeds of FRISSON and P2 were sown in single row plots of about 4 x 0.5 m. Fertilization was 22 kg ha^{-1} P as triple phosphate and 42 kg ha^{-1} K as K_2SO_4. Additionally, only mutant P2 received 500 kg ha^{-1} nitrogen as NH_4NO_3 in a fivefold split application every 4 weeks from the sowing date onwards.

In 1995, the experiment was repeated and started at the same date. However, two 10 m long rows of 100 peas of each line were sown. While P and K fertilization was the same, 450 kg ha^{-1} N was given in a threefold split application on P2 rows every 6 weeks from the sowing date onwards.

Seeds were treated in both years with the fungicides CARBENDAZION® and TIRAME® (1:1 w/w) as a standard method at the station to prevent emergence reduction by soil borne phytopathogenic fungi. These fungicides may have an impact on mutualistic AM fungi (JOHNSON & PFLEGER, 1992). In 1996, heavy rainfall during the winter and spring time disturbed the experiment (A-Tab. 8). The trial suffered from *damping-off* syndrome and herbicide application did not successfully control weeds. In consequence, weed pressure was very high. For harvest on 29 May, four plants of both isolines were dug out from less disturbed areas at random with a spade and a screw driver. AMF colonization of attached fine roots was estimated as a mixed sample (section 2.5.4).

4.4.3 Results and discussion

In 1994/95, FRISSON achieved a grain yield of 9 g, P2 6 g and the 120 Portuguese accessions an average of 6.5 g per plant. For more details, see A-Tab. 21. The second year, no comparison with the Portuguese pea lines was possible because the trial was abandoned by the station. In 1995/96, grain yield of P2 was similar to the year before whereas yields of FRISSON were nearly 3 times higher than those in 1995. In the second year the difference between the two isolines was much higher (Tab. 24). AMF had colonized roots of FRISSON at a rate of 64 % whereas no colonization in P2 roots was observed.

Tab. 24: Yields and yield components of two pea isolines sampled in field experiment PF 96 a in Elvas (**means** of 4 plants ± SD)

Isoline	Shoot dm [g]	Straw dm [g]	Grain dm [g]	Harvest Index	100 grain weight	Grain pod^{-1} [no.]	Pods [no.]	Grain [no.]
Frisson	**45** ± 28	**19** ± 11	**26** ± 16	**57** ± 2	**15** ± 1	**4.0** ± 0.7	**41** ± 21	**166** ± 96
P2	**11** ± 5	**5** ± 1	**5** ± 3	**43** ± 20	**13** ± 1	**3.0** ± 0.4	**13** ± 8	**40** ± 26

Phosphorus and N concentration of mixed samples were higher in mutant P2 than in FRISSON (A-Tab. 27) in both years. Although the analyses were not repeated for single plant samples, N and P concentrations in mutant P2 cannot explain the 5-fold higher yield of FRISSON.

Unfortunately, the evaluation experiment of the plant breeding station had no statistical design but the unexpected prominently higher yields of FRISSON in comparison to mutant P2

provoked some speculation that the growth of the mutant P2 is limited by other genetic factors than those of symbiotic deficits. Besides, late drought stress may also have played a role.

4.5 Mycorrhizal response assayed as isoline effect at three sites (PF 96)

4.5.1 Introduction

Previous field trials at Évora and Elvas revealed that it is generally possible to grow the forage pea variety FRISSON and the non-symbiotic isomutant P2 under relatively unfavorable Mediterranean dry land farming conditions in the Alentejo. Yields of FRISSON were about 37 to 80 % higher, compared to mutant P2. However, at high fertilization with N and P, mutant P2 could not reach the yield levels of FRISSON at Elvas. At site Évora, where no P fertilizer was applied, not only reduced P uptake but also drought stress or drought restricted nutrient uptake were supposed to be reasons for the growth depression of P2 plants.

In a new experiment, the following hypotheses should identify mycorrhiza as the driving force behind enhanced drought resistance in peas.

(1) N fertilization up to a level of no response ensures that the lacking biological N_2 fixation in mutant P2 does not limit yields.
(2) Relatively high P fertilization excludes P effects without suppressing mycorrhiza.
(3) Both isolines perform equally when irrigated.
(4) Lower yields of non-mycorrhizal mutant P2 under drought stress indicate an intrinsic mycorrhiza effect independent of fertilization (see section1.5.5).
(5) The yield differences between the two isolines are useful to characterize the efficiency of native AMF of different sites under field conditions.

Three sites were chosen for direct comparison of mycorrhizal effects. In addition to Évora (Vertic Luvisol), a Ferric Luvisol under pasture land near Portel was selected in expectation of a strong mycorrhizal impact. This soil is characterized by high P absorption and low water holding capacity. The third soil at the relatively fertile horticultural site of Mitra (Humic Cambisol) was chosen because of its control properties: very low mycorrhization was found here in a pre-study and soil was regularly fertilized at high levels (see section 2.1 for further information about sites).

4.5.2 Materials and methods

It was planned to investigate following experimental factors:

(1) Sites (Évora, Portel, Mitra)
(2) Water (irrigated, drought stress)

(3) N fertilization (75, 150, 300, 450 kg ha^{-1})

(4) Isolines (FRISSON, mutant P2)

Factors (3) and (4) were arranged as a randomized block design split by the factor water within sites at 3 replications. The Irrigation treatment was planned as a drip irrigation by tubes between rows. Pressure valves every 40 cm of tube length should let through 10 mm water every 2-3 days in early morning or late evening hours during drought period. However at flowering, soil was still water saturated and therefore the irrigation system (Irrifruta Lda., Portugal) was not installed. Therefore, hypothesises (3) and (4) could not be tested. Extreme climatic conditions in 1996 affected also the second experimental factor. Nitrogen fertilization as NH_4NO_3 (26 %) was reduced *de facto* to the two lower levels of 75 and of 150 kg ha^{-1} N, respectively. The second fraction for the treatments 300 and 450 kg ha^{-1} N was not incorporated into the soil after the application on 21 May because there was no precipitation later. Unfortunately, the installation of the now preferable irrigation system was no longer possible. Consequently, replications of the experiment were increased to 6 for N75 and 18 reps for N150 on 144 plots in total. The experimental factors achieved were:

(1) Sites (Évora, Portel, Mitra)

(2) N fertilization (75, 150 kg ha^{-1})

(3) Isolines (FRISSON, mutant P2)

Soil tillage was done by single-axle rotary hoal at Portel and Évora and tractor rotary hoal at Mitra. At Portel, natural pasture vegetation (A-Tab. 23) was killed with herbicide (glyphosate 2 %) sprayed 8 days before tillage. Phosphorus (44 kg ha^{-1} P as triple phosphate), K (71 kg ha^{-1} K as K_2SO_4) and N were incorporated into the top soil before sowing.

Plot size was 2 x 3 m at 20 cm row distance. Higher quantities of seed material enabled, in contrast to years before, a seed density of 40 per m². However, because seeds of mutant P2 were still limited in stock they were sown only in inner 2 m² of plots. The remaining random half meter or two outer rows were sown always with three seeds per mound of provided commercial FRISSON (courtesy AGRI OBTENTIONS, Guyancourt, France). Within plots, two pea seeds, having multiplied in pots or trials the year before, were sown per mound.

The sowing procedure was facilitated by rectangular aluminum profiles (300 x 5 x 5 cm) perforated at the edge every 10 cm. A metal stick was drilled through these perforations exactly 3 cm into the soil. Seeds were dropped through the openings into the sowing mound and the correct depth was ensured by putting the stick in a second time. The procedure was

very efficient, enabled straight and equidistant rows, avoided sowing bed perturbation and guaranteed a safe change of germplasm at defined position even within rows.

Sowing was carried out on 30 March at Évora and 2 April 1996 at Portel. Unfortunately, high soil moisture delayed soil tillage at Mitra. Sowing was therefore delayed here until 11 April. Where seeds did not emerge or seedlings died, the blanks were re-sown at Évora and Mitra, but these plants developed late and were not taken in account at final harvest. However at Portel, random plants of the thinning procedure after 25 to 30 days, could be transferred successfully into the Frisson plots.

Trials were protected with nets against birds at the beginning and with 1 m high fences made from blue plastic fabric against rabbits. Green aphids, attacking peas at florescence at the start of June at Évora and Portel, were controlled by spraying pyrethrum solution. Weed control was done by hand. Weed pressure was particularly high at Mitra.

At 50 DAS, 4 plants of each isoline were taken at random from each block from N150 treatments for dry matter estimation, N and P analysis. Data for P were based on sample mixtures of 4 plants from each of 3 replications, whereas data for N were based on single plants (n=12). Fine roots of pea plants obtained at 25 and 50 DAS were stained with trypan blue to determine AMF colonization. Alkaline phosphatase staining was done for the later sampling date at site Évora and Portel (see section 2.5.4).

Soil water content was determined by the TDR (IMCO, Germany) method at 11-cm depth (18 replications per site or 6 per block, respectively) from 43 to 66 DAS. Unfortunately, no further measurements were possible because of a broken pre-drill made of stainless steel. This tool was needed to drill two equidistant holes before a mechanically sensitive TDR sensor could be put into the soil.

The inner 2 m² of plots were harvested at maturity at the end of June and at Mitra in early July, respectively. After harvest, soil samples from 0-20 and 20-40 cm soil depth were taken with a 8 cm ∅ core sampler from N75 plots (12 replications of 2 mixed samples per site) for analysis of N_{min} and P (Bray I). At Portel, the very stony soil only made it possible to take samples from the top 20 cm soil with a spade, and fine soil (< 2 mm) content was quantified gravimetrically. Plants of each plot were counted, harvested, separated into straw and grain, dried at 60 °C, and weighed. Plant material from N75 and N150 treatments was analyzed for N and P. Differences of grain constancy weight between 35 and 60 °C of 24 FRISSON samples were determined at **5.5** ± 1.0 % and dry matter for P2 grain samples, dried at 35 °C to enable further utilization, were corrected accordingly.

4.5.3 Results and discussion

4.5.3.1 Emergence

Emergence was low due to *damping-off* disease at Évora and Mitra but was significantly better at Portel (Tab. 25). Furthermore, it was possible at Portel to replace missing FRISSON plants by transplanting. Emergence of P2 plants was significantly less as compared to FRISSON at all sites, however only as a tendency at Mitra. Plant density was decreasing up to harvest, especially for mutant P2. Emergence at Évora was much better the two years before.

Tab. 25: Emergence at 14 DAS and final plant density of two pea isolines at harvest (PF 96) **Means** ± SD in (%) per sowing mound. Different letters indicate significant differences (Tukey's HSD) of both isolines between sites in a nested design (horizontal, capital) and between isolines (vertical, small).

	Évora		**Portel**		**Mitra**		**All**	
Isoline	Emer-gence	Plant density	Emer-gence	Plant density	Emer-gence	Plant density	Emer-gence	Plant density
Frisson	**62**±7.7**a**	**61**± 6.6**a**	**74**±9.1**a**	**93**± 3.0**a**	**58**±14.7**a**	**52**±12.8**a**	**65**±13**a**	**69**±20**a**
P2	**54**±9.9**a**	**48**±12.1**a**	**67**±7.2**b**	**64**±12.1**b**	**52**±13.4**a**	**43**±13.6**a**	**57**±12**b**	**52**±16**b**
All	**58**±9.7	**54**±11.5	**70**±9.0	**79**±16.8	**55**±14.4	**48**±13.9	**61**±13	**60**±19
	A	**B**	**A**	**A**	**B**	**B**		

4.5.3.2 Soil water content

The year 1996 was extraordinarily wet until mid May (A-Tab. 8). Soil water content (**means** ± SD of % v/v) at 11 cm depth was highest at Mitra, at **20** ± 5 (43 DAS) and **17** ± 6 (52 DAS), followed by Évora, at **13** ± 3 (55 DAS) and **9** ± 2 (66 DAS). At Portel, growing conditions may have been water restricted by a soil water content of only **9** ± 3 at 52 DAS during last the month of the growth period, in particular considering the very stony top soil (A-Tab. 9 and A-Tab. 12).

4.5.3.3 Biomass, P and N relation at first harvest

FRISSON grew better than P2 plants till first harvest (50 DAS) significantly at Portel and Mitra (Fig. 15). At Évora, biomass differences were not significant, as was also the case for N and P plant tissue concentrations. However, mutant P2 had a higher N but lower P concentration at Portel as compared to FRISSON.

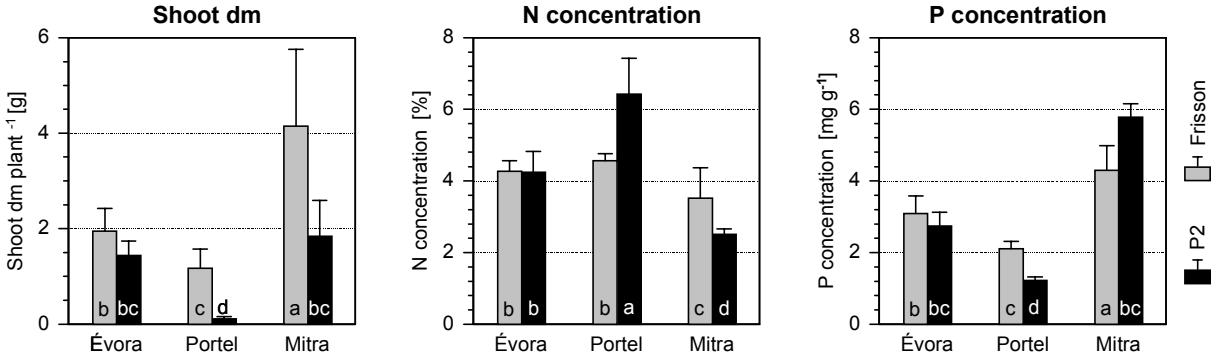


Fig. 15: Shoot dry matter, N and P plant tissue concentrations at 50 DAS in pea field experiment PF 96
Different letters show significant differences among isolines and sites (nested design; data based on 4 plants from each of 3 replications only of N150 treatment; Tukey's HSD after test for outliers and ln transformation for variable shoot dm; errors bars show SD)

This may indicate a sufficient N supply but P deficits. In contrast, the higher P but lower N concentration found in mutant P2 as compared to FRISSON may indicate the opposite for site Mitra: till 50 DAS, the P concentration was significantly highest, but the N deficit may have limited growth of P2 mutants of which some showed visible chlorosis at this time.

4.5.3.4 Mycorrhizal colonization and plant response till first harvest

AMF colonization was negligible at Mitra (Tab. 26) and confirmed the preliminary estimation in fine roots of lettuce in November 1995. The lack of AMF colonization may be related to a suppression by high soil P (compare section 4.5.3.8) and/or to former horticultural practice at this site, i.e. intensive use of pesticides. However, biomass between FRISSON and P2 differed by a factor of 2, which may be explained by the N concentration data in Fig. 15.

At Portel with a very high mycorrhizal status, biomass was very low but the isoline response (see in analogy definition of mycorrhizal response in section 1.3) was highest, with a relative difference of 90 %. The mutant P2 had obvious difficulties surviving, probably in the context of P deficits. No mycorrhiza was detected in P2 roots of all sites.

At Portel, AMF colonization increased strongly between 25 and 50 DAS and was characterized by a high abundance of arbuscules and few vesicles (Tab. 26).

Tab. 26: AMF colonization (chitin staining) of FRISSON at 25 and 50 DAS (PF 96) Estimation by grid line method (see section 2.5.4) with n=100 (**means** ± SD) arb. = arbuscules, ves. = vesicles.

Sample date		**25** days after emergence						**50** days after emergence					
Site		**Évora**		**Portel**		**Mitra**		**Évora**		**Portel**		**Mitra**	
AMF colonization [%]		**7** ± 7		**18** ± 4		**4** ± 4		**17** ± 6		**91** ± 3		**3** ± 3	
Frequency [%] arb./ves.		arb.	ves.	arb.	ves.	arb.	ves.	arb.	ves.	arb.	ves.	arb.	ves.
Classified	*low*	2	0	11	0	1	0	5	1	11	7	2	0
	medium	4	0	7	0	2	0	6	1	29	17	1	0
	high	0	0	1	0	0	0	6	0	48	0	1	0

The vital functional staining of the mycorrhizal alkaline phosphatase was rated for mycorrhizal colonization in Évora twice as high as the chitin staining (Tab. 27 compared to Tab. 26). The first was examined under a stereo microscope at double to 8-fold higher magnification as compared to the latter, which was observed by a dissection microscope (see section 2.5.4). Supposedly, this is caused more by the sensitivity of the respective microscopic technique on colonization rating than by the staining method itself (Tab. 27). However, the very dense colonization and very high abundance of arbuscules in fine roots of FRISSON from Portel indicated a highly active and well developed native mycorrhiza for this site. Although glyphosate was applied at a rate twice as high as recommended, AMF colonization seemed not to be impaired as reported for soybeans (MUJICA et al., 1999).

Tab. 27: AMF colonization (alkaline phosphatase staining) of FRISSON (PF 96) Estimation by stereo microscope (section 2.5.4) at 50 DAS (n=30 root pieces; **means** ± SD) Vesicles were seldom and not classified

Site	**Évora**	**Portel**
AMF colonization [%]	**46** ± 26	**100** ± 0
[%] *of arbuscules classified:*		
(none)	10	0
very low	15	6
low to medium	11	12
medium to high	6	31
very high	4	52

4.5.3.5 Yields at final harvest

Hypothesis (1) could be proven by examining for the two lower levels of N fertilization, which were, however, the most sensitive treatments regarding a possible N response. It was a prerequisite of the experiment that the growth of the mutant P2 as the non-mycorrhizal control in the field bioassay was independent of possible N deficits. Although only two of four planned N fertilization levels were realized in the field experiment, the ANOVA contrast analysis allows a comparison of the production of mutant P2 with a N fertilization of 75 and 150 kg ha^{-1}, respectively (A-Tab. 24). Differences were acceptably low concerning the objective of the experiment, as indicated by high p-values, i.e. for grain production over all sites as high as 0.98. No N effect was detected for plant and plot related production variables.

However, this interpretation is restricted by the results obtained at first harvest (50 DAS) at Mitra, although no N fertilization effect on straw N concentration was detected at final harvest (A-Tab. 25). Consequently, hypothesis (1) can only partly be affirmed regarding those restrictions concerning site Mitra. Nevertheless, the data in the following tables and figures were presented for better comprehensibility as reanalysis across all N treatments.

The differences of yields at final harvest were similar to the estimation of the intermediate harvest. Significant differences in grain yield varied in relation to reference unit. Calculated per plant, sites follow the order Mitra > Évora > Portel with FRISSON significantly better than mutant P2 (Fig. 16). Calculated per area unit at final plant stand, differences between Mitra and Évora are omitted across isolines (Tab. 28) and for variable straw yield (data not shown) within isolines. The plant specific variable considers the low production at the final plant stand and may be more relevant for the objectives of the experiment. Emergence was influenced by phyto-sanitary problems which were not the focus of that experiment although interaction of AMF with other phytopathogenic fungi responsible for *damping-off* disease are possible. The sites Évora and Mitra were much more susceptible to *damping-off* than the marginal nature near site Portel, supposedly due to the cropping history and soil moisture.

Hence, the shoot production per plant (Fig. 16) did not differ between isolines significantly at Mitra where almost no AMF colonization was found in contrast to the other two sites. However, the interpretation of the non-significant growth difference between isolines at Mitra is restricted because of the high variation and conflicts with the data obtained at 50 DAS.

Simultaneously with the grain yield differences, the harvest index for mutant P2 was lower at Mitra (Tab. 28), not at Évora. At Portel, the difference is the clearest on very low

levels for both isolines, and hypothesis (5) can be confirmed in the context of high mycorrhizal colonization.

Inefficiently controlled yield losses by aphids may have contributed to the fact that peas at Portel achieved only about half of the harvest index at the other two sites. Another reason for the low yield and harvest index at Portel was grain size, which was 20 % smaller than that at the other two sites (Tab. 28).

Tab. 28: Yields and yield components of two pea isolines in field experiment PF 96
Means ± SD. Small letters indicate significant differences between isolines at each site and capital letters across isolines. (Tukey's HSD after ln transformation for dm, n=24 plots). Hundred grain weight (HGW) was determined by subsamples of mixtures from each isoline of 3 sites.

Site	Isoline	Grain dm [g m^{-2}]	Shoot dm [g m^{-2}]	Harvest Index [%]	HGW
Évora	Frisson	**72.0** ± 15.1 **a**	**173.5** ± 47.4 **a**	**42.5** ± 4.7 **a**	**12.7**
	P2	**40.1** ± 13.6 **b**	**99.1** ± 33.3 **bc**	**40.2** ± 2.1 **ab**	**11.9**
Portel	Frisson	**20.1** ± 5.9 **c**	**74.5** ± 21.4 **c**	**28.5** ± 11.1 **c**	**10.7**
	P2	**3.8** ± 1.9 **d**	**15.5** ± 5.8 **d**	**23.7** ± 4.4 **d**	**10.3**
Mitra	Frisson	**97.8** ± 46.9 **a**	**210.6** ± 101.6 **a**	**46.3** ± 4.7 **a**	**12.9**
	P2	**52.2** ± 21.8 **b**	**127.0** ± 53.5 **b**	**40.9** ± 3.2 **b**	**12.3**
Évora	all	**56.3** ± 21.2 **B**	**136.3** ± 55.3 **A**	**41.6** ± 3.6 **A**	**12.3**
Portel	all	**12.0** ± 9.3 **C**	**45.0** ± 33.4 **B**	**26.1** ± 8.7 **B**	**10.5**
Mitra	all	**76.0** ± 42.7 **A**	**168.8** ± 91.3 **A**	**44.1** ± 4.6 **A**	**12.6**

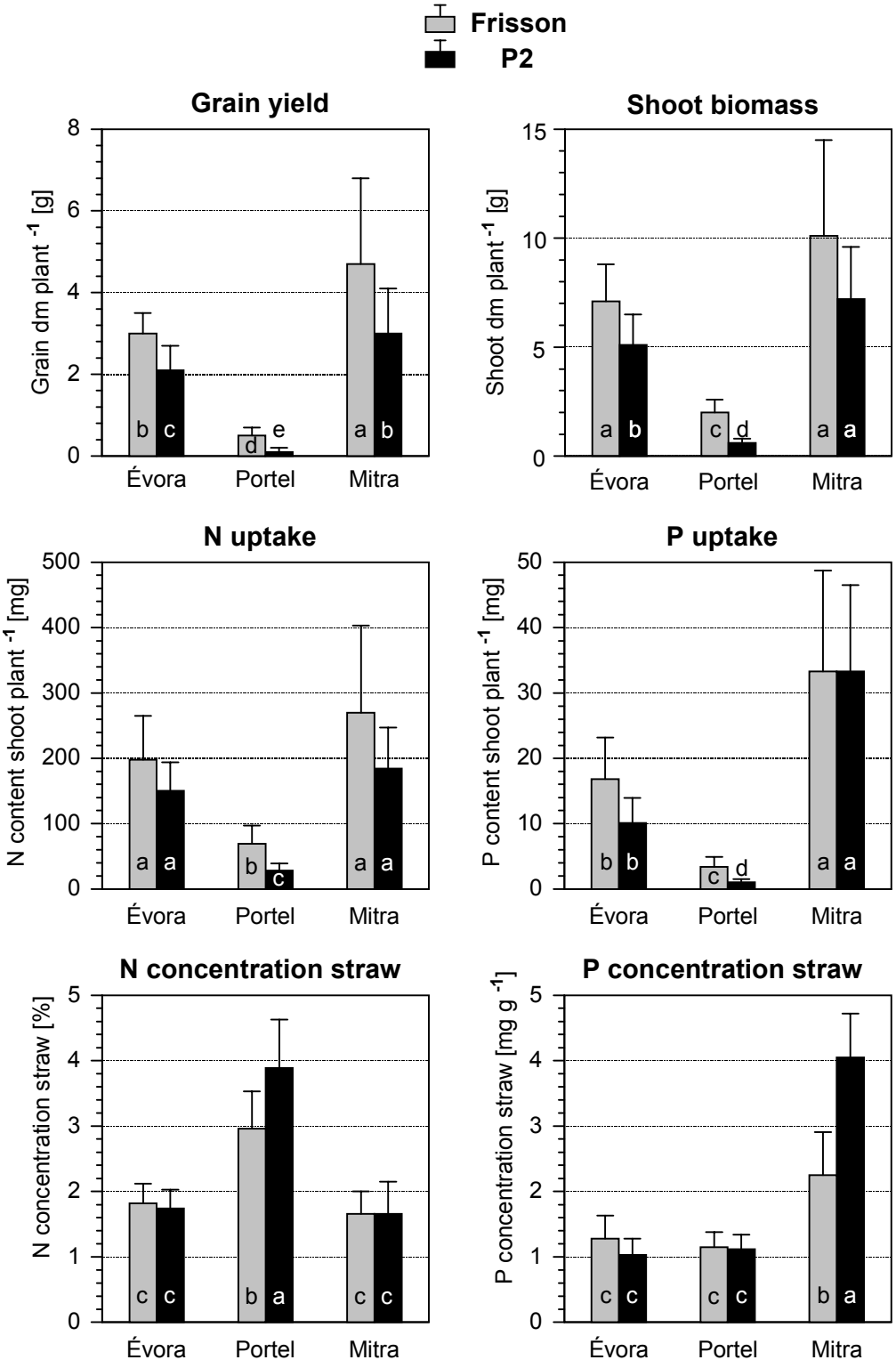


Fig. 16: Grain yields and shoot dry matters, shoot uptakes and straw concentrations of N and P of two pea isolines in field experiment PF 96 at three sites

Different letters indicate significant differences between FRISSON and P2 at maturity (Tukey's HSD after ln transformation; reanalysis across N levels: n = 24 plots of 2 m² at means of 8 to 78 plants). Error bars are SD. For more detailed data, see A-Tab. 27.

4.5.3.6 Relation of N and P in plants at final harvest

The concentrations of N and P in grain and straw, and the shoot content, respectively, differed between sites and isolines (A-Tab. 27). Within pea lines, there was no response to N fertilization (data not shown), with only one exception: at Portel, the straw N concentration in FRISSON was significantly increased (Tukey's HSD) by N fertilization from 2.5 % to 3.8 % straw N concentration. Nodulated roots of FRISSON were scarcely observed at the thinning procedure and first harvest. Therefore, it can be assumed that also FRISSON depended on mineral N at Portel.

The impact of N fertilization on grain N concentration of mutant P2 was significant in contrast analysis (A-Tab. 25), however not when tested by Tukey's HSD for each site. Across sites and isolines, the grain N concentration was significantly increased by N fertilization from 4.6 % to 4.9 % grain N concentration (no other significant differences; data not shown). Concerning sites independent of isoline, the order both of grain and of straw N concentration showed the opposite pattern as compared to yields.

Grain N concentration : Portel (5.2 % a) > Évora (4.6 % b) > Mitra (4.4 % c)

Straw N concentration : Portel (3.4 % a) > Évora (1.8 % b) ≈ Mitra (1.7 % b)

(different letters indicate significant differences according Tukey's HSD; other data not shown)

Although straw N concentration at Portel was the highest, N uptake of mutant P2 was significantly lower by 60 % as compared to FRISSON due to extremely low biomass. At the two other sites, differences between isolines were not significant either for N or for P uptake (Fig. 16).

The straw and grain P concentration and consequently P uptake were clearly highest at Mitra (A-Tab. 27) and differences between isolines in straw P concentration were significant (Fig. 16). Évora was the only site where FRISSON was higher in grain P concentration as compared to mutant P2 (A-Tab. 27). Better growth enhanced P uptake in comparison to site Portel at similar P concentration levels. In contrast to the other two sites, FRISSON could absorb more P than the mutant P2 at Portel.

4.5.3.7 Discussion with other mycorrhiza field-pea studies

The first field trial with FRISSON and mutant P2 of LOVATO (1994) in plots of 3 rows of 80 cm length with 3 replications at a fertilization of 500 kg N ha^{-1} and 41 µg g^{-1} plant available P resulted in a mycorrhizal response of 28 %, measured as relative growth difference between isolines. The differences lost significance at a fertilization of 44 kg ha^{-1} P which

increased available P to 60 µg g^{-1}. The isoline effect was interpreted as compensation of P deficits by native AMF. In contrast, introduced *G. intraradies* and *G. mosseae* had no effect in steam disinfected re-inoculated plots in which the same fertilization resulted in a rise from 36 to 42 µg g^{-1} available P. However, no response on P fertilization either of FRISSON or of mutant P2 was observed. Grain yields achieved other dimensions with 437 to 670 g m^{-2}, at 3-fold for FRISSON and even 10-fold for P2. However, the French experiment was irrigated.

In consideration of JAKOBSEN (1987), who also reported no effects of AMF inoculation on pea yields in disinfected and non-disinfected plots, the amount of applied P was regarded as too low for substituting the mycorrhizal benefit of P uptake. However, JAKOBSEN found a significant response of 10 % yield difference on P fertilization of 60 kg ha^{-1} P which increased available P ($NaHCO_3$ extraction) from 16 to 29 µg g^{-1} and 19 to 32 µg g^{-1} in DAZOMET® disinfected plots, respectively. The P uptake, especially in seeds, tended to be higher in AMF inoculated treatments.

4.5.3.8 Experimental difficulties

The soil at Mitra was regularly fertilized over years with high amounts of manure in fall. Unfortunately, no information about composition and amount of the fertilizer was available. Although P soil concentration after harvest was very low (A-Tab. 26), high P uptakes and best yield suggested that P supply was probably not the limiting factor at this site. As a consequence, this result raises questions about the Bray I method used to determine the plant available soil P here. It can be speculated that mineralization of organic P was a considerable source of P for the pea plants.

Emergence problems and weed pressure were very serious at Mitra despite enormous efforts which were undertaken to control weeds by hand.

Growing conditions were affected by late sowing, especially at Mitra. The resulting faster growth may have reduced the mycorrhizal response (see section 1.7.2). Growing rate may interact with mycorrhiza effects during the process of (over-) compensating possible early parasitic relations (SMITH & READ, 1997) by a plant effective mutual relation of symbiosis.

A general conceptual problem of comparing the two isolines for the determination of the mycorrhizal effect may be the exudation of H^+ into the rhizosphere. This results from N reduction during the process of biological N_2 fixation (BNF) and subsequent nitrate assimilation (MARSCHNER, 1995), when N_2 fixation cannot be suppressed in the wild type. Symbiotic peas usually receive about 70 % of their N from this source under field conditions

(WERNER, 1987). However, the theory conflicts with data obtained by BETHLENFALVAY and co-workers (1997): a highly significant elevation of soil pH was found in pots cultivated with mycorrhizal N_2 fixing peas as compared to mineral N supplied peas. The AMF colonization itself increased also pH in comparison to non-mycorrhizal. The availability of mineral soil P for plants depends on pH within a large range of soil acidity (MARSCHNER, 1995; HINSINGER, 2001). The use of mycorrhizal isolines which do not fix any N_2 could help to elucidate this problem.

4.5.3.9 Conclusion

Differences in P uptake mirrored in a way the mycorrhizal status at the three sites. At Portel, highly colonized FRISSON took up more than three times the P as isoline P2. The AMF resistant mutant suffered here from P deficits and hypothesis (2) must be rejected for this site. At Évora, with little AMF colonization, the difference was still 50 %, but was not significant. At Mitra, the site with control properties with almost no mycorrhiza, the P uptake of the two isolines was equal (Fig. 16). Hypothetically, no differences between the two isolines were also to be expected because there was neither a potential nor a need for mycorrhiza symbiosis at least concerning the P nutrition. Strikingly, only the P uptake unambiguously confirms this hypothesis, whereas the information value of shoot biomass is restricted by high CV. Finally, hypothesis (5) must be rejected here regarding the variable grain yield (Fig. 16). However, this is related to the rejection of hypothesis (1) for site Mitra in context with possible N deficits in P2 mutants during early growth (Fig. 15).

In any case, it is advisable to interpret not only the yield data but also to include the difference in P uptakes between isolines as an efficiency criterion and N uptakes and plant tissue concentrations as control criteria. The evaluation of an efficient mycorrhiza functioning in crop production must be completed by AM staining. In the case of site Portel the combined data supported each other and confirmed that AMF are very efficient under field conditions.

4.6 Comparison of mutants with symbiotic deficiencies of the vars. FRISSON and SPARKLE in a field experiment at site Mitra (PF 96 b)

4.6.1 Introduction

The utilization of mutants of var. SPARKLE is a potential solution for the nitrogen dilemma which may occur when FRISSON is compared to non-fixing mutant P2 as a non-mycorrhizal control. The variety SPARKLE is an early flowering garden pea originating from

Idaho, USA, and is cropped for human nutrition (so called *freezer* pea) in contrast to the forage pea variety FRISSON which originates from France, near Paris.

The mutant E135 (*sym* 13: *myc* $^{+}$ *nod* $^{+}$ *fix* $^{-}$) is mycorrhizal, in contrast to R25 (*sym* 8: *myc* $^{-1}$ *nod* $^{-}$), although both isolines are not capable of BNF (LaRUE & WEEDEN, 1994; BALAJI et al., 1994). In a first pot test trial SPARKLE and E135 were well colonized by AMF, whereas mutant R25 grew less well and no mycorrhizal structures were detected (A-Tab. 20). Site Mitra was chosen for the best control conditions and highest soil fertility in order to obtain good seed multiplication. The isolines FRISSON and mutant P2 were included in the experiment to allow a direct comparison. The following question should be answered:

Are mutants R25 and E135 of var. SPARKLE suitable indicator plants for evaluating mycorrhiza efficiency under field conditions, without being influenced by N effects?

4.6.2 Materials and methods

The experimental layout was planned as a 2-factorial randomized block design split by first factor water (irrigated, drought stress) with 4 replications. As in the field experiment PF96 at three sites, an irrigation treatment was omitted and the design was reduced to the only experimental factor pea line at 8 replications. The five lines from two varieties were:

(1) FRISSON parental line symbiotic character *myc* $^{+}$ *nod* $^{+}$
(2) FRISSON mutant P2 symbiotic character *myc* $^{-1}$ *nod* $^{-}$
(3) SPARKLE parental line symbiotic character *myc* $^{+}$ *nod* $^{+}$
(4) SPARKLE mutant E135 symbiotic character *myc* $^{+}$ *nod* $^{+}$ *fix* $^{-}$
(5) SPARKLE mutant R25 symbiotic character *myc* $^{-1}$ *nod* $^{-}$

Soil tillage was done by tractor rotary hoal. Fertilizers of N (26 %) as NH_4NO_3 at 300 kg ha^{-1} N, P (20 %) as triple phosphate at 44 kg ha^{-1} P, and K (17 %) as K_2SO_4 at 71 kg ha^{-1} K, respectively, were incorporated into the top soil before sowing. Plot size was 1 x 2 m and plant density was 25 seeds per square meter. Due to lack of seed material, mutants P2 and R25 were sown only in the inner 1 m². The remaining random margin rows, as well as first and last 50 cm of all rows were sown with FRISSON. Sowing was done on 11 April 1996 with the help of rectangular aluminum profiles at 3 cm depth (see section 4.5.2). Distance within rows was 20 cm. All seeds were treated with fungicides CARBENDAZION® and TIRAME® (1:1) because of previously observed emergence difficulties in multiplication pot cultures of SPARKLE which is characterized by a crinkled grain surface. The trial area was protected with 1 m high fences made of blue plastic fabric against rabbits and with nets against birds in the first 6 weeks. Weed control was repeatedly done by hand. After 50 DAS, two plants of each

isoline were taken at random from each block for dry matter estimation and fine roots of FRISSON, SPARKLE and E135 were stained with trypan blue to determine AMF colonization. At 82 DAS, plants of the inner 1-m² plot were counted, harvested, separated into straw and grain, dried at 35 and 60 °C, and weighed. All data were adjusted to constant weight 60 C°. Soil water content (v/v) was determined by the TDR (IMCO, Germany) method at 11 cm depth (8 replications respectively 2 per block) at 43 and 52 DAS.

4.6.3 Results and discussion

Soil water content (**means** ± SD of % v/v) was higher, at **24** ± 2 (43 DAS) and **21** ± 5 (52 DAS), than in the large experiment PF 96, which may be related to lower plant density. Especially the mutant R25 emerged badly. The shoot dry matter at 50 DAS of about 1.4 g from SPARKLE isolines did not differ significantly and AMF colonization was negligible, at about 3 % both for SPARKLE and E135, as for FRISSON in experiment PF 96. Compared with experiment PF 96, emergence was 10 percent points higher for FRISSON, while only 3 for mutant P2, each at a high level of variation. Also, FRISSON grew by 2.5 g shoot biomass per plant better at the lower plant density and at a double rate of N fertilization. Grain yield per plant was almost the same in both trials.

Tab. 29: Emergence and yields in pea field experiment with 5 lines (PF 96 b)
Different letters show significant differences (Tukey's HSD for unequal Spjotvoll/Stoine after test for outliers and ln transformation except harvest index. Dry matter for plants are **means** ± SD of 6 to 40 plants from 2 m²) Additionally letters in frames concern separate ANOVA models (FRISSON vs. SPARKLE in second column and P2 vs. E135 in third column). Better homogeneity of variance makes it possible to do without ln transformation in part here*.

Pea line	Emergence [%]		Grain yield [g m^{-2}]			Grain dm plant^{-1} [g]		Shoot dm [g m^{-2}]			Shoot dm plant^{-1} [g]			Harvest Index [%]	
*Transformation**	ln		ln					ln ln			ln				
Frisson	**69** ±11	a	**67.6** ±23.1	a	a	**5.35** ±1.45	a	**133.0** ±42.0	a	a	**9.57** ±1.84	a	a	**51.1** ±2.3	a
P2	**47** ±13	b	**28.0** ±12.0	bc	a	**2.81** ±0.63	b	**58.1** ±17.2	bc	a	**5.42** ±1.06	b		**48.2** ±6.9	ab
Sparkle	**35** ± 6	b	**30.2** ± 7.4	ab	b	**2.77** ±0.64	b	**65.1** ±13.4	ab	b	**6.69** ±1.62	ab	b	**42.1** ±5.1	bc
E135	**40** ± 2	c	**15.0** ± 6.4	c	b	**1.32** ±0.47	c	**35.8** ±12.4	c	b	**2.95** ±0.57	c		**41.9** ±3.2	bc
R25	**20** ± 6	b	**5.4** ± 2.5	d		**1.23** ±0.39	c	**12.3** ± 5.9	d		**3.09** ±0.70	c		**38.9** ±4.3	c

The parental symbiotic line of the forage variety FRISSON exceeded SPARKLE in most production variables by a factor of two, and by one third for shoot biomass per plant. The biomass production per plant of SPARKLE mutants did not differ, whereas threefold higher grain yield and double shoot dry matter per m² were noted as caused by half emergence rate of R25 compared to E135. Differences between wild type and *myc* $^{-1}$ mutant were more extreme for the var. SPARKLE than for var. FRISSON.

Generally, SPARKLE was not adapted for the growing conditions, in particular when sown too late. Mutant E135 and R25 should be tested for possible genetic defects other than symbiotis under more favorable conditions.

Under ample N supply in a pot experiment carried out by MARKWEI and LaRUE (1992), R25 reached only 80 % of the plant dry matter of wild type SPARKLE after 5 weeks. This difference was not significant, although it was significant 2 weeks before. The defect of the *sym* 13 gene in mutant E135 already has a multiple effect (KNEEN et al., 1990).

KAHILUOTU and co-workers (2000) used mutants R72 (*sym* 10: *myc* $^{-}$ *nod* $^{-}$) and N15 (*sym* 9: *myc* $^{+}$ *nod* $^{-}$) of var. SPARKLE in greenhouse experiments and a field trial in Finland in a similar approach to that in the work presented. However, they reported only a small mycorrhizal growth response of N15 and, unfortunately, not absolute biomass production. In contrast to E135 (KNEEN et al. 1990), R25 and R72 (MARKWEI & LaRUE, 1992), the mutant N15 is not referred to so far in the literature. Reports about symbiosis deficient pea mutants must be treated with some care. For historical reasons, plant breeders and genetic engineers were focusing on the symbiosis with *Rhizobia* in their work with pea mutants. The fact of suppressed arbuscular mycorrhiza in non-nodulating pea mutants, firstly reported by DUC and co-workers 1989, was sometimes withheld either out of ignorance (MARKWEI & LaRUE, 1992) or to avoid a discussion about unwanted implications (SAGAN et al., 1993).

However, LaRUE and WEEDEN suggested in 1994 a hypothesis that nodulation evolved, at least in part, from the much more ancient arbuscular mycorrhizal association. As AMF, *Rhizobia* do not induce defense responses. They are very heterogeneous with probably only the formation of the *nod* factors in common. These oligo-glucosamines of a “chitin-like” structure are very rare in bacteria (LaRUE & WEEDEN, 1994) but not in fungal walls. “The evolution of the nod factors may have permitted free-living nitrogen fixing bacteria to invade plant roots without provoking a defense response if the nod factor was perceived by the plant as a signal from compatible mycorrhiza.” Put plainly, this means that during evolution *Rhizobia* “stole” the “password” from AMF to enter the protected plant root. Very recently,

molecular biology research of on *Rhizobia* became aware of the common genetic mechanism of the two symbioses (ALBRECHT et al., 1999; RUIZ-LOZANO et al., 1999; MARSCH & SCHULTZE, 2001).

The methodological problems in the experiments presented underline the importance of not just studying the symbiotic defects in isolation for the micro-symbiont AMF or *Rhizobia*.

Unfortunately, existing *myc* $^{+}$ *fix* $^{-}$ mutants of var. FRISSON were not available even on personal request due to a very restrictive distribution policy of the Station de Génétique et Amélioration des Plantes INRA, Dijon (see also KAHILUOTU et al., 2000).

4.7 Soil borne pathogens at experimental sites

4.7.1 Introduction

Soil borne pathogens were a serious problem for pea cropping at the studied sites. At Elvas, Évora and Mitra, emergence in 1996 was reduced by *damping-off* disease caused by not further specified fungi, putatively *Pythium* spec. (section 4.4.2, section 4.5.3 Tab. 25, section 4.6.3 Tab. 28.). Generally, peas were susceptible to the following soil borne phytopathogenic fungi: *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium solani*, *Fusarium oxysporum* (KRAFT et al., 1994), and *Aphanomyces euteiches* (SLEZACK et al., 1998). At Évora, in September 1996, Red Crown Rot caused by *Calonectria crotalariae* was identified (by courtesy of McGAWLEY) in volunteers from a former pea field trial. Very common in high quantities was *Rhizoctonia solani* at all sites, especially at Portel. Their microsclerotia formed a large (undesirable) part in soil extracts beside the AMF spores. *R. solani* was identified in petri dishes with water agar by its characteristic rectangular branching patterns of septated hyphae (AGRIOS, 1978) growing from surface sterilized microsclerotia (by courtesy of McGAWLEY). *Rhizoctonia* is known for being the main soil borne phytopathogenic fungus in the Alentejo (per. comm. GONÇALVES, ENMP). However, isolates of other anastomosis groups, also called binucleate *Rhizoctonia* (BNR) are soil saprophytes or colonize as endomycorrhizal fungi orchids (POPE et al., 1998). Most BNR are considered non-pathogenic or weakly pathogenic to cultivated plants (BURPEE & GOULTY, 1984). Severe damage is supposedly caused by phytopathogenic nematodes. They should be examined quantitatively and qualifiedly with the help of a proved expert at two sites.

4.7.2 Materials and methods

Soil samples were taken on 18 November 1996 with a mechanical hand planter and processed the following days. At Évora, soil from 8 mixed subsamples in 4 blocks (n=4) were taken of a chickpea trial at sowing near the pea field of experiment PF 96. At Portel, 12 samples were taken directly from the former pea field of PF 96 as a transect from 3 blocks (n=12) and the soil was sieved at 2 mm due to high rock contents.

For nematode extraction, 500 g of field fresh soil were wet sieved on 45 µm and centrifuged in tap water at 1500 rpm for 5 min. After decanting, the pellet was subsequently homogenized in sugar solution (456 g in 1000 ml water) and centrifuged again for 1 minute at 1500 rpm gently running out without brake. The supernatant was washed in a 45 µm sieve and transferred into graduated petri dishes for identification and counting under a dissection microscope (courtesy McGAWLEY).

4.7.3 Results

Very high levels of phytopathological nematodes were found in Évora, whereas both quantities and qualities of nematodes were barely harmful in the soil from Portel (McGAWLEY per. comm., 1996) as shown in Tab. 30.

Tab. 30: Quantification of nematodes at the genus level in soils from Évora and Portel
Counts were per kg field fresh soil (**means** ± SD: at Évora with n=4, at Portel with n=12). *Rotylenchulus** were juveniles.

	Criconemella	*Helicotylencus*	*Tylenchorynchus*	*Hoplolaimus*	*Pratylenchus*	*Paratylenchus*	*Xiphinema*	*Rotylechulus**	**All**
Évora	**31** ± 29	**98** ± 70	**47** ± 34	**12** ± 12	**6** ± 6	**6** ± 11	**0.5** ± 0.9	**0**	**200** ± 133
Portel	**3** ± 5	**42** ± 24	**5** ± 6	**0.4** ± 1.3	**0**	**0**	**0**	**16** ± 26	**67** ± 48

5 Pea experiments in a Ferric Luvisol of a high AMF potential

5.1 Introduction

From previous pot and field experiments, it was concluded that soil from site Portel constitutes the most interesting environment for evaluating mycorrhizal effects by growth differences between FRISSON and the AMF resistant isomutant P2. Native AMF, which had not yet been characterized, were found to be effective at low yield levels and good emergence of field-peas. Low pressure by phytophagous nematodes was favorable. However, peas are susceptible to the root rot induced by *Rhizoctonia solani* (KRAFT et al., 1994) which was strongly abundant in the soil (see section 4.7). Root damage may reduce N uptake of mutant P2 more than of FRISSON. Consequently, such an impact on growth difference between isoline could occur independently of focused mycorrhiza effects. Root damage can be caused by soil acidity. As long as N is not limited in mutant P2 by a reduced root surface, possible isoline growth differences would indicate a benefit of the mycorrhizal symbiosis against soil acidity. The ferric properties of the soil have a high potential to absorb fertilized P from the plant available P pool (see A-Tab. 11 and A-Tab. 15). Consequently, P uptake may be decisive for growth differences of isolines in field experiment PF96 at Portel, although P fertilization was high. The mutant P2 hardly survived at much higher straw N concentrations, as compared to FRISSON. Additional pot experiments on soil acidity, soil borne pathogens, N and P fertilization should explain why.

5.2 Estimation of AMF spores

5.2.1 Introduction

In contrast to other sampled soils, site Portel was not investigated for AMF spores by other participants of the EU project (section 2.5.1). Therefore, characterization of AMF and enumeration of spores was caught up on comparing two different extraction methods. The objective was to obtain data for comparison with the other examined soils.

5.2.2 Materials and methods

The soil samples were the same as for the quantification of nematodes at Portel (section 4.7.2). The procedures for AMF spore extraction according to the sugar gradient and split centrifugation method are described in sections 2.5.2 and 4.7.2. The latter was the same as the

processing used for nematodes. The typification and counting of AMF spores was according to the method explained in section 2.5.3.

5.2.3 Results and discussion

The typification resulted in 6 different classes (Tab. 31). At species level, *Glomus mosseae* and *Glomus invermaium* could be determined. The dominating spore type was probably the same hyaline *Glomus spec.* which was frequent in other soils of the region (see section 2.5.1). Apart from others, a hyaline *Scutellospora spec.* and yellow to brown colored spores of genus *Acaulospora* were found. For further taxonomic details see section 2.5.1 and A-Tab. 1.

Tab. 31: Enumeration of AMF spores by two different extraction methods in a Ferric Luvisol

Gradient method is sugar gradient centrifugation whereas split method is split centrifugation in tap water followed by centrifugation in sugar solution (see section 2.5.2). Numbers of spores are **means** ± SD from 12 soil samples. Total numbers of spores are calculated for 50 g field fresh soil and g dry soil, respectively.

Spore type	*Glomus mosseae*	*Glomus invermaium*	*Glomus spec.*	*Scutellospora spec.*	*Acaulospora spec.*	others	**total** $(50\ g)^{-1}$	**total** g^{-1} dry soil
Gradient	**3.8** ± 3.0	**11** ± 11	**63** ± 36	**6.8** ± 3.3	**3.9** ± 1.8	**19** ± 13	**95** ± 35	**2.6** ± 1.2
Split	**4.8** ± 3.5	**56** ± 19	**116** ± 44	**13.2** ± 7.5	**13.1** ± 9.7	**59** ± 27	**263** ± 67	**6.1** ± 1.3

However, number of spores was not regarded as a valuable variable to differentiate mycorrhiza efficiency between sites. Firstly, accuracy of quantitative spore extraction was limited as shown in Tab. 31 by high CV and large differences between the two centrifugation methods in the extraction procedure. Secondly, taxonomy was in some cases so difficult that even experts could not achieve uniform results (see section 2.5.1) and errors in literature are very abundant (see section 1.5.1). Thirdly, the information of those counts is ambiguous: viability of spores is difficult to distinguish and spore size can vary by a factor of around 15. Nevertheless, the two features have high ecological importance (BRUNDRETT et al., 1996).

Similar (total) AMF spore numbers of about 2 to 5 ml^{-1} soil were counted from soils out of pots in which chickpea and safflower plants were cultivated for a bioassay (DIEDERICHS et al., 1996). Counts from the Ferric Luvisol (cf. Chromic Luvisols) were the highest compared to the other soils examined in the presented work.

Spore populations do not necessarily reflect the contribution of the micro symbiont AM fungi on AM symbiosis (SMITH & READ, 1997). The absolute proof of which AMF is colonizing the plants in the field is a challenge for molecular biological techniques such as immunological (HAHN et al., 1999), PCR-based (CHELIUS & TRIPLET, 1999) and fatty acid methyl ester profiles (GRAHAM et al., 1995), but it was not possible to perform them within the framework of the presented research.

5.3 Germination bioassay on liming (PP 97 a)

5.3.1 Introduction

The soil from site Portel (section 2.1, Tab. 1) is characterized by low pH and high Fe, Al, and Mn concentration (A-Tab. 10, A-Tab. 15, and A-Tab. 18). While manganese toxicity may be limited by relatively high magnesium stocks (A-Tab. 11; MARSCHNER, 1995), aluminum could create serious problems for root development of pea roots, especially of root hairs and apical meristem (RUNGE & RODE, 1991). Root surface may be crucial for N uptake of non-symbiotic mutant P2. Root variables can be assayed independently of photosynthesis by a simple germination experiment. The objective was to check possible (but undesirable) side effects of soil acidity on the two isolines in relation to N uptake. To prove whether toxic cations can harm pea roots germinating into the Ferric Luvisol, liming was chosen as a control treatment.

5.3.2 Materials and methods

Experiment was arranged as a 2-factorial complete randomized block design with isolines FRISSON and P2 as 1st and liming (1.67 % w/w $CaCO_3$) as 2nd factor at 4 replications. The pH of untreated fine soil in H_2O was 5.5, and 4.5 in KCl. The addition of 1.67 % (w/w) calcium carbonate, corresponding to 5 t $CaCO_3$ ha^{-1}, increased pH to 7.0, both in water and KCl. The double dose, equivalent to 10 t ha^{-1}, did not change pH further after magnetic stirring for 24 hours. Cone pots were filled with 350 g air dried soil screened on 1.25 cm, corresponding to 160 g fine soil, due to a content of pebbles and cobbles of about 46 %. Two peas of a uniform selection of the two isolines were sown per pot. The soil was moistened every 2-3 days with 25 ml demineralized water. Germination was allowed for 14 d at 25°C in a dark incubation oven. Peas were separated subsequently for shoots and roots. Roots were stained in 1 % methyl blue solution for contrast improvement and scanned on an HP Desk Scan II (Hewlett Packard, USA) using CIAS Image Analysis Software (CID Inc., USA) for root length and area

calculation. Dry matter was determined for roots and shoots. Root hairs were observed qualitatively at 50 x magnification under a dissection microscope.

5.3.3 Results and discussion

No damage of roots or root hairs was detected which would otherwise indicate Al toxicity (RUNGE & RODE, 1991). No differences were found in biometric variables of germinated peas, neither affected by liming nor by isoline (Tab. 32). Consequently, no severe decrease of pea root growth or function by high soil acidity of Ferric Luvisol can be assumed during the early growing period.

Tab. 32: Germination bioassay on liming with two pea isolines in a Ferric Luvisol (PP 97 a) Biometric variables (**means** ± SD, n=4) after 14 d dark germination at 25°C. Experimental factors were isolines (**I**) FRISSON and mutant P2 and liming (**L**).

Isoline	Liming [% $CaCO_3$]	Plant dm [mg]	Root dm [mg]	Root length [cm]	Root surface [cm²]
Frisson	0	**64.7** ± 2.3	**6.7** ± 0.7	**84.0** ± 14.7	**10.7** ± 1.4
Frisson	1.67	**67.3** ± 4.7	**6.8** ± 0.8	**81.4** ± 9.3	**10.7** ± 1.4
P2	0	**67.7** ± 3.3	**6.6** ± 0.7	**96.9** ± 10.5	**11.4** ± 1.4
P2	1.67	**66.8** ±12.3	**7.4** ± 1.3	**91.4** ± 10.4	**11.3** ± 1.2
All		**66.6** ± 7.0	**6.9** ± 1.0	**88.4** ± 12.9	**11.0** ± 1.4

ANOVA	Factors:	**I**	**L**	**I x L**	**I**	**L**	**I x L**	**I**	**L**	**I x L**	**I**	**L**	**I x L**
	F-value:	0.10	0.05	0.19	0.20	0.85	0.50	3.01	0.38	0.05	0.67	0.01	0.01
	P-level:	0.76	0.83	0.67	0.66	0.38	0.49	0.11	0.55	0.82	0.43	0.94	0.94

5.4 Interrelation of mutualistic and phytopathogenic fungi with *myc* $^{+}$ and *myc* $^{-1}$ peas (PP 97 b)

5.4.1 Introduction

Arbuscular mycorrhiza fungi may function as bioprotectors against soil borne phytopathogenic fungi (section 1.3, LINDERMAN 1994). Field experiments with peas in 1996 were strongly affected by *damping-off* disease (section 4.7.3). Root rot by *Rhizoctonia solani* may create severe damage for pea cropping (section 5.1). Although emergence difficulties in 1996 were not as dramatic as at other sites, high amounts of microsclerotia of

Rhizoctonia built up a high infection potential at site Portel (section 4.7.1). *Rhizoctonia* multiplies readily and root rot can be detected macroscopically. An AMF inoculum with *Glomus manihot* was chosen due to the lack of a pure culture of an autochthonous AMF species.

The objective of the experiment was to prove whether AMF can reduce root rot in peas and mutant P2 is a suitable non-mycorrhizal control for a bioassay on the interrelation between mutualistic and phytopathogenic fungi.

5.4.2 Materials and methods

As a consequence of the non-efficient AMF inoculum in experiment PP 95, a new pure culture of *Glomus manihot* (IAT 78-1 orig. SIEVERDING C-1-1) with *Plantago lanceolata* as the host plant was chosen for multiplication. Core samples (10 ml) close to roots were used as an inoculum in sowing mounds of a peat-soil 1:1 mixture (Vertic Luvisol) which was heated in an oven twice for 24 h at 120°C. Five maize plants were cultivated in 10-liter pots, the first 6 weeks in a climate chamber and subsequently in a greenhouse. Fine roots were positively controlled for colonization with *Glomus manihot*. At maturity, the soil-peat-root mixture was first homogenized with a spade and subsequently with a blender.

Tab. 33: NPK fertilization in pot experiments PP 97 b-e

Due to different soil filling of pots, concentrations vary between experiments (exp.) PP 97 **b**, **c**, **d** and proceeding experiment PP 97 **e**. Data must be corrected by 46 % skeletal phase for element concentration in fine soil (< 2 mm). Element concentration in applied nutrient solution is calculated for one third of the total amount (100 ml).

	Code	Equivalent field fertilization [kg ha^{-1}]		Reagent	Element [mg plant^{-1}]	Element [µg soil g^{-1}]		Element [mmol soil g^{-1}]		Nutrient solution [mmol l^{-1}]
						exp. **b**, **c**, **d**	exp. **e**	exp. **b**, **c**, **d**	exp. **e**	
N	N150	150	N	NH_4NO_3	375	68 **d**	55	4.9 **d**	4.3	268
	N450	450			1125	205	-	14.6	-	89
P	P100	100 (44)	P_2O_5 (P)	$Ca(H_2PO_4)_2 \cdot H_2O$	109	20	16	0.6	0.6	12
	P300	300 (131)			327	59 **d**	48	1.9 **d**	1.7	35
Ca	P100	28	Ca		71	13	10	0.3	0.3	6
	P300	85			212	39 **d**	31	1.0 **d**	0.9	18
K	All	100 (83)	K_2O (K)	K_2SO_4	208	38	30	1.0	0.9	18

Isolates of *Rhizoctonia solani* (see section 4.7) were multiplied in petri dishes with dextrose potato (3.9 %) yeast (0.65 %) agar (DIFCO) for 18 d at 25° C.

A combined *Rhizoctonia*-AMF inoculum was prepared. Well developed cultures including about 100 ml growth medium were homogenized with 300 ml *aqua dest.* to 400 ml of suspension. Half of it was autoclaved for 60 min as done with 100 ml of the soil-peat-root mixture of AMF inoculum. Factorial combinations of sterile and infectious aliquots of both inocula were blended. Subsequently, 75 ml of the respective suspension was collocated in a sowing bed and covered with substrate soil. The Ferric Luvisol from Portel was screened on 1.25 cm but still contained 46 % w/w of gravel. It was heated twice for 24 h at 120 °C in an oven.

NPK fertilization was applied as 100 ml of threefold split nutrition solution (Tab. 33) before sowing (1/3 NPK), 21 DAS (1/3 NK, 2/3 P) and 44 DAS (1/3 NK). Five-liter pots were filled with 5.5 kg soil, which consisted of 2525 g fine soil, and arranged as a complete randomized block design in a greenhouse. Pots were put with plates on a gravel ground. Three surface sterilized seeds were sown per pot. The experiment was started at the same time (13 May) as the experiments PP 97 c and d described below. However, re-sowing after 10 d was necessary due to emergence problems. Plant stands were thinned to one plant per pot at 20 DAS and a mixed sample of fine roots was stained for AMF colonization. Plants were irrigated with tap water every 2 days *ad lib.* and moved one position forward within blocks. Leaf attacking fungi were controlled with 1 % TIRAME® spray at 22 DAS. Plants were harvested at maturity at 60 DAS. The air temperature in the greenhouse was regulated by a cooling system, which operated automatically at temperatures above 25°C. One wall of the greenhouse was made of perforated cardboard which was wetted constantly. A cool air stream was built up by a ventilator on the opposite wall. The blocks of the experiment were orientated in the direction of this air stream. A shadow screen made of aluminum allowed 25 % of sunlight to enter.

Exemplarily, the light regime outside (1300 µE m^{-2} s^{-1} PAR) and inside (640 µE m^{-2} s^{-1}) the greenhouse was compared with a Quantum sensor (LiCor, USA) around midday on a bright day in October 1996. When the screen was closed, as was necessary to additionally control temperature during summertime, the irradiance decreased to 200 µE m^{-2} s^{-1}. The temperature was recorded during last 2 weeks of the experiment; the mean temperature was 23.5° C during the measuring period (A-Tab. 29).

5.4.3 Results and discussion

At 20 DAS, *Glomus manihot* had well colonized FRISSON fine roots of inoculated treatments (A-Tab. 28) whereas no mycorrhiza was observed in controls.

Tab. 34: Effect of factorial inoculation with *G. manihot* and *Rhizoctonia* on yields of two pea isolines (PP 97 b)
Inocula treatments were factorial with *Glomus manihot* (+AMF) or without (-AMF) and with *Rhizoctonia solani* (+Rhizoc.) or without (-Rhizoc.), respectively. Significant differences of **means** ± SD according to LSD test were indicated by different letters for p-levels inferior 0.05 and 0.10.

Isoline	Inocula		Grain dm [g]		LSD p>		Shoot dm [g]		LSD p>		Harvest		LSD p>	
					0.05	0.10			0.05	0.10	Index		0.05	0.10
Frisson	+AMF	+Rhizoc.	**0.54**	±0.29	a	a	**1.38**	±0.49	ab	a	**37**	± 9	a	a
Frisson	+AMF	-Rhizoc.	**0.56**	±0.19	a	a	**1.58**	±0.33	a	a	**35**	± 5	a	a
Frisson	-AMF	+Rhizoc.	**0.25**	±0.20	bc	bc	**0.76**	±0.43	bc	b	**24**	±16	ab	bc
Frisson	-AMF	-Rhizoc.	**0.49**	±0.42	ab	ab	**1.42**	±0.90	ab	a	**31**	±12	ab	ab
P2	+AMF	+Rhizoc.	**0.09**	±0.08	c	c	**0.65**	±0.17	c	b	**16**	±13	b	c
P2	+AMF	-Rhizoc.	**0.13**	±0.06	c	c	**0.57**	±0.16	c	b	**22**	± 3	ab	c
P2	-AMF	+Rhizoc.	**0.11**	±0.07	c	c	**0.64**	±0.28	c	b	**14**	± 8	b	c
P2	-AMF	-Rhizoc.	**0.17**	±0.06	c	c	**0.73**	±0.16	bc	b	**23**	± 4	ab	c

All plants developed poorly and at a high variation in growth variables. Irregular leaf damage caused by insufficiently controlled unknown air borne fungi disturbed the experiment. However, as nonsignificant trend according to Tukey's HSD, the wild type grew better with the AMF inoculum (Tab. 34). Inoculated only with *Rhizoctonia*, biomass and grain production did not differ from mutant P2, which did not respond to inoculation. Consequently, mutant P2 could not serve as a control for damage caused by *R. solani* under such experimental conditions. However, the AMF inoculum seemed to alleviate the damage caused by *R. solani*. All *Rhizoctonia* treated peas were infected as indicated by macroscopically visible black colored necro-tissue on stunted roots and black stem lesions (AGRIOS, 1978).

KASIAMDARI and co-worker very recently (2002) carried out factorial inoculation experiments with *R. solani*, BNR, and *Glomus coronatum* in mung bean at two levels of P supply. Although *G. coronatum* enhanced P uptake and growth of the host plant, the AMF

inoculation reduced lesion symptoms caused by *R. solani* independently of the P nutrition. Therefore, the authors concluded that other factors than P status of the mycorrhizal plant are involved in the disease suppression of the two rhizoctoina types.

Roots of *myc* $^{-1}$ pea mutants did not respond differently to inoculation with BNR than the wild type (MORANDI et al, 2002). The evidence suggests that there is also no higher sensitivity to pathogenic *R. solani.*

All *Rhizoctonia* treated peas were infected as indicated by macroscopically visible black colored necro-tissue on stunted roots and black stem lesions (AGRIOS, 1978). Progressed senescence did not allow quantitative root washing and, consequently, fine roots of harvested plants were not stained for AMF colonization.

5.5 Test of a new soil fungicide to control *Rhizoctonia* disease in peas (PP 97 c)

5.5.1 Introduction

As an alternative approach to inoculation experiments, the impact of soil borne phytopathogenic fungi should be estimated by the application of soil fungicides. RIDOMIL® GOLD (Ciba Geigy corp. USA) was tested in soil from Portel on efficacy to control diseases caused by *Rhizoctonia* (i.e. root and stem rot) and on possible effects of AM development. RIDOMIL® based on the active ingredient metalxyl, is indicated for *Pytium* damping-off and root rot. The new formula of RIDOMIL® GOLD is twice as effective and the active ingredient (a.i.) is double the concentration. Unfortunately, a fungicide explicitly indicated for *Rhizoctonia* root and stem rot, namely TERRACLOR® (Ciba Geigy corp. USA) was neither available in Portugal nor in Germany. Therefore, RIDOMIL® GOLD was applied to examine how AMF colonization is influenced and whether possible diseases by *Rhizoctonia* in peas can be reduced.

5.5.2 Materials and methods

The recommended application for metalxyl as RIDOMIL® GOLD per ha is 147 g a.i. in a volume of 131 liters of water as 1124 µg g^{-1} soil spray. Calculated for 20 cm top soil, the recommendation corresponds to 44 µl solution per kg soil. The concentration was lowered to 269 µg g^{-1} and 1000 µl were applied with an Eppendorf pipette one day before sowing on the central soil surface in the sowing area of each pot filled with 5500 g air dried soil. The concentration of metalxyl in the soil was accordingly 0.05 mg a.i. kg^{-1}. Fertilization, experimental layout and other conditions were the same as in experiment PP 97 b (section

5.4.2, Tab. 33). However, emergence was better and no re-sowing was necessary. In consequence, fungi induced leaf damage occurred later and was better controlled. The experiment started on 13 May and lasted for 76 d to 28 July 1997.

5.5.3 Results and discussion

The soil fungicide application had no significant effect on AMF colonization in FRISSON fine roots estimated by both trypan blue staining of chitin and functional vital staining of alkaline phosphatase. However, high variations allow only a restricted interpretation of the obtained data (Tab. 35).

Plant growth of the two isolines was also not significantly effected by RIDOMIL although the fungicide application apparently increased biomass of the symbiotic peas (Fig. 17, more details see A-Tab. 30). Shoot dry matter was slightly but not significantly higher than in parallel experiment PF 97 b (Tab. 34 and A-Tab. 30).

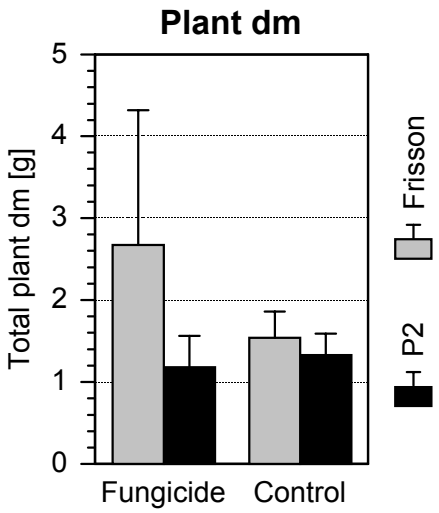


Fig. 17: Impact of fungicide application on dry matters of pea isolines FRISSON and mutant P2 (PP 97 c)
Means (n=4) ± SD (error bars) were not significant different (Tukey's HSD).

Metalxyl was reported to be harmless against AMF (JOHNSON & PFLEGER, 1992) with only one exception (JABAJI-HARE & KENDRICK, 1987). However, vital staining may reveal a more differentiated reaction of mycorrhiza function (KOUGH et al., 1987) which surprisingly tended to be enhanced in the experiment presented.

Tab. 35: Impact of fungicide application on AMF colonization of FRISSON (PP 97 c)
Mixed sample at 20 DAE and from 4 replications at 60 DAE (**means** ± SD). Differences between treatments were not significantly different according to Turkey's HSD. For comparison with other pea pot trials and more details, see A-Tab. 28 and A-Tab. 30.

Sample date	**20** DAE		**60** DAE (days after emergence)			
AM staining	**Trypan blue**		**Trypan blue**		**Alkaline phosphatase**	
Treatment	Fungicide	Control	Fungicide	Control	Fungicide	Control
AMF colonization [%]	**7**	**9**	**48** ± 16	**42** ± 15	**66** ± 14	**48** ± 20

Recent studies of SUKARNO et al. (1994) and SUKARNO et al. (1996) demonstrated clearly a reduction of AMF colonization in onion and extraradical hyphae. Furthermore, metalxyl had phytotoxic effects at an application rate of 12.5 mg a.i. kg^{-1}. However, even when a factor of 4 is calculated for the more efficient new formula, the latter rate exceeded the amount of metalxyl applied in the experiment presented by a factor of 64.

However, whether or not new RIDOMIL® GOLD, which is claimed to be effective against *Rhizoctonia solani* (McGAWLEY, per. comm.), ultimately did reduce *Rhizoctonia* diseases deriving from the Ferric Luvisol cannot be answered honestly by the data obtained. A complicating circumstance is that the microsclerotia found in the soil could not be classified to more or less phytophatogenic types (i.e. *Rhizoctonia solani* or BNR, compare preceding section 5.4) within the framework of the experiment presented.

5.6 N and P fertilizing experiment (PP 97 d)

5.6.1 Introduction

A high N level was chosen in both preceding experiments PP 97 b and PP 97 c to exclude possible N limitation in mutant P2, whereas a moderate P fertilization with 20 µg g^{-1} triple phosphate plus the content of 7 µg g^{-1} soil P (Bray I) was supposed to allow a normal development of arbuscular mycorrhiza in Frisson. However, such a P supply may be sub-optimal for peas in the substrate used. The ferric properties of the soil (A-Tab. 15) imply the capacity of P sorption. Conversely, the application of high amounts of plant available P must be regarded with caution due to possible suppression of AM in pea plants. Therefore a further experiment was conducted to examine whether the non-symbiotic mutant P2 can achieve the growth potential of mycorrhizal wild type by greatly increasing N and/or P supply in the Ferric Luvisol.

5.6.2 Material and method

The experiment was arranged as a 2-factorial complete randomized block design with N and P fertilization on two levels of 150 or 450 kg N ha^{-1}, and 100 (44) or 300 (131) kg ha^{-1} P_2O_5 (P), respectively. Exact amounts and concentrations of the respective treatment are listed in section section 5.4.2, Tab. 33. All experimental conditions were the same as in parallel experiment PP 97 c and pots without fungicide application of the latter were the same as the N450 P100 treatment. The lower N fertilization treatment N150 was oriented on the lower N level in the field experiment PF 96.

5.6.3 Results and discussion

P fertilization had the strongest effect on the growth of both isolines (Fig. 18), as indicated by high F-values for all yield variables (A-Tab. 30). The harvest index (HI) of mutant P2 was reduced by half with the lower P supply. For FRISSON, a significant reduction of HI was only observed in poorly growing plants of N450 P100 treatment. The differences of plant-to-shoot ratio followed the pattern of the variable HI.

Surprisingly, the high N supply caused a significant growth decrease in FRISSON. The, certainly very high, dose of 1.1 g N applied to plants of only 1.5 g biomass resulted in more than 7 % straw N concentration (Fig. 18) which was the highest of all pea trials in the study presented (compare section 4.1.3, Tab. 20; section 4.5.3.6, Fig. 16; A-Tab. 31). A disturbance of the experiment may serve as an explanation: insufficiently controlled leaf attacking fungi might have been attracted more than proportionally by the very high N concentrations in FRISSON leaves. Impairing salt effects were not yet likely at such a soil N concentration. Also possible is a suppression of the mycorrhizal response by high N fertilization (SMITH & READ, 1997) which was, however, not observed at the low P fertilization level.

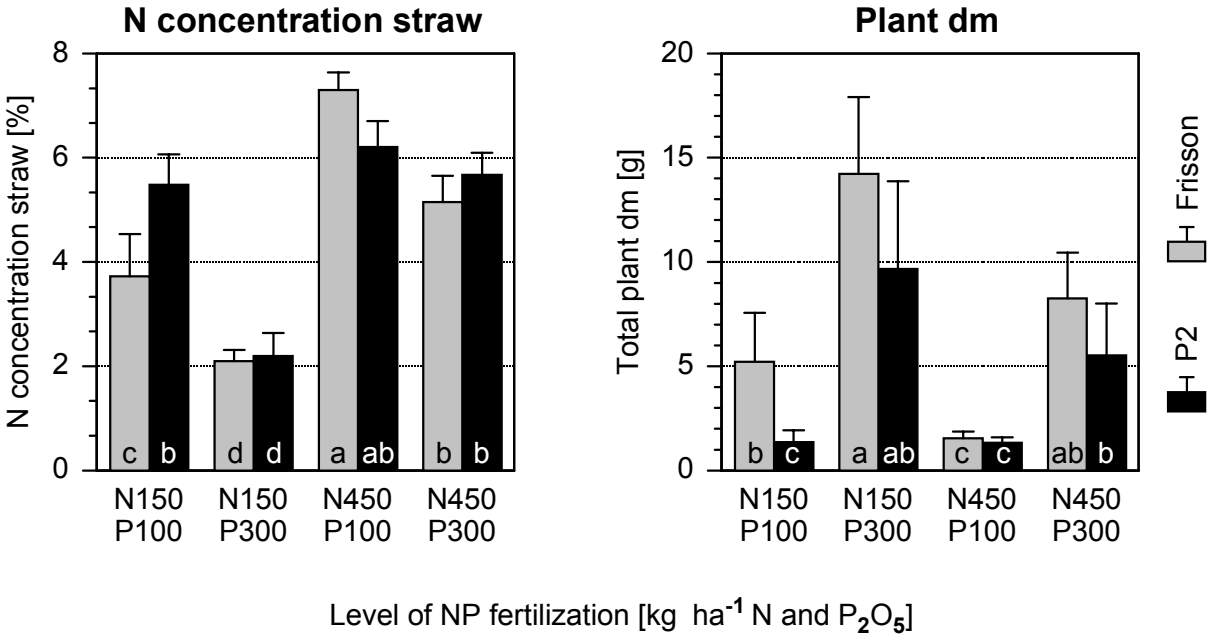


Fig. 18 Straw N concentration and total plant dry matters of pea isolines FRISSON and mutant P2 at different P and N fertilization (PP 97 d)
Treatments N150, N450, P100 and P300 correspond to equivalent kg ha^{-1} N and P_2O_5 (44 and 131 kg ha^{-1} P) Means (n=4) ± SD (error bars) Significant differences according Tukey's HSD (ln transformation for variable plant dm) are indicated by different letters.

In contrast to N, P straw concentrations were very low, except in FRISSON of N150 P300 treatment (A-Tab. 31). An important result to be emphasized is that mutant P2 plants were clearly sufficiently supplied with 0.4 g N, as indicated by no growth response to triple the amount of N fertilizer. The hypothesized reduction of AMF colonization at higher P supply could be confirmed, as indicated by a significant F-value (A-Tab. 30).

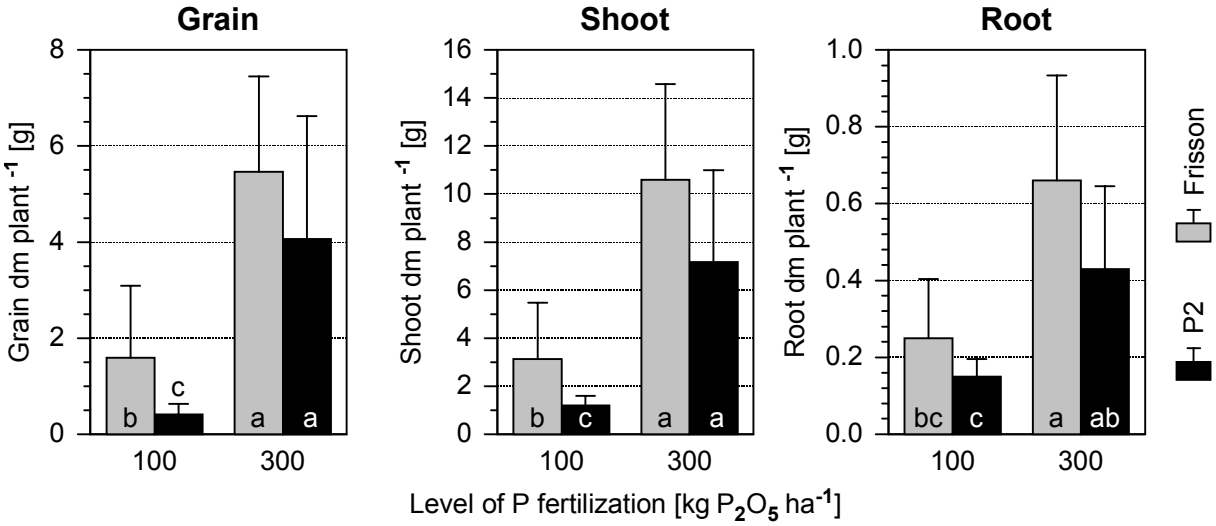


Fig. 19: Grain yields, shoot and root dry matters of pea isolines FRISSON and mutant P2 at different P fertilization (PP 97 d)
Treatments P100 and P300 accordingly to equivalent kg ha^{-1} P_2O_5 (44 and 131 kg ha^{-1} P) Means ± SD (error bars) and significant differences (Tukey's HSD after ln transformation, indicated as different letters) of dry matters. Re-analysis across two N fertilization of 150 and 450 kg N ha^{-1} (n=8).

Across the two N treatments, biomass differences between isolines were not significant. However, growth of mutant P2 was significantly reduced in comparison to FRISSON at the lower P supply (Fig. 19). The interpretation of the obtained data in the experiment presented, is similar to that in preceding pot trials PP97 b and PP97 b, restricted by high variation within treatments. The perturbation derived from irregular plant damage and possibly from insufficiently standardized irrigation.

5.7 Repeated P fertilizing experiment (PP 97 e)

5.7.1 Introduction

The preceding experiment had identified a high sensitivity of mutant P2 to phosphorus supply between 44 and 131 kg ha^{-1} P. However, a repetition of the experiment at the lower N supply of 150 kg N ha^{-1} was indicated for three reasons. Firstly, both isolines grew poorly in experiment PP 97 d compared to the growth potential observed in other pot trials before. Secondly, CV was unacceptably high, and thirdly, FRISSON reacted in a phytopathological context negatively to a high N dose. Therefore, experimental conditions were optimized in order to identify P deficits for growth depression in mutant P2 at even relatively high P fertilization.

5.7.2 Materials and methods

Growing conditions differed in the following aspects. Pots contained 20 % more soil and were filled consequently with 6850 g of 1.25-cm-screened soil or 3145 g fine soil. The complete amount of P fertilizer and one third of the NK fertilizer was applied at sowing as nutrient solution at high accuracy using a 50 ml dispenser instead of a 100 ml cylinder.

Second and third NK doses, as one third of the total amount, were applied at 24 DAS and 48 DAS, respectively. The absolute amount of NPK fertilizer per plant was the same as in experiment PP 97 b, c and d (section 5.4.2, Tab. 33). The soil water content was maintained at 17 % (w/w). In order to determine the water replenished, pots were weighed routinely at least every two days. Calculated for fine earth, target water content was about 33 % (w/w). TDR measurements (IMCO, Germany) ca. 1 h after irrigation at 30 DAS at 11 cm depth determined a soil water content of **21** ± 1.6 % (v/v, n=3 per 16 pots). Generally, it must be considered that irrigation was much more regular and accurate, which was possible with only 16 pots in this trial as compared to 72 in the trial session before. Pots were placed on a bench instead of graveled greenhouse ground.

Climate conditions were similar (Tab. 36) as in previous experiment. Only night temperatures were 4 degrees lower (compare A-Tab. 29). The air temperature was still within an optimal range for peas, although the experiment was carried out in autumn. Consequently, the light regime was different due to smaller irradiance angle and shorter day length. However, a more temperate climate outside of the greenhouse made it possible to open the shadow screen at 41 DAS, in contrast to experiments PP 97 b, c, d, and the light regime was at

least half of outdoor irradiance (compare section 5.5.2). Numbers of flowers and pods were noted during the experiment. Pea plants were harvested at maturity around 77 DAS.

Tab. 36: Temperature and air humidity in greenhouse during experiment PP 97 e
Records of thermo-hydrograph from 15 September to 18 November 1997 greenhouse at Mitra located below experimental bench at 1 m above ground. Data were calculated from measures of every 2 h (Day: 6 to 20 h, night 20 to 6 h GMT)

	Temperature [C°]					**Relative air humidity** [%]				
	min	max	**mean**	day	night	min	max	**mean**	day	night
Mean	**15.6**	**28.8**	**20.9**	**23.3**	**17.5**	**58.3**	**91.6**	**79.4**	**71.9**	**89.7**
SD	3.0	2.3	2.5	2.6	2.8	9.2	2.8	5.8	8.0	3.8
Min	8.0	22.0	14.3	13.0	10.8	42.0	53.0	65.5	56.6	76.4
Max	20.0	35.0	24.9	27.4	21.4	92.0	98.0	94.3	94.0	96.0

5.7.3 Results and discussion

Grain production of all treatments was almost equal, except for low P fertilized mutants which produced only a fifth of that level, at 1.5 g per plant (Fig. 20). However, shoot biomass of FRISSON was significantly higher than P2 also at the high level of P supply. Root biomass of mutant P2 was significantly lower than of FRISSON at the two P treatments, which may be related to root growth promoting properties of mycorrhizal colonization. The symbiotic peas did not show a single significant response to P supply in all yield variables (A-Tab. 30). However, the mycorrhiza was decreased with triple P supply by a factor of 2 (Tab. 37).

Tab. 37: AMF colonization at 77 DAS of FRISSON at two levels of P fertilization (PP 97 e)
Means ± SD of 4 plants were estimated by grid line method (chitin staining) with n=100. Highly significant p-values of t-test are marked ***.

P fertilization [kg ha^{-1} P_2O_5 (P)]		**100 (44)**	**300 (131)**	t-test p-value
AMF colonization [%]		**77** ± 3	**48** ± 5	0.0002***
frequency [%] of arbuscules				
classified	*low*	**24** ± 8	**19** ± 3	0.3675
	medium	**38** ± 4	**22** ± 4	0.0031***
	high	**15** ± 8	**7** ± 4	0.2298

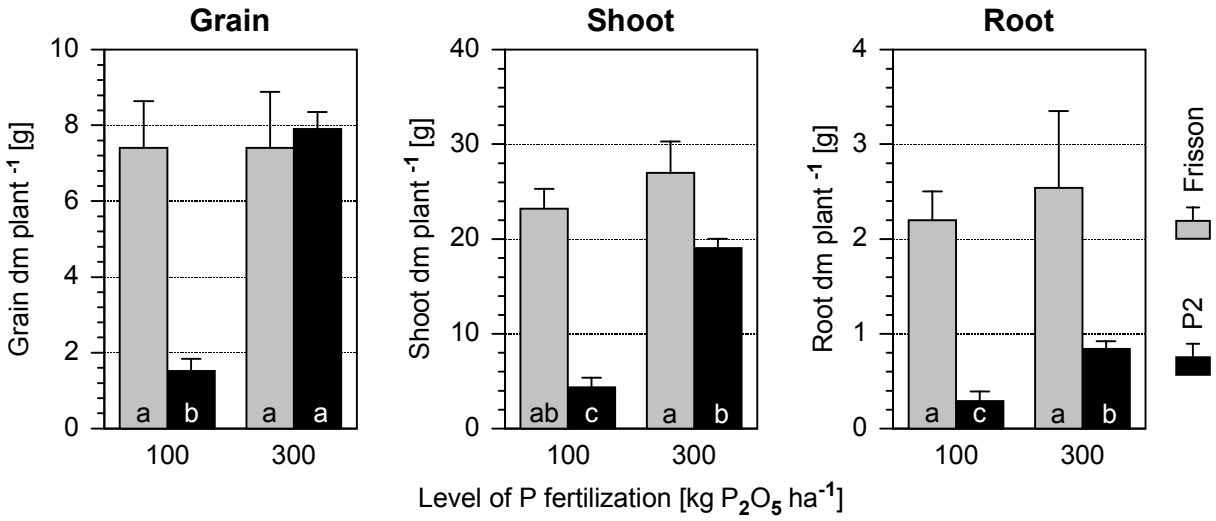


Fig. 20: Grain yields, shoot and root dry matters of pea isolines FRISSON and mutant P2 at different P fertilization (PP 97 e)
Treatments P100 and P300 are equivalent to kg ha^{-1} P_2O_5 (44 and 131 kg ha^{-1} P). Means (n=4) ± SD (error bars) and significant differences (Tukey's HSD as indicated by different letters) of dry matters. More detailed data in A-Tab. 30.

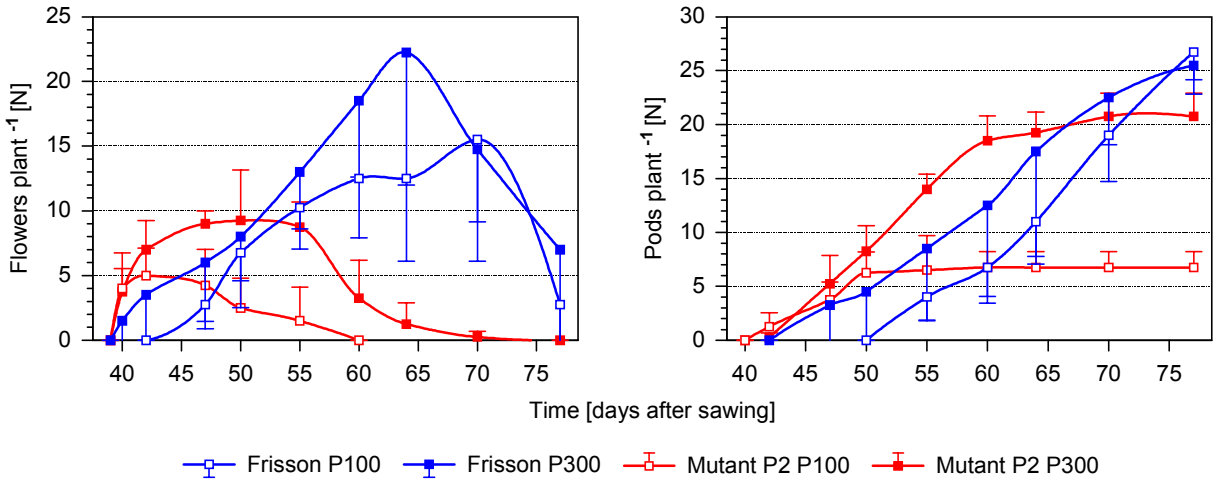


Fig. 21: Flowering and pod building of two pea isolines at different P fertilization (PP 97 e)
Means (n=4) ± SD (error bars) of FRISSON and Mutant P2 at 100 (44) (treatment P100) and 300 (131) (treatment P300) kg ha^{-1} P_2O_5 (P) equivalent field fertilization.

Mutant P2 flowered earlier than FRISSON (Fig. 21) which was also noted in the field trial PF 95 a at Elvas (A-Tab. 21) and was reported by LOVATO (1994). Therefore, the later maturing of the symbiotic peas may imply an underestimation of grain production of FRISSON in the pot trials presented because that could not carried out until optimal maturity. This problem underlines the importance of field experiments. The peak of inflorescence of FRISSON plants was apparently delayed by lower P supply. Regarding pod building, the relation was similar but less pronounced.

The N concentration (Fig. 22) of plant tissue was the highest, but the N uptake (Fig. 22) was the lowest in the pea mutants grown at 16 µg g^{-1} P as triple phosphate. Straw N concentration decreased in the mutant by half as a result of the triplication of P fertilization, whereas the wild type remained unaffected (Fig. 22). Grain concentration was still lower by 1 % N (A-Tab. 31). Thus, N uptake was obviously not the reason for the growth depression of mutant P2 at the low phosphorus level and probably not for the slightly lower biomass production at the higher P level in comparison to FRISSON (Fig. 20). The differences in P concentrations underline the sensitivity of the *myc* $^{-}$ mutant to P supply. Only 12 % of the amount of P which was taken up by mycorrhizal wild type was found in shoots of the non-mycorrhizal mutant at the lower P supply, whereas 64 % were achieved at the higher level (Fig. 22). The slightly (not significantly) higher P concentration of straw in P300 fertilized *myc* $^{-}$ peas compared to *myc* $^{+}$ peas indicates that P status was barely a limiting growth factor here, although P contents of shoots were significantly less due to lower biomass production. Nitrogen and P uptake of roots were not considered but probably negligible at root dry matters in the range of standard deviation of shoots (A-Tab. 30). However, on the basis of the obtained data, it cannot be excluded that P efficiency of mutant P2 may have been reduced in comparison to the wild type FRISSON.

Finally, the following conclusion can be drawn: In the studied soil, the main benefit of arbuscular mycorrhiza in peas was unambiguously enhanced P uptake and could be precisely identified by yield differences of isolines genetically different in symbiotic properties.

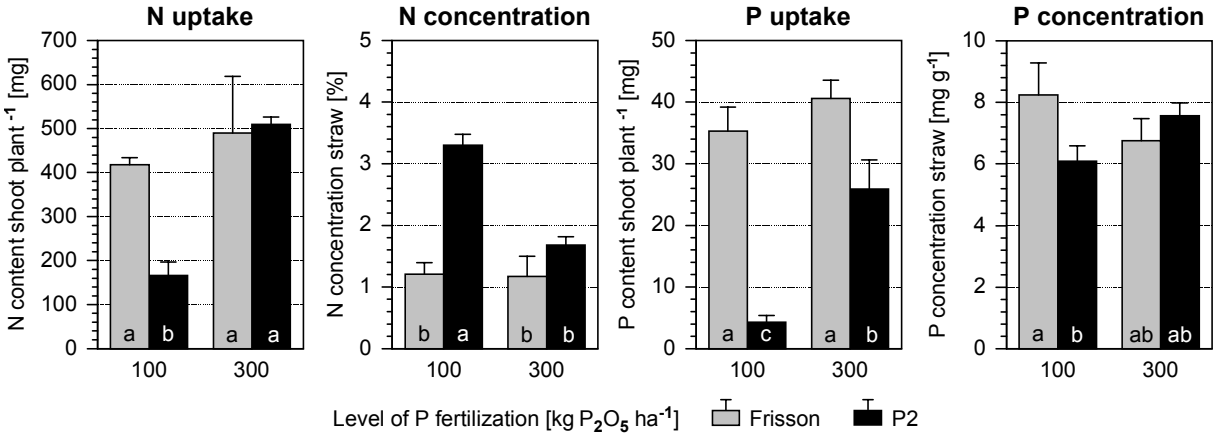


Fig. 22: Shoot uptake and straw concentration of N and P in mycorrhizal FRISSON and mutant P2 at different P fertilization (PP 97 e)

Treatments as in Fig. 21. Means ± SD (error bars); significant differences (Tukey's HSD) were indicated as different letters. More detailed variables see in A-Tab. 31.

6 General discussion

Mycorrhiza research is popular but difficult and methodological improvements are therefore still a challenge. The MPN test in particular was an inefficient start-up. A fundamental critical analysis of its limitations is urgently needed. Although the mycorrhiza MPN test no longer seems to be adequate, the evaluation of mycorrhizal propagules in soils remains a fundamental methodological problem. There is a general tendency in applied science such as crop science to focus on results, rather than paying attention to the problems that arise in the process, which may lead to the application of inadequate methods.

Mycorrhiza experiments are fascinating because they produce large plant effects (SMITH & READ, 1997). Safflower is surely a suitable test plant in this sense and was found to be highly mycotrophic. It is a considerable oil seed with 0.2 million Mt production worldwide 1998 (FAO, 1999a) and yield improvements by mycorrhiza management would be of economic interest.

Unfortunately, the safflower experiments suffered from insufficiently defined AMF inoculum, which was simultaneously objective of the investigation. However, already the production of a reproducible pure inoculum is difficult (SMITH & READ, 1997) and *G. manihot* as an exotic AMF inoculum was not efficient under growth chamber and green house conditions with two different soils, respectively.

Despite enormous efforts, the isolation of native AMF found in pure cultures failed in the associated project, as shown by a comparison of the project proposal (CARVALHO, 1992) and final report (CARVALHO, 1997). The screening of natural AMF populations for their effectiveness in the sense of maximized plant benefit constitutes a first important step in the management of mycorrhiza for crop production. The fact of regular non-specificity of AMF against host plants (see section 1.2) should allow conclusions to be drawn from results with pea as a screening crop for other mycotrophic crops. Additionally, pea itself is already an important legume crop throughout the world, with 13 million Mt grain yield on 7.2 million ha in 1998 (FAO, 1999b). Restrictions may arise in those cases when the host plant specific variation of the mycorrhizal response is significant to different AMF (MOSSE, 1975; GRAW et al., 1979; BETHLENFALVAY, 1992). *Pisum sativum* has a comparably high mycorrhizal dependency (PLENCHETTE, 1983). However, differences in their response to AMF were found even with genetically related leafless pea cultivars (ESTAÚN et al., 1987) and

colonization rates of 398 different pea cultivars including garden, forage, and (semi) leafless cultivars varied over a broad range (JENSEN, 1985).

The coarse and sparse root system of peas is usually easy to wash out and to stain for mycorrhizal colonization. A conceptual advantage of peas is their high dependency on phosphorus supply as a grain legume in context with the enhanced P uptake as a generally accepted main plant beneficial mycorrhizal effect (BETHLENFALVAY, 1992).

The question arises as to whether non-fixing peas have the same P dependency as N_2 fixing peas. Bacteroides, those *Rhizobia* which are associated in the root nodules, are very sensitive to inorganic P (McDERMOTT, 2000). Phosphorus is highly concentrated in nodules (MARSCHNER, 1995). However, this is due more to a high proportion of meristematic tissue with metabolic active membranes and frequent cell division. Consequently, high contents of phospholipids, DNA and RNA in nodules are primarily responsible for the high P tissue concentration rather than, as is is often argued, the P of the metabolic pool as required by ATP consuming N reduction (REINHARD, 1995; MARSCHNER, 1995).

The low mycorrhizal response reported by KAHILUOTO et al. (2000) for the *nod* $^{-}$ *myc* $^{+}$ pea mutant of var. SPARKLE in comparison to the *myc* $^{-1}$ may be related to the lack of nodules and a consequent lower need for P.

However, experiment PP97e demonstrated that the non-fixing mutant P2 has an extreme dependency on P supply. More specific experiments would be worthwhile to elucidate whether P dependency is the same for the wild type under non-symbiotic growing conditions, or whether mutant P2 is more sensitive to P supply. In the latter case, further genetic defects are to be assumed and would restrict the utilization of such indicator plants for the assessment of AM symbiosis.

BURLEIGH and HARRISON (1997) found a gene in *Medicago truncatula* which was suppressed in response to early AMF colonization processes but also at high phosphate supply. Its expression was down regulated even in *myc* $^{-}$ alfalfa mutants (BURLEIGH & HARRISON, 1997). It shows similarity to a gene encoding P-starving in tomato. These findings may provoke speculation about genetic relationships or plagiotropic effects concerning a putatively altered P uptake efficiency in pea mutant P2 and its mycorrhiza resistance encoding gene.

The pea mutant P2 has been intensively studied for its defense reactions against AMF (RUIZ-LOZANO et al., 1999). Although the mutation concerns probably only one gene (FRANKEN, per. comm., 1994), a plagiotropic effect is exerted on two extremely different

symbioses (ALBRECHT et al., 1999). The suspicion that more than only symbiotic properties were affected can not be dispelled by the experiments presented. Plant hormonal interaction may be involved in the mutation, as suggested by enhanced mycorrhizal hyphal growth in mutant P2 treated with an auxin inhibitor (MÜLLER, 1999).

The sterility found in three plants (see section 4.1.3) of several thousand P2 mutants which were cultivated in the course of this work, may be of importance in this context because this was never observed in the wild type. Peas are self-pollinating. However, in rare cases (0.1-0.5 %) pollen may be transferred by bumble-bees (STALLING, per. comm.) which implicates the risk of genetic pollution in the germplasm of the mutant and, to a lesser extent, the undesirable transfer of the symbiotic defects to pea crops in the range of the insect. Although the gene defect causes a strong disadvantage in competition strength of the plant, it must be treated with caution because peas serve as human nutrition. There is no guarantee that these peas are not harmful due to changed composition. This should be thoroughly investigated before these mutants are applied as a regular bioassay in field experiments.

The sensitivity of peas to drought stress might be ambivalent. As a main limiting production factor in the Mediterranean environment studied, possible benefits from mycorrhiza may be effective. Conversely, water supply and air humidity are far from optimum conditions for pea cropping.

Effectively screened native AMF may potentially serve for inoculum production. However, also other prerequisite conditions must be considered. Pure cultures must be established free of pathogens. Although aeroponic culture techniques have been developed (JARSTFER & SYLVIA, 1999; MOHAMMAD et al., 2000), multiplication of AMF usually requires suitable substrates (HEINZEMANN, 1994). Consequently, species or isolates of *Glomales* need to have properties which can exceed the demands of previously determined effectiveness. As long as the multiplication of AMF cannot be achieved independently of symbiosis, a high level of infectiousness of the AMF is necessary to establish a certain amount of radical and extra radical hyphae, spores and other propagules in roots and mycorhizosphere of host plant for multiplication. The infectivity strength which enables a fungus to colonize plants under interspecific competition is of little importance in these multiplication cultures. However, when the inocula of exotic or natural AMF are applied in the field, infectivity in the sense of competition strength may be crucial (WILSON & TOMMERUP, 1992).

The two, under certain circumstances, opposing properties of AMF, on the one hand the infectiousness in a sense of high colonization rates, and on the other hand the infectivity in a

sense of high competitive strength (BETHLENFALVAY, 1992; JOHNSON & PFLEGER, 1992), may heavily disturb the sequential approach by SIEVERDING (1991).

Conversely, the integrated approach with a screening and evaluation crop pursued in this work may help to identify, independently of such perturbation, those AMF with adequate properties which are crucial for the effectiveness under field conditions. Interfungal variation to the host plant response is an established (BETHLENFALVAY, 1992) but not a well understood fact (JAKOBSEN, 1999).

Additionally, the field conditions themselves are increasingly becoming the focus of mycorrhiza research: the strategy of how to benefit from AM symbiosis in agriculture is changing. While inoculation becomes less important, the successful management of indigenous or introduced AMF populations in the field is gaining priority (VLEK et al., 1996, KAHILUOTO et al., 2000; KAHILUOTO & VESTBERG, 2000). For this purpose, appropriate non-mycorrhizal controls for a field bioassay are essential.

It is worth noting that KAHILUOTO and co-workers presented as late as 2000 the first systematic study on developing a uniform, reproducible, and comparable bioassay to assess mycorrhiza efficiency under relevant greenhouse and field conditions. They concluded that BENOMYL® application is the most appropriate non-myorrhizal control for field AMF communities, because responsiveness of pea mutants to AMF was low (compare section 4.6.3). However, they only achieved single-row plots with 8 pea plants each and did not include the wild type in their experimental setup. In contrast to the view presented in the introducing section 1.7.3., the risk of undesirable side effects of benzyladenine were seen by KAHILUOTO and co-workers (2000) as tolerable.

Despite all justified focusing on P uptake by mycorrhizal and AMF resistant peas, the possible alteration of N uptake by AM also deserves attention. The methodological problem of the experiments presented can turn to an excellent tool under BNF suppressed conditions. Highly N sensitive mycorrhizal and *myc* $^{-}$ pea plants are worth studying in combination with different AMF for their ability to take up different forms of N in comparison to P uptake efficiency. However, similar to P, suppression of mycorrhiza development also can be caused by high N supply (JENSEN & JAKOBSEN, 1980). These aspects are rarely studied in context with AM management, although N is most times the major plant nutrient in an agro-ecosystem. Drought is playing a more and more prominent role in worldwide cropping conditions, and scientific interest in the interaction of AMF with N nutrition may increase simultaneously.

Further investigation on “how to control” the non-mycorrhizal control for undesirable side effects are necessary for AMF resistant plant mutants, particularly as more mutants other than those of *Pisum sativum* will possibly be used as non-mycorrhizal controls in the future. The same attention should be paid to the utilization of mutants for studying other important features of plant growth, such as the recently found mutants of root hairless barley (GAHOONIA et al., 2001). Generally, genetically modified crops may also be altered in unknown properties which are expressed only under certain environmental conditions. For example, changed leaf properties such as form, senescence, growth, assimilation and transpiration rates were found in transgenous potatoes which apparently had no enzymatic linkage to the introduced yeast-derived invertase gene (HEINEKE et al., 1992). For genetically modified plants, as for plant mutants, it is worth considering that it is particularly difficult to search for the “needle in a haystack” if researchers do not know what this “needle” looks like and whether there might be more than one of them.

7 Summaries

7.1 **Summary:** Mediterranean dryland farming in the Alentejo (Portugal) is limited by P deficiency in soils and by drought. The utilization and management of arbuscular mycorrhiza (AM) symbiosis may improve production and sustainability of the cropping system. For this purpose, native arbuscular mycorrhiza fungi (AMF) were sought and tested for their efficiency to increase plant growth by enhanced P uptake and by alleviation of drought stress.

Pot experiments (always one plant per pot) with safflower (*Carthamus tinctorius*) and pea (*Pisum sativum*) in five soils (mostly sandy loamy Luvisols) and field experiments with peas were carried out during three years at four different sites. Host plants were grown in heated (100 °C) soils inoculated with AMF or the respective heat sterilized inoculum. In the case of peas, mutants resistant to AMF colonization were used as non-mycorrhizal controls. The mycorrhizal impact on yields and its components, transpiration, and P and N uptake was studied in several experiments, partly under varying P levels and water supply.

Preliminary efficiency screening by most probable number bioassays was found to be of limited value for discriminating species and isolates of native AMF.

Monoliths of undisturbed soil (32 kg) were placed in the open to simulate field conditions. Inoculation with a native AMF mix improved grain yield, shoot and leaf growth variables as compared to control. Exposed to drought from 60 DAE onwards, higher soil water depletion of mycorrhizal plants resulted in a haying-off effect. The growth response to this inoculum could not be significantly reproduced in a subsequent open air pot (8 kg) experiment at two levels of irrigation (80 and 40 % water holding capacity) and P fertilization (0 and 50 mg kg^{-1}), however, safflower grew better at higher P and water supply by multiples. The water use efficiency was improved by the AMF inoculum for shoot but not for grain dry matter per liter in the two experiments. Transpiration rates (mmol cm^{-2} d^{-1}) were not significantly affected by AM but as a tendency were higher in non-mycorrhizal safflower.

A fundamental methodological problem in mycorrhiza field research is providing an appropriate (negative) control for the experimental factor arbuscular mycorrhiza. Soil sterilization or fungicide treatment have undesirable side effects in field and greenhouse settings. Furthermore, artificial rooting, temperature and light conditions in pot experiments may interfere with the interpretation of mycorrhiza effects. Therefore, the *myc* $^{-1}$ pea mutant P2 was tested as a non-mycorrhizal control in a bioassay to evaluate AMF under field

conditions in comparison to the symbiotic isogenetic wild type of var. FRISSON as a new integrative approach. However, mutant P2 is also of *nod* $^-$ phenotype and therefore unable to fix N_2. A 3-factorial experiment was carried out in a climate chamber at high N/P/K levels of 1.8/0.26/0.33 g per (5 kg) pot to examine the two isolines under non-symbiotic and symbiotic (inoculation with local strain of *Rhizobium* and *G. manihot*) conditions. The AMF mutant achieved the same (or higher) biomass as wild type both under good and poor water supply (80 % and 40 % water holding capacity). However, *G. manihot* did not improve plant growth. Differences of grain and straw yields in field trials were large (up to 80 %) between those isogenetic pea lines mainly due to higher P uptake under P and water limited conditions. The lacking N_2 fixation in mutants was compensated for by high mineral N supply as indicated by the high N status of the pea mutant plants. This finding was corroborated by the results of a major field experiment at three sites with NPK fertilization (75 or 150 kg ha^{-1} N as NH_4NO_3, 44 kg ha^{-1} P as triple phosphate, 71 kg ha^{-1} K as K_2SO_4). The higher N rate did not affect grain or straw yields of the non-fixing mutants.

Very efficient AMF were detected in a Ferric Luvisol on pasture land as revealed by yield levels of the evaluation crop and by functional vital staining (alkaline phosphatase) of highly colonized roots. Generally, levels of grain yield were low, at between 40 and 980 kg ha^{-1}. An additional (7 kg) pot trial was carried out to elucidate the strong mycorrhizal effect in the Ferric Luvisol. A triplication of the plant equivalent field fertilization of 44 kg ha^{-1} P from 0.11 to 0.33 g P as calcium mono phosphate per pot was necessary to compensate for the mycorrhizal benefit: at the lower P rate, grain yield of the wild type was found to be five times higher as compared to mutant P2, very similar to that found in the field experiment.

However, the differences in grain and shoot dry matter between the two isolines were not always plausible as the evaluation variable because differences were also found in (small) field test trials with apparently sufficient P and N supply and furthermore in a soil of almost no AMF potential. This similarly occurred for pea lines of var. SPARKLE and its non-fixing mycorrhizal (R25) and non-symbiotic (E135) isomutants, which were tested in order to exclude experimentally undesirable benefits by N_2 fixation. In contrast to var. FRISSON, SPARKLE was not a suitable variety for Mediterranean field conditions.

This raises suspicion putative genetic defects other than symbiotic ones may be effective under field conditions, which would conflict with the concept of an appropriate control. It was concluded that AMF resistant plants may help to overcome fundamental problems of present research on arbuscular mycorrhiza, but may create new ones.

7.2 Zusammenfassung: Untersuchungen zur arbuskulären Mykorrhiza (AM) im Alentejo (Portugal) unter Verwendung von mykorrhiza-resistenten Erbsen-mutanten als Kontroll-Instrument für Feldbedingungen

Mediterraner Trockenfeldbau im Alentejo (Portugal) wird allgemein begrenzt vom Phosphormangel der Böden und Trockenheit. Der Gebrauch und das Management der Symbiose von arbuskulärer Mykorrhiza (AM) Pilzen mit Kulturpflanzen kann die Produktion und Nachhaltigkeit dieses Feldbausystems verbessern. Zu diesem Zweck wurde nach nativen AM-Pilzen gesucht, um sie auf ihre Effizienz zu testen, das Pflanzenwachstum mittels verbesserter Phosphoraufnahme und Trockenstress-Resistenz zu steigern.

Gefäßexperimente mit jeweils einer Pflanze pro Gefäß wurden mit Färberdistel (*Carthamus tinctorius*) und Erbse (*Pisum sativum*) in fünf Böden, zumeist sandig-lehmige Luvisole, durchgeführt. Erbsen wurden in Feldexperimente während dreier Jahre an vier verschieden Orten untersucht. Die Wirtspflanzen wuchsen in hitzesterilisierten (100°C) Böden, die mit AM-Pilzen oder dem entsprechendem sterilisierten Inokulum beimpft waren. Im Falle der Erbsen wurden Mutanten mit einer Resistenz gegen die Besiedlung von AM-Pilzen als mykorrhiza-freie Kontrolle eingesetzt. Der Einfluss der Mykorrhiza auf Ertrag und seine Komponenten, Transpiration, Phosphor- und Stickstoffaufnahme wurde in verschiedenen Experimenten, zum Teil unter variierender Phosphordüngung und Bewässerung, untersucht.

Ein vorhergehendes Screening auf Effizienz mittels MPN-Tests war nur von einge-schränkter Aussagekraft hinsichtlich der Unterscheidung verschiedener Arten und Isolaten der nativen Mykorrhiza-Pilze.

Ungestörte Boden-Monolithe (32 kg) wurden zur Simulation von Feldbedingungen für Färberdistelanbau im Freiland aufgestellt. Die Beimpfung mit einer Mischung nativer AM-Pilze erhöhte Kornertrag sowie Spross- und Blattwachstum im Vergleich zu unbeimpften Kontrollen. Austrocknung von 60 Tage nach Auflauf an führte aufgrund des höheren Wasserentzugs durch die mykorrhizierten Pflanzen zu einem “haying-off“-Effekt. Die Wachstumswirkung dieses Inokulums konnte aber in einem weiteren Freilandexperiment in Gefäßen (8 kg) nicht signifikant bestätigt werden. Auf die beiden anderen Versuchsfaktoren, zwei Bewässerungsstufen (40 und 80 % Wasserhaltekapazität) und P-Düngung (0 und 50 mg kg^{-1}), reagierten die Färberdisteln jedoch mit einer Wachstumssteigerung um ein Vielfaches von der jeweiligen niedrigen zur höheren Stufe. Die Wasserausnutzungseffizienz war durch

das AM-Pilz-Inokulum verbessert, allerdings nur bezogen auf die Sprossbiomasse, nicht jedoch bezüglich des Kornertrags. Die Transpirationsraten (mmol cm^{-2} d^{-1}) waren nicht signifikant verändert und nur in der Tendenz höher bei den nicht-mykorrhizierten Färberdisteln.

Ein grundsätzliches Problem der Mykorrhiza-Forschung liegt in einer geeigneten (negativen) Kontrolle für den experimentellen Faktor arbuskuläre Mykorrhiza speziell unter Feldbedingungen. Bodensterilisierung oder Fungizidbehandlung haben unerwünschte Nebeneffekte bei der Installation von Feld- und Gewächshausexperimenten. Weiterhin können die künstlichen Bedingungen hinsichtlich Durchwurzelung, Temperatur und Licht bei Topfexperimenten die Interpretation des Mykorrhiza-Effekts stören. Aus diesem Grunde wurde die *myc* $^{-1}$ Erbsenmutante P2, die nicht fähig ist Mykorrhiza auszubilden, als Kontrollreferenz eines Bioassay im Vergleich zum symbiotischen Wildtyp var. FRISSON getestet. Ziel war es, die arbuskuläre Mykorrhiza unter Feldbedingungen in einem neuen integrativen Forschungsansatz zu evaluieren. Jedoch zeichnet sich die Mutante P2 auch durch einen *nod* $^{-}$ Phänotyp aus und ist daher nicht in der Lage Luftstickstoff zu fixieren.

In einem 3-faktoriellen Experiment wurden zur Voruntersuchung die beiden Isolinien unter nicht-symbiotischen und symbiotischen Bedingungen, d. h. nach Beimpfen mit einem lokalen Stamm von *Rhizobium* und dem AM-Pilz *G. manihot*, in einer Klimakammer bei hoher N/P/K Düngung von 1.8/0.26/0.33 g pro (5 kg) Gefäß kultiviert. Die Mykorrhiza-Mutante erreichte dieselbe oder sogar eine höhere Biomasseproduktion als der Wildtyp sowohl unter guter als auch schlechter Wasserversorgung (80 % oder 40 % Wasserhaltekapazität). *G. manihot* war aber nicht geeignet, das Pflanzenwachstum zu steigern.

In den Feldversuchen waren die Unterschiede im Korn- und Strohertrag zwischen beiden isogentischen Erbsenlinien dagegen groß. Die bis zu 80 % höhere Ernte beim Wildtyp FRISSON schien hauptsächlich auf eine höhere Phosphoraufnahme unter P- und wasserlimitierten Wachstumsbedingungen zurückzuführen zu sein. Das Fehlen der Luftstickstofffixierung konnte offenbar durch hohe Gaben mineralischen N ausgeglichen werden, wie die hohen N-Konzentrationen in den Erbsenmutanten belegten. Dieses wurde durch die Ergebnisse des Hauptfeldversuch an drei Orten mit NPK Düngung (75 oder 150 kg ha^{-1} N als NH_4NO_3, 44 kg ha^{-1} P als Super-Phosphat, 71 kg ha^{-1} K als K_2SO_4) bestätigt, da hier die höhere N-Rate weder Korn- noch Strohertrag der nicht-fixierenden Mutanten beeinflusste.

In einem Ferric Luvisol auf Weideland wurden sehr effiziente AM-Pilze entdeckt. Dies zeigten sowohl die Ertragsunterschiede zwischen den beiden Isolinien als auch die funktionale Vitalfärbung (basische Phosphatase) in hochgradig mykorrhizierten Wurzeln. Allgemein lag das Ertragsniveau mit 40 bis 980 kg ha^{-1} niedrig. Mit einem zusätzlichen (7 kg) Gefäßexperiment sollte geklärt werden, worauf die starke Mykorrhizawirkung im Ferric Luvisol zurückzuführen ist. Eine Verdreifachung der Phosphordüngung des vorangegangen Feldversuch von 44 auf 131 kg ha^{-1} P, entsprechend von 0.11 auf 0.33 g P als Calciummonophosphat pro Gefäß, war notwendig, um den Vorteil der Mykorrhiza zu kompensieren: bei der geringeren P-Stufe lag der Kornertrag des Wildtyps fünffach höher im Vergleich zur Mutante, ganz ähnlich wie im Feldexperiment.

Einschränkend muss aber ergänzt werden, dass die Unterschiede in Korn- und Sprossproduktion zwischen den beiden Isolinien nicht immer als plausible Bewertungs-massstäbe gelten konnten, da auch Unterschiede in (kleinen) Test-Feldversuchen mit offensichtlich ausreichender P und N Versorgung sowie in einem Boden mit einem äußerst geringen Potential an AM-Pilzen vorgefunden wurden. Letzteres wurde für die Erbsenlinien der var. SPARKLE festgestellt. Diese wurde gegenüber ihrer Isomutanten E135, die kein N_2 fixiert, aber zu Mykorrhiza fähig ist, und R25, die beide Symbiosen ebenso wie die Mutante P2 nicht ausbildet, getestet. Hier wäre im direkten Vergleich der experimentell unerwünschte Vorteile der N_2-Fixierung genetisch auszuschließen. Im Gegensatz zu Sorte FRISSON war allerdings SPARKLE für mediterrane Feldbedingungen wenig geeignet.

Eine gewisse Skepsis wird daher gegenüber möglichen genetischen Defekten über die beiden Symbiosefunktionen hinaus hervorgerufen. Würden diese unter Feldbedingungen Bedeutung gewinnen, wäre das Konzept einer wirksamen 0-Kontrolle gefährdet. Als Schlussfolgerung mag bemerkt werden, dass mykorrhiza-resistente Pflanzen helfen können, fundamentale Probleme der gegenwärtigen Forschung an arbuskulärer Mykorrhiza zu überwinden, aber ebenso neue bereiten.

7.3 Resumo: Estudos com micorizas arbusculares (AM) no Alentejo (Portugal) usando mutantes de ervilhas resistentes para fungos AM como uma ferramenta de controle em condições de campo

A agricultura de sequeiro no Alentejo (Portugal) é limitada pela deficiência de P nos solos e pela seca. A utilização e manejo da simbiose de micorizas arbusculares (AM) pode aumentar a produção e a sustentabilidade do sistema agrícola. Para este propósito, fungos nativos de AM foram procurados e testados na sua eficiência para aumentar o crescimentos das plantas pela melhoria na absorção do P e pela resistência ao estresse hídrico.

Foram realizados ensaios com vasos (sempre uma planta por vaso) com cártamo (*Carthamus tinctorius*) e ervilha (*Pisum sativum*) em cinco solos (a maioria areno-argiloso Luvisols) e experimentos no campo com ervilhas durante três anos em quatro diferentes lugares. Plantas hospedeiras foram cultivadas em terra esterilizada (100 °C) com AMF (fungos micorízicos arbuscular) ou com seu respectivo inoculo estéril. No caso das ervilhas, mutantes resistentes à colonização AMF (*myc* $^-$) foram usados como controle não-micorízico. O impacto das micorizas na produção e seus componentes, transpiração, absorção de P e N foram investigados em vários ensaios, em parte sob vários níveis de P e suprimento de água.

A selecção preliminar com uma bioanálise segundo do número mais provável (MPN) revelou esta ser uma metodologia pouco fiável para discriminar espécies eficazes dos fungos AM nativos.

Monólitos não perturbados de solos (32 kg) foram colocados abertos ao ar com o objectivo simular as condições de campo para a produção de cártamo. Inoculação com uma mistura nativa de AMF aumentou a produção de grãos, palha e parâmetros de crescimento de folhas em comparação ao controle. Exposição a seca, 60 dias depois da emergência, resultou em um efeito “haying-off” em plantas com micorizas. A resposta de crescimento nestes inoculos não puderam ser reproduzidas significamente em um subsequente experiência com vasos abertos ao ar (8 kg) com dois níveis de irrigação (80 e 40 % da capacidade do recipiente) e fertilização com P (0 e 50 mg kg^{-1}), entretanto, cártamo desenvolveu-se melhor com os suprimentos mais elevados de P e água. A eficiência do uso da água aumentou com os inóculos AMF para palha mas não para matéria seca de grãos por litro nos dois ensaios. Razão de transpiração (mmol cm^{-2} d^{-1}) não foi afectada significativamente por AM assim como uma tendência mais alta para cártamo sem micorizas.

Um problema metodológico fundamental nas pesquisas de campo com micorizas é fornecer um controle apropriado (negativo) para o factor experimental AM. Tratamentos de esterilização ou fumigação do solo ou com fungicidas tem vários efeitos indesejáveis para ensaios no campo e em vasos. Além disso, as condições artificiais de enraizamento, de temperatura e luz em ensaios com vasos podem interferir na interpretação dos efeitos das micorizas. Neste sentido, o mutante P2 *myc* $^{-1}$ de ervilha foi testado como planta não-hospedeira para avaliar as AM sob condições de campo em comparação à linhagem parental isogenética, var. FRISSON, como uma nova abordagem integrativa. Entretanto, o mutante P2 é também do *nod* $^{-}$ fenótipo e, consequentemente, incapaz de fixar N_2.

Um experimento factorial 3 foi conduzido em câmera de crescimento com alta fertilização N/P/K de 1,8/0,26/0,33 g por vaso (5 kg) para examinar as duas mesmas linhagens sob condições não-simbiose e simbiose (inoculação forçada do *Rhizóbium* local e a variedade exótica *Glomus manihot*). O mutante AMF alcançou a mesma (ou mais elevada) biomassa que a linhagem normal sob bom (80 % capacidade retenção de aqua WHC) e pobre (40 % WHC) suprimento de água. *Glomus manihot* verificou-se não eficiente para aumentar o cresimento das plantas.

As diferenças de produção de grãos e de palha nos ensaios de campo foram grandes (até 80 %) entre aquelas linhagens isogenéticas de ervilha principalmente devido à mais elevada absorção de P sob condições limitantes de P e água. O factor falta de fixação de N_2 em mutantes foi compensada pelo factor de elevação no suprimento de N mineral indicado pela alta concentração de N nas plantas mutante de ervilha. Isto foi confirmado com os resultados da experiência de campo principal em três locais com fertilização NPK (75 ou 150 kg N como NH_4NO_3, 44 kg P como fosfato triplo, 71 kg K como K_2SO_4 ha^{-1}). O aumento da dose de N não afectou a produção de grãos e de palha dos mutantes não-fixadores.

Fungos AM muito eficazes foram detectadas em Luvisol Ferroso (Ferric Luvisol) em pastagens como foi revelado pelos níveis de avaliação da colheita e pela coloração funcional vital (fosfatase alcalina) das raízes altamente colonizadas. Em geral, níveis de produção de grãos foram baixos entre 40 e 980 kg ha^{-1}. Um ensaio adicional com vasos (7 kg) foi conduzido em casa de vegetação para averiguar o alto efeito micorízico em Luvisol Ferroso. O equivalente de uma triplicação da fertilização das plantas no campo de 44 kg ha^{-1} P a partir de 0.11 à 0.33 g P por vaso na forma de cálcio mono fosfato foi necessário para compensar o benefício das micorizas: na taxa baixa de P, a produção de grãos pela variedade paternal foi

cinco vezes maior em comparação com o mutante P2, muito similar como os resultados do experimento no campo.

Entretanto, as diferenças na produção de grãos e palha entre as duas mesmas linhagens, utilizado como um valor de selecção, não foram sempre plausíveis porque também foram encontradas em (pequenas) ensaios no campo com aparente suficiente suprimento de P e N e além disso em um solo com quase nenhum potencial AMF. Similar ocorreu para as isolinhas de ervilhas da var. SPARKLE: (1) linha simbiótica parental, (2) isomutante E135 não-fixadoras mas com micorizas e (3) isomutante R25 não-simbiótico, os quais foram testados com o objectivo de excluir os benefícios indesejáveis pela fixação de N_2. Em contraste à ervilha de forragem FRISSON, a ervilha de jardim SPARKLE não foi uma variedade apropriada para o campo sob condições Mediterrâneas. Suspeitas são deste forma motivadas pelo outros putativos defeitos genéticos, além dos simbióticos, as quais oporiam, efectivo sob condições de campo, com o conceito de um apropriado controle. Com isto concluiu-se que as plantas resistentes as AMF podem auxiliar a superar problemas fundamentais na presente pesquisa das micorizas abusculares, entretanto pode criar novos problemas.

8 Appendix

A-Tab. 1: Characterization of Glomalean species found in soils of experimental sites (from HEINZEMANN in CARVALHO, 1995; modified) Muronym from the outer to inner most wall according to WALKER (1986). Wall types are: U = unit, L = laminate, A = amorphous, M = membranous, index $_o$ = ornamentation

Name	Color	Spore size (µm)	Muronym	Wall groups	Wall width (µm)	Surface	Ornamen-tation	Melzer's reaction
Glomus sp. Y120	Yellow-brown	120-150	A(LUU)	1	18.0/1.0/0.5	Rough & dirty	No	No
Glomus mosseae	Hyaline-yellow	150-180	A(LU)	1	2.0/1.0		No	No
Glomus aggregatum	Hyalin	50-70	A(LL) or A(L)	1	1.0/1.0 or 1.5	Smooth	No	
Glomus sp. B50	Brown	50-70	A(ULU)	1	1.0/6.0/0.5		No	No
Glomus sp. B300	Brown	300-350	A(LU)	1	12/1	Rough & dirty	No	No
Acaulospora sp. Y150	Yellow	150-230	A(U_oL)	1	2.0/6.0		Fine spines	No
Acaulospora sp. HY90	Hyaline-yellow	90-100	A(U)	1	1.0		No	No
Scutellospora sp. H130	Hyalin	130-200	A(L)B(U)C(M)	3	3.0/0.5/0.5			Red
Scutellospora sp. H300	Hyaline-yellow	300-380	A(L)B(U)C(M)	3	3.0/0.5/0.5	Smooth	No	Deep red

A-Tab. 2: Formula for calculating the MPN test according to FISHER and YATES (1970) Example is experiment MPN 94. Cells in formulas (MS Excel®) refer to columns marked as *A* to *E*, and rows of spread sheet, respectively. Value for *S* is corrected (0.181 instead of 0.201).

A	*B*	*C*	*D*	*E*
3	Dilution series	Infected pots		
4	4^{0}	"	5	
5	4^{-1}	"	5	
6	4^{-2}	"	5	
7	4^{-3}	"	5	
8	4^{-4}	"	5	
9	4^{-5}	"	4	
10	4^{-6}	"	3	
11	4^{-7}	"	3	
12	4^{-8}	"	2	
13	4^{-9}	"	1	
14	4^{-10}	"	1	
15	4^{-11}	"	0	
16	4^{-12}	"	0	
	Symbol	**Signification**	**Value**	**Formula**
17	x	Sum of infected pots replication^{-1}	7.8	$\frac{\sum(D4:D16)}{5}$
18	s	No. of dilution series	13	
19	y	s – x	5.2	13-D17
20	k	Tab. VIII$_2$ FISHER & YATES (1970)	0.552	
21	a	Factor of dilution	4	
22	log Ω	x · log a – k	4.1441	(D17·LOG(D21)-D20)
23	Ω	$10^{\log \Omega}$	13934	$10^{(D17\ LOG(D21)\ -\ D20)}$
24	S ***(corrected)***	Tab. VIII$_2$ FISHER & YATES (1970)	0.4254	$\sqrt{0.181}$ ***(not*** $\sqrt{0.201}$ ***!)***
25	z	for 95 % probability	1.6450	
26	log Ω superior	$\log\Omega - \frac{S}{\sqrt{n}} \cdot z$	4.4571	$D22 - \frac{\sqrt{0.181}}{\sqrt{5}} \cdot 1.645$
27	log Ω inferior	$\log\Omega + \frac{S}{\sqrt{n}} \cdot z$	3.8311	$D22 + \frac{\sqrt{0.181}}{\sqrt{5}} \cdot 1.645$
28	Ω superior	$10^{\log \Omega\ s}$	28645	10^{D26}
29	Ω inferior	$10^{\log \Omega\ i}$	6778	10^{D27}
30	ξ	Soil density dry soil in g ml^{-1}	1.239	
31	V	Pot volume in ml	240	
34	Infectious propagules per g dry soil		7193	$\frac{D23}{1.239 \cdot 240}$
35	Upper limit of confidence at 95 % probability		9633	$\frac{D28}{1.239 \cdot 240}$
36	Lower limit of confidence at 95 % probability		2279	$\frac{D29}{1.239 \cdot 240}$

A-Tab. 3: Formula for calculating the MPN test according to COCHRAN (1950)
Example is experiment MPN 94. Calculation (MS Excel®) is based on no. of not infected pots s_x and the corresponding volume of infectious soil v_x. Variable *d* was calculated by solving equation (1) with the solver function.

Dilution series	not infected pots		Volume of parental soil in pots	
	Symbol in formula	[n]	Symbol in formula	[ml]
4^{-5}	s_a	1	v_a	0.23437500
4^{-6}	s_b	2	v_b	0.05859375
4^{-7}	s_c	2	v_c	0.01464844
4^{-8}	s_d	3	v_d	0.00366211
4^{-9}	s_e	4	v_e	0.00091553
4^{-10}	s_f	4	v_f	0.00022888

Variables		
Symbol in formula	Value	Signification
n	5	Replications.
e	2.71828	No. of Euler
d	5994.25133	Propagules ml^{-1}

Equation (1):

$$\frac{(n-s_a)\cdot v_a\cdot e^{-(v_a\cdot d)}}{1-e^{-(v_a\cdot d)}}+\frac{(n-s_b)\cdot v_b\cdot e^{-(v_b\cdot d)}}{1-e^{-(v_b\cdot d)}}+\frac{(n-s_c)\cdot v_c\cdot e^{-(v_c\cdot d)}}{1-e^{-(v_c\cdot d)}}+\frac{(n-s_d)\cdot v_d\cdot e^{-(v_d\cdot d)}}{1-e^{-(v_d\cdot d)}}+\frac{(n-s_d)\cdot v_d\cdot e^{-(v_d\cdot d)}}{1-e^{-(v_d\cdot d)}}+$$
$$\frac{(n-s_e)\cdot v_e\cdot e^{-(v_e\cdot d)}}{1-e^{-(v_e\cdot d)}}+\frac{(n-s_f)\cdot v_f\cdot e^{-(v_f\cdot d)}}{1-e^{-(v_f\cdot d)}}-(s_a\cdot v_a+s_b\cdot v_b+s_c\cdot v_c+s_d\cdot v_d+s_e\cdot v_e+s_f\cdot v_f)=-6\cdot 10^{-10}\cong 0$$

Symbol	Signification	Value	Formula
d	Propagules ml^{-1}	5994.25	
ξ	Soil density g ml^{-1}	1.239	
Ω	Propagules per g dry soil	7427	ξ · d
c	Factor for limit of confidence on 95 % probability	2.41	
Ω l	Lower limit of confidence on 95 % probability	3082	Ω · c^{-1}
Ω s	Upper limit of confidence on 95 % probability	17899	Ω · c

A-Tab. 4: Detailed harvest data of safflower monolith trial SAF 94

Treat-ment	Shoot dm	Shoot height	Cum. shoot length	Shoot bran-ches	Coarse root dm > 2 mm	Grain plant^{-1}	Grain yield dm	1000 grain weight	Harvest Index	Grain loss by insects	Grains flower^{-1}	No. of Flowers plant^{-1}	Flower dm	Petal dm	Leaf dm	Leaf shoot ratio
	[g]	[cm]	[cm]	[no.]	[g]	[no.]	[g]	[g]	[%]	[%]	[no.]	[no.]	[g]	[g]	[g]	[%]
M1W1	**55**±10	**68**±5	**345**±67	**26**±8	**2.8**±0.7	**491**±130	**12.9**±1.9	**28**±9	**24**±6	**4**± 3	**21**	**24**	**34**	**2.08**	**7.1**	**13**
M0W1	**17**± 7	**66**±4	**122**±32	**10**±4	**1.0**±0.3	**130**± 90	**5.2**±2.6	**44**±7	**30**±5	**26**± 8	**16**	**8**	**11**	**0.69**	**1.9**	**11**
M1W0	**15**± 1	**61**±5	**192**±17	**12**±1	**1.0**±0.2	**66**± 11	**1.7**±0.5	**25**±5	**11**±3	**37**± 7	**9**	**8**	**6**	**0.51**	**4.0**	**27**
M0W0	**10**± 2	**66**±6	**111**±35	**7**±2	**0.6**±0.2	**66**± 26	**2.3**±0.7	**37**±9	**24**±9	**6**±11	**13**	**6**	**5**	**0.50**	**1.4**	**14**

A-Tab. 5: P, N, C and ^{13}C analyses of safflower plants in monolith trial SAF 94
Different letters indicate significant differences (Tukey's HSD). The ^{13}C isotope discrimination is calculated as $\delta^{13}C$ for a PDB standard.

Treatment	P conc. grain	P content grain ^{ln}	P conc. straw	P content straw	P content shoot ^{ln}	C conc. straw	N conc. straw	C/N straw	$\delta^{13}C$ PDB straw
	[mg g^{-1}]	[mg]	[mg g^{-1}]	[mg]	[mg]	[%]	[%]		
M1W1	**2.78**±0.44 **b**	**35.7**±7.5 **a**	**1.87**±0.24 **a**	**78.2**±16.9 **a**	**114**±21 **a**	**42.1**± 0.8	**1.72**±0.30	**25.5**±5.7	**-25.5**±0.32 **b**
M1W0	**4.38**±0.55 **a**	**7.4**±2.2 **c**	**1.27**±0.38 **b**	**16.7**± 5.5 **b**	**24**± 5 **b**	**41.3**± 0.7	**1.97**±0.21	**21.2**±2.0	**-24.5**±0.24 **a**
M0W1	**2.83**±0.68 **b**	**14.1**±4.7 **b**	**0.55**±0.20 **c**	**6.7**± 2.8 **c**	**21**± 7 **b**	**46.8**±10.2	**1.91**±0.35	**24.9**±4.4	**-26.0**±0.34 **b**
M0W0	**2.00**±0.28 **b**	**4.4**±0.9 **c**	**0.43**±0.08 **c**	**3.4**± 1.5 **c**	**8**± 2 **c**	**42.5**± 1.1	**1.75**±0.31	**24.9**±3.7	**-25.7**±0.44 **b**

A-Tab. 6: Detailed harvest data and P uptake of safflower plants in pot trial SAF 95

Treat-ment	Shoot dm	Shoot height	Cum. shoot length	Coarse root dm >2 mm	No. of grain plant^{-1}	Grain yield dm	1000 grain weight	Harvest Index	Flower plant^{-1}	Leaf dm	Leaf area	Leaf shoot ratio	P content grain	P content straw	P content shoot
	[g]	[cm]	[cm]	[g]	[no.]	[g]	[g]	[%]	[no.]	[g]	[cm²]	[%]	[mg]	[mg]	[mg]
M1P1W1	**60**±7.6	**65**±4.2	**393**±11	**3.05**±0.63	**512**±121	**23.7**±3.7	**49**±12	**40**±1.6	**30**±1.0	**8.9**±0.8	**1032**±93	**14.9**± 1.6	**37.8**± 9.6	**37** ±13	**75** ±21
M1P1W0	**12**±2.1	**48**±2.1	**77**± 7	**0.47**±0.06	**95**± 22	**3.4**±0.9	**36**± 6	**28**±6.3	**7**±1.7	**2.5**±0.5	**223**±18	**21.3**± 6.7	**6.0**± 1.8	**12** ± 5.1	**18** ± 5.4
M1P0W1	**16**±6.4	**47**±6.4	**87**±27	**0.76**±0.29	**146**± 62	**6.5**±2.8	**46**±11	**41**±4.7	**8**±2.4	**2.7**±1.0	**244**±52	**17.4**± 3.7	**10.5**± 6.4	**12** ± 7.9	**23** ±14
M1P0W0	**3**±0.5	**39**±4.4	**41**± 6	**0.12**±0.04	**25**± 9	**0.6**±0.2	**23**± 1	**18**±4.0	**2**±0.5	**0.8**±0.2	**52**± 9	**27.7**±11	**0.8**± 0.4	**2.6**± 1.2	**3.3**± 1.4
M0W1P1	**59**±5.0	**65**±4.2	**378**±55	**3.00**±0.37	**612**±170	**23.7**±4.0	**41**± 7	**40**±3.9	**27**±2.4	**9.1**±0.7	**1010**±89	**15.4**± 1.1	**36.7**±11	**47** ±17	**84** ±21
M0P1W0	**17**±3.8	**52**±1.6	**120**±14	**0.97**±0.35	**146**± 38	**5.9**±1.3	**42**± 8	**34**±1.4	**12**±3.8	**3.4**±1.0	**336**±70	**19.5**± 0.9	**9.6**± 4.0	**11** ± 4.9	**20** ± 8.3
M0P0W1	**11**±3.7	**47**±4.3	**87**±26	**0.48**±0.14	**97**± 50	**3.7**±1.7	**39**±10	**34**±4.4	**6**±2.3	**1.7**±0.4	**175**±39	**16.6**± 2.2	**5.7**± 2.6	**8.3**± 2.9	**14** ± 3.9
M0P0W0	**3**±0.4	**40**±0.4	**46**± 4	**0.12**±0.02	**26**± 3	**0.6**±0.2	**21**± 3	**17**±2.9	**3**±0.5	**0.9**±0.3	**56**± 9	**26.9**± 7.1	**1.0**± 0.1	**3.6**± 1.1	**4.6**± 1.1

A-Tab. 7: P, N, C, and ^{13}C analyses of pea plants in field experiment PF 94
as means of 2 x 6 sub samples ± SD. The ^{13}C-isotope values are given as $\delta^{13}C$ for a PDB standard. Different letters show significant differences (Tukey's HSD).

	P concentration grain [mg g^{-1}]	**P** concentration straw [mg g^{-1}]	**P** content grain [mg]	**P** content straw [mg]	**P** content shoot [mg]
Frisson	**3.19** ± 0.21 b	**0.475** ± 0.050 a	**18.67** ± 6.42 a	**7.36** ± 1.52 a	**26.03** ± 6.50 a
P2	**4.12** ± 0.18 a	**0.512** ± 0.008 a	**6.42** ± 1.54 b	**2.69** ± 0.83 b	**9.69** ± 3.38 b
	C concentration grain [%]	**C** concentration straw [%]	**C** content grain [g]	**C** content straw [g]	**C** content shoot [g]
Frisson	**42.35** ± 0.14 a	**41.39** ± 0.52 a	**2.53** ± 0.57 a	**1.74** ± 0.39 a	**4.27** ± 0.96 a
P2	**41.69** ± 0.37 a	**40.73** ± 0.52 a	**0.60** ± 0.17 b	**0.67** ± 0.22 b	**1.27** ± 0.39 b
	N concentration grain [%]	**N** concentration straw [%]	**N** content grain [mg]	**N** content straw [mg]	**N** content shoot [mg]
Frisson	**3.38** ± 0.06 a	**0.85** ± 0.001 a	**201** ± 43 a	**36** ± 8 a	**237** ± 51 a
P2	**2.70** ± 0.14 b	**0.83** ± 0.145 a	**38** ± 9 b	**13** ± 6 b	**52** ± 15 b
	C/N grain	**C/N** straw	**C/N** shoot	δ^{13}**C** PDB grain	δ^{13}**C** PDB straw
Frisson	**12.66** ±0.13 b	**49.39** ± 0.61 a	**18.08** ±0.13 b	**-23.33** ± 0.29 a	**-26.51** ± 0.15 a
P2	**15.63** ±0.90 a	**53.30** ± 5.92 a	**25.90** ±0.99 a	**-26.64** ± 0.43 b	**-28.48** ± 0.33 b

A-Tab. 8: Precipitation at site Évora from 1993 to 1996
From final Project Report (CARVALHO, 1997), modified. Monthly precipitation in (mm)

	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Season
Long term	26	66	88	93	96	84	99	57	46	24	5	3	687
1992/93	21	70	6	88	24	41	30	64	81	12	0	0	436
1993/94	44	157	95	8	78	97	4	27	97	0	0	0	608
1994/95	1	56	64	37	43	50	17	26	19	28	36	0	376
1995/96	37	14	192	297	263	54	56	33	117	9	2	0	1074

A-Tab. 9: Profile description of Ferric Luvisol (Portel)
(from STAUSS & KALLIS, 1992; modified)

Ap	0-20 cm	(5YR 4/6), silty to loamy sand (uS-lS), very gravelly (rounded quartzite with red patina), singular to subangular blocky, aggregate stability weak to very weak, moderate high bulk density, strongly rooted, undulated horizon boarder line to
AB	20-29 cm	(2.5 YR 5/8), loamy sand, very gravelly (rounded quartzite with red patina), subangular to angular blocky, aggregate stability moderate, high bulk density, aggregate stability moderate, high bulk density, no clear horizon boarder line to
2 Bt	29-55 cm	(10 R 4/8), clay (T), moderate gravely (rough-edged quartzite), angular blocky, cracks by drying, slickensides, aggregate stability moderate, high bulk density, moderately rooted, clear transition to
2 Btg 1	55-93 cm	colored (10 R 4/8, 7.5 YR 5/8), loamy clay (lT), very few gravel, coarse angular polyhedron, cracks by drying, slickensides, aggregate stability weak, high bulk density, weakly rooted, fluid transition to
2 Btg 2	>93 cm	colored (10 R 4/8, 10 R 8/1, 7.5 YR 5/8), sandy clayey loam (stL), few gravel, coarse angular polyhedron, cracks by drying, slickensides, aggregate stability moderate, high bulk density, very weakly rooted

A-Tab. 10: Chemical soil analysis of horizons in Ferric Luvisol
(from STAUSS & KALLIS, 1992; modified)
SOM= soil organic material, indices $_{fix}$ (fixed), $_{org}$ (organic) $_{pot}$ (potential), Conduc.= electric conductivity. H-value is the proportion of H and Al ions of CEC calculated by CEC_{pot} minus CEC_{total}; sat. =saturation. S-value is the stock of basic cations

Horizon	Depth [cm]	C_{total} [mg g^{-1}]	SOM [%]	N_{total} [mg g^{-1}]	N_{fix} [mg g^{-1}]	N_{org} [mg g^{-1}]	C/N	pH $CaCl_2$	pH H_2O	Conduc. [µS]
Ap	0-20	8.42	1.45	0.46	0.01	0.45	19	4.7	5.8	20
AB	20-29	4.42	0.73	0.25		0.25	17	4.5	5.7	18
Bt	29-55	4.73	0.81	0.34	0.04	0.30	16	4.4	5.3	86
Btg 1	55-93	3.22	0.55	0.20	0.03	0.17	19	4.3	5.5	37
Btg 2	>93	2.00	0.34	0.21	0.07	0.14	14	3.9	5.2	44
Btg 2	(red)	1.98	0.34	0.16						
Btg 2	(white)	2.59	0.45	0.25						

		Na [%]	K [%]	Mg [%]	Ca [%]	P [µg g^{-1}]	Si [%]	Al [%]	Fe [%]	Mn [µg g^{-1}]
Ap	0-20	0.34	0.05	0.08	0.11	185	35.5	2.67	2.53	294
AB	20-29	0.39	0.65	0.13	0.08	179	35.0	4.36	3.28	207
Bt	29-55	0.05	0.74	0.30	0.12	286	22.6	13.94	10.38	149
Btg 1	55-93	0.04	0.73	0.33	0.11	260	21.6	14.37	11.15	167
Btg 2	>93	0.05	0.84	0.38	0.06	208	23.6	12.92	10.72	197
Btg 2	(red)	0.06	0.86	0.34	0.06	289	22.2	12.61	13.94	241
Btg 2	(white)	0.13	0.95	0.41	0.04	99	26.8	13.73	4.52	153

		Exchangeable cations [cmol per kg fine soil]							Base	S-value
		Na	K	Mg	Ca	CEC_{total}	H-value	CEC_{pot}	sat. [%]	[mol m^{-2}]
Ap	0-20	0.02	0.06	0.70	1.16	1.9	2.5	4.4	44.3	4.7
AB	20-29	0.03	0.04	1.17	0.97	2.2	2.8	5.0	44.2	2.2
Bt	29-55	0.26	0.10	6.03	4.08	10.5	9.0	19.5	53.6	38.8
Btg 1	55-93	0.27	0.09	4.89	4.05	9.3	7.8	17.1	54.4	50.6
Btg 2	>93	0.30	0.09	4.06	2.12	7.6	9.9	17.5	43.4	49.0
Btg 2	(red)	0.30	0.09	4.50	1.94	6.8	9.7	16.5	41.4	
Btg 2	(white)	0.50	0.18	3.73	1.36	5.8	13.7	19.5	29.6	

A-Tab. 11: Stock of nutrients in physiological depth of Ferric Luvisol
(from STAUSS & KALLIS, 1992; modified) Ap- AB- Bt- horizon 0-55 cm in (g m^{-2})
Indices $_{CAL}$ refers to calcium-acetate-lactate extraction

N_{org}	Ca_{total}	Mg_{total}	K_{total}	K_{CAL}	P_{total}	P_{CAL}
240	600	1200	3800	16	144	0.5

A-Tab. 12: Physical properties of Ferric Luvisol
(from STAUSS & KALLIS, 1992; modified) FC = field capacity

Horizon	Depth [cm]	Bulk density [g cm^{-3}]	Density of solid [g cm^{-3}]	Volume of pores [% v/v]	Water content [% v/v] at pF of 0.6	1.8	2.5	4.2	Available FC [% v/v]	Air capacity [% v/v]
Ap	0-20	1.69	2.6	36.2	33	24	14	4	20.0	12.6
AB	20-29	1.82	2.7	33.6	29	17	10	3	14.7	16.2
Bt	29-55	1.52	2.8	45.2	41	37	33	30	7.1	7.8
Btg 1	55-93	1.47	2.8	48.0	46	44	42	36	7.9	4.4
Btg 2	>93	1.53	2.8	46.2	43	40	37	32	8.9	5.8

A-Tab. 13: Soil texture of Ferric Luvisol
Percentage (w/w) of textural fractions in (from STAUSS & KALLIS, 1992; modified)

Horizon	Depth	Sto-	Silicatic fine soil						Sand	Silt	Clay	class
	[cm]	nes	gS	mS	fS	gU	mU	fU				
Ap	0-20	50	23.3	12.6	22.9	20.5	8.0	4.5	58.8	33.0	8.2	uS-lS
AB	20-29	60	22.6	10.3	19.2	15.8	11.1	5.1	52.1	31.9	16.0	lS
Bt	29-55	12	5.2	3.8	6.3	5.9	6.6	6.4	15.3	18.9	65.8	T
Btg 1	55-93	5	1.7	2.9	8.0	7.3	10.1	6.8	12.5	24.2	63.3	tL
Btg 2	>93	16	8.2	10.8	13.8	8.7	10.5	6.0	32.9	25.1	42.1	stL

A-Tab. 14: Composition of clay minerals in Ferric Luvisol
(from STAUSS & KALLIS, 1992; modified, in % w/w)

Horizon	Depth [cm]	Kaolinite	Illite	Transition minerals	Vermiculite	Chlorite	Smectite
Ap	0-20	60	14	13		13	
AB	20-29	68	16	10		6	
Bt	29-55	80	16	3		1	
Btg 1	55-93	80	14	4	2		5
Btg 2	>93	77	11	7			7
Btg 2	(red)	79	15	4	3		
Btg 2	(white)	76	7	7	2		

A-Tab. 15: Oxalate and dithionite soluble oxides from Ferric Luvisol
Values are (mg g^{-1}) and their ratios; n.dec. are inferior to the detectable limit (from STAUSS & KALLIS, 1992; modified)

Horizon	Depth	Al_o	Al_d	Al_d/Al_o	Fe_o	Fe_d	Fe_d/Fe_o	Mn_d	Mn_o	Mn_d/Mn_o
Ap	0-20	0.7	0.9	0.74	0.5	8.5	0.07	0.13	0.18	0.71
AB	20-29	0.7	1.3	0.51	0.4	14.6	0.03	0.01	0.07	0.22
Bt	29-55	0.9	4.2	0.21	1.6	49.6	0.03	n.dec.	0.07	
Btg 1	55-93	1.6	3.3	0.49	2.4	48.1	0.05	n.dec.	0.07	
Btg 2	>93	1.3	2.9	0.46	2.2	44.8	0.05	n.dec.	0.07	
Btg 2	(red)	1.1	2.5	0.42	3.6	62.0	0.06	n.dec.	0.12	
Btg 2	(white)	1.9	2.3	0.83	0.6	15.7	0.04	n.dec.	n.dec.	

A-Tab. 16: Soil chemical analyses of top soils at Évora and Elvas 1996
From EGER in CARVALHO (1996) and CARVALHO (1997), modified. CAL = calcium-acetate-lactate extraction

Site	Évora	Elvas	
(Source)	(CARVALHO, 1996)		(CARVALHO, 1997)
Variable			n=54
C_{total} [%] Lichterfelde	0.48	1.66	
N_{total} [%] Kjelldahl	0.053	0.105	
P [µg g^{-1}]	18 (CAL)	322 (CAL)	127
K [µg g^{-1}]	56 (CAL)	139 (CAL)	119
pH	4.9 ($CaCl_2$)	7.6 ($CaCl_2$)	8.4 (H_2O)

A-Tab. 17: Profile description of Vertic Luvisol (Évora)
Site was not cultivated at the time of description and, consequently no rooting properties were estimated (courtesy STAHR & co-worker, 1994)

Depth [cm]	Tex-tural class	Gravel [%] v/v	Color (moist)	pH $CaCl_2$	SOM [%] w/w	Carbonate [%] w/w	Soil structure shape	Comments	Pores	Stability of aggregates	Bulk density [g cm^{-1}]	Horizon transi-tions	Horizon
-20	sL	2 - 3	104R 4/4	5.0	1.5	0	Subangular blocky to granular	Reduction of harvest residues	Few meso-pores	Moderate to poor	1.4	Not clear	Ap 1
-35	sL	2 - 3	104R 3/4	5.3	< 1	0	Subangular blocky	Mn concretion	Few meso-pores	Poor	1.4	Diffuse	Ap 2
-65	tL	2 - 3	104R 3/4	7.0	< 1	0	Angular blocky	Slicken-sides	Many cracks	Poor	1.4	Diffuse	Bt 1
-105	lT	2 - 3	104R 3/3	7.0	< 1	0	Angular blocky to prismatic	Slicken-sides	Many cracks	Moderate	1.4	Clear	Bt 2
-120	sT	< 5	104R 4/3	7.0	< 0.5	0.5 - 2	Subangular blocky to granular		Few	Poor	1.6		Bw

A-Tab. 18: Chemical analyses of soils from sites of field and pot experiments
Data from DIEDERICHS (unpublished) [1] refer to numeration below. Soil samples were taken in June 1993 with a spade from fist 30 cm top soil and consist of 10 mixed replicates. [2] Mitra Montado site. [3] Elvas project site near Vila Boim. [4] Soil type characterization according to HOLLAND (1991). [5] Soil type characterization courtesy of STAHR & co-workers (A-Tab. 9). [7] Characterization STAUSS & KALLIS (1992) and STAHR (per. comm.) A-Tab. 9. [8] Soil type characterization according to CARVALHO (1997). [9] after steam sterilization for 48 h at 100°C (DIEDERICHS unpublished data). SOM = soil organic matter, CEC = cation exchange capacity, µeq = micro equivalents

Site	Soil no. [1]	Soil type FAO	N [%]	C [%]	C/N	SOM [%]	P Bray I [µg g^{-1}] air dry	P Bray I [µg g^{-1}] sterile [9]	P_{total} [µg g^{-1}]
Mitra [2]	1-4	Leptosol / Cambic Arenosol [4]	0.20	2.31	12	3.97	5	12	319
Évora	6	Vertic Luvisol [5]	0.05	0.49	10	0.84	15	19	303
Beja	8	Vertisol [6]	0.09	0.84	9	1.44	4	4	280
Portel	10	Ferric Luvisol [7]	0.09	1.06	12	1.82	19	24	222
Elvas [3]	14	Luvisol [8]	0.13	1.32	10	2.27	41	54	637

Site	Soil no. [1]	Soil type FAO	CEC [µeq g^{-1}]	Exchangeable cations [µeq g^{-1}] Na	K	Ca	Mg	Mn	Al	pH ($CaCl_2$)
Mitra [2]	1-4	Leptosol / Cambic Arenosol [4]	99	1.9	3.3	74	18	1.2	0	5.2
Évora	6	Vertic Luvisol [5]	86	1.8	1.5	59	23	0.8	0	5.3
Beja	8	Vertisol [6]	256	2.6	4.4	227	22	0	0	6.9
Portel	10	Ferric Luvisol [7]	115	2.8	1.7	59	46	0.9	3.6	4.5
Elvas [3]	14	Luvisol [8]	191	1.7	8.0	169	12	0	0	7.2

A-Tab. 19: Results from first pea pot test trial 1995 with FRISSON and mutant P2
Pre-germinated peas were transplanted after 3 d in pots filled with 2 kg. Vertic Luvisol from Évora autoclaved for1 h. Paper filtered suspension from native soil was added. N (0.9 g per plant) was applied as 9 x 100 ml 0.1 % N solution (NH_4NO_3) twice per week from 15 d onwards. Four replicates with one plant per pot were grown for 54 d in a climate chamber (section 2.2). Fine roots of FRISSON were controlled for absence of AMF colonization. The p-level of t-test qualifies differences between isolines.

Isoline	Shoot dm [g]	Straw dm [g]	Grain dm [g]	Grain $plant^{-1}$ [no.]	Pod $plant^{-1}$ [no.]	Grain pod^{-1} [no.]	Harvest Index [%]
Frisson	**3.3** ± 0.2	**1.5** ± 0.1	**1.8** ± 0.2	**14.3** ± 1.5	**4.3** ± 1.0	**3.5** ± 0.7	**55** ± 2.1
P2	**2.8** ± 0.8	**1.3** ± 0.4	**1.4** ± 0.4	**11.4** ± 3.3	**3.6** ± 1.1	**3.3** ± 0.9	**51** ± 2.2
t-test p-level	0.19	0.40	0.10	0.15	0.19	0.98	0.06

A-Tab. 20: Results of first pea pot test trial 1995 with SPARKLE and mutants E135 and R25
One plant in 2 kg pots (2 replications) with native Vertic Luvisol were grown up to maturity in climate chamber (section 2.2).

Isoline	Shoot dm [g]	Straw dm [g]	Grain dm [g]	Grain plant^{-1} [no.]	100 grain weight	Harvest Index [%]
Sparkle	**4.6** ± 0.1	**1.9** ± 0.2	**2.7** ± 0.3	**10** ± 2	**27** ± 2	**58** ± 5
E135	**5.3** ± 0.3	**3.0** ± 0.8	**2.2** ± 0.5	**12** ± 2	**20** ± 7	**43** ± 12
R25	**3.6** ± 0.5	**2.0** ± 0.5	**1.7** ± 0.1	**10** ± 1	**17** ± 1	**47** ± 5

A-Tab. 21: Results from pea field experiment PF 95 a at Elvas
Means of 30 plants and 120 Portuguese lines, respectively (courtesy PERREIRA, ENMP)

Agronomic measure	**Frisson**	**Mutant P2**	**Portuguese lines**
Days to emergence	19	23	18.4
Days to bloom	97	106	104.3
Days to fructification	108	115	112.9
Days to maturity	158	151	153.3
Days of bloom	32	25	25.4
Plant height [cm]	45	49	91.7
Height of 1st pod	24	23	57.8
100 grain weight [g]	11.8	12.6	25.5
Grain per plant [g]	9.08	5.74	6.54

A-Tab. 22: Plant species list from site Évora (Vertic Luvisol)
(seed bank experiments 1992-94, courtesy Luís Dias, Dep. of Biology, UE)

Agrostis pourretii Willd.
Anagallis arvensis L.
Avena sp.
Brassica napus L.
Briza minor L.
Catapodium rigidum
Cerastium sp.
Chamaemelum nobile (L.) All.
Chenopodium opolifolium Schrader ex Koch & Ziz
Chrysanthemum segetum L.
Chrysanthemum sp.
Conyza canadensis (L.) Cronq.
Cyperus sp.
Diplotaxis catholica (L.) DC
Epilobium hirsutum L.
Galinsoga parviflora Cav.
Gnaphalium luteum-album L.
Helianthus annuus L.
Heliotropium europaeum L.
Herniaria lusitanica Chaudhri
Hirschfeldia incana (L.) Lagrèze-Fossat
Juncus bufonius L.
Lactuca serriola L.
Linaria viscosa (L.) Dum.-Courset
Logfia gallica (L.) Cosson & Germ.
Lythrum hyssopifolia L.
Lythrum sp.
Lythrum tribracteatum Salzm. ex Sprengel
Medicago rugosa Desr.
Mentha suaveolens Ehrh.
Misopates orontium (L.) Rafin.
Ornithopus compressus L.
Papaver sp.
Phalaris brachystachys Link
Phalaris minor Retz.
Phalaris paradoxa L.
Poa annua L.
Polycarpon tetraphyllum (L.) L.
Polygonum aviculare L.
Reseda luteola L.
Rumex conglomeratus Murray
Silene gallica L.
Sonchus sp.
Spergula arvensis L.
Spergularia purpurea (Pers.) Don
Stellaria media (L.) Vill.
Triticum aestivum L.
Urtica urens L.

A-Tab. 23: Plant species list of natural vegetation from site Portel (PF 96)
Determination was done on 13 April 1997 by courtesy of Helge Bruelheide and Ute Jandt according to VALDÉS et al. (1987).

Anarrhinum bellidifolium (L.) Willd.
Andryala integrifolia L.
Avena barbata Pott ex Link
Brachypodium distachyon (L.) Beauv.
Bromus mollis = hordeaceus L.
Bromus rigidus Roth (cf. *diandrus* Roth)
Calendula arvensis L.
Cerastium sp.
Chamaemelum mixtum (L.) All.
Colchicum sp.
Daucus cf. *setifolius* Desf.
Echium plantagineum L.
Eruca sativa Miller
Euphorbia segetalis
Galactitis tomentosa Moench
Hedypnois cretica (L.) Dum.-Courset
Hordeum leporinum Link.
Hypochoeris glabra L.
Iris filifolia Boiss.
Jasione montana
Lamarckia aurea Moench
Leontodon longirostris (Finch & P.D. Sell) Talavera
Logfia gallica (L.) Cosson & Germ.
Lotus cf. *angustissimus* L.
Misopates orontium (L.) Rafin.
Papaver pinnatifidum Moris
Poa annua L.
Reseda undata L.
Rumex bucephalophorus L.
Sanguisorba minor Scop. ssp. *magnolii* (Spach) Briq.
Sheradia arvensis L.
Silene gallica L.
Spergula arvensis L.
Spergularia rubra (L.) J. & C. Presl
Trifolium stellatum (L.)

A-Tab. 24: Probability of an effect by N fertilization on production of mutant P2 (PF 96)
P-level of contrast analysis in ANOVA for factor N (treatment 75 kg N ha^{-1} with 6 replicates against treatment 150 kg N ha^{-1} with 18 replicates) All dependent variables were ln transformed.

Site	Grain dm m^{-2}	Shoot dm m^{-2}	Grain dm $plant^{-1}$	Grain dm $plant^{-1}$
Évora	0.921	0.949	0.678	0.547
Portel	0.711	0.844	0.999	0.969
Mitra	0.674	0.585	0.845	0.599
All	0.977	0.870	0.725	0.501

A-Tab. 25: Probability of an effect by N fertilization on N and P concentrations and contents in plant material of mutant P2 (PF 96)
P-level of contrast analysis in ANOVA for factor N (treatment 75 kg N ha^{-1} against treatment 150 kg N ha^{-1} with 6 replicates each). Dependent variables indexed ^{ln} were transformed so.

Site	N I T R O G E N			P H O S P H O R U S		
	Concentration		Content	Concentration		Content
	Grain	Straw	shoot ^{ln}	Grain ^{ln}	Straw ^{ln}	shoot ^{ln}
Évora	0.023	0.298	0.366	0.093	0.092	0.932
Portel	0.022	0.509	0.482	0.801	0.322	0.955
Mitra	0.536	0.658	0.975	0.914	0.313	0.467
All	0.003	0.467	0.363	0.370	0.323	0.662

A-Tab. 26: N and P soil concentrations after harvest in pea field experiment PF 96
Samples were taken from **N150** treatment (mixture of 2 core samples from 6 plots per site)

Site	Depth [cm]	**N**-NH_4 [µg g^{-1}]	**N**-NO_3 [µg g^{-1}]	**N**-min [µg g^{-1}]	**P**-Bray I [µg g^{-1}]
	0-20	**9.9** ± 8.9	**33.9** ±13.3	**44.0** ±20.6	**0.62** ± 0.49
Évora	20-40	**3.3** ± 4.1	**11.1** ± 6.4	**14.4** ± 8.3	**0.52** ± 0.45
	0-40	**6.6**	**22.5**	**29.1**	**0.57** ± 0.29
Portel	0-20	**73.8** ±27.9	**123.8** ±54.8	**197.7** ±39.0	**7.18** ± 5.35
	0-20	**2.9** ± 4.1	**29.4** ± 5.4	**32.2** ± 6.6	**0.06** ± 0.03
Mitra	20-40	**0.6** ± 2.2	**16.0** ± 5.4	**16.6** ± 6.1	**0.04** ± 0.02
	0-40	**1.8**	**22.7**	**24.4**	**0.05** ± 0.01

A-Tab. 27: P and N concentrations and uptakes in pea plants of all field experiments
Replicates: 2 x 6 plants in exp. PF 94, 4 plots of 3.6 m² in exp. PF 95, 24 plots of 2 m² in exp. PF 96, mixed samples of 4 plants in exp. PF 96 a. Within double lines, significant differences are indicated by different letters for exp. PF 94 and PF 95 according to t-test, in exp. PF 96 to Tukey's HSD.

		N I T R O G E N			**P H O S P H O R U S**		
		Concentration		Content	Concentration		Content
Site Trial	**Isoline**	Grain [%]	Straw [%]	shoot $plant^{-1}$ [mg]	Grain [mg g^{-1}]	Straw [mg g^{-1}]	shoot $plant^{-1}$ [mg]
Évora	Frisson	**3.38** ±0.06 a	**0.85** ±0.00 a	**237** ± 51 a	**3.19** ±0.21 b	**0.48** ±0.05 a	**26.0** ±6.5 a
PF94	P2	**2.70** ±0.14 b	**0.83** ±0.15 a	**52** ± 15 b	**4.12** ±0.18 a	**0.51** ±0.01 a	**9.7** ±3.4 b
Évora	Frisson	**4.94** ±0.16 a	**2.06** ±0.14 b	**256** ± 10 a	**3.19** ±0.22 a	**0.56** ±0.09 a	**13.0** ±1.1 a
PF95	P2	**5.28** ±0.25 a	**2.65** ±0.20 a	**83** ± 13 b	**2.39** ±0.14 b	**0.45** ±0.05 a	**2.5** ±0.5 b
Évora	Frisson	**4.52** ±0.47 b	**1.82** ±0.30 c	**198** ± 67 a	**4.06** ±0.54 b	**1.28** ±0.35 c	**16.8** ±6.4 b
PF96	P2	**4.66** ±0.29 b	**1.74** ±0.29 c	**150** ± 44 a	**3.42** ±0.57 c	**1.03** ±0.25 c	**10.1** ±3.8 b
Portel	Frisson	**5.15** ±0.27 a	**2.96** ±0.57 b	**69** ± 28 b	**3.19** ±0.57 cd	**1.15** ±0.23 c	**3.4** ±1.5 c
PF96	P2	**5.27** ±0.24 a	**3.89** ±0.74 a	**28** ± 11 c	**2.83** ±0.47 cd	**1.12** ±0.22 c	**1.0** ±0.5 d
Mitra	Frisson	**4.44** ±0.29 b	**1.66** ±0.34 c	**270** ±133 a	**5.32** ±0.20 a	**2.25** ±0.66 b	**33.3** ±15.4 a
PF96	P2	**4.29** ±0.24 b	**1.66** ±0.49 c	**184** ± 63 a	**5.74** ±0.15 a	**4.05** ±0.67 a	**33.3** ±13.2 a
Elvas	Frisson	**3.40**	**1.54**	**1170**	**4.27**	**1.46**	**138.0**
PF96a	P2	**3.70**	**2.38**	**319**	**4.38**	**2.31**	**35.1**

A-Tab. 28: AMF colonization in fine roots of FRISSON at 20 DAS (PP 97 b-d)
Estimation (chitin staining) by grid line method with n=100 in mixed samples of 2 x 4 plants.

AMF		*G. manihot*			natural AMF population			
P fertilization treatment		P100			P100		P300	
N fertilization treatment		N450			N150	N450	N150	N450
Treatment concerning phytopathological fungi		+*Rhizoc tonia*	-*Rhizoc-tonia*	+Fungi-cide	Nil			
AMF colonization 20 DAS [%]		**34**	**45**	**7**	**29**	**9**	**8**	**25**
Frequency [%] *of arbuscules*								
classified	*low*	3	19	2	10	4	3	10
	medium	2	13	1	4	2	0	6
	high	3	7	1	0	0	0	0

A-Tab. 29: Temperature during last 2 weeks of experiment PP 97 b-d
Records of thermo-hydrograph from 22 July to 5 August 1997 greenhouse at Mitra at 1 m above ground. Data were calculated from measures of every 2 h (Day: 6 to 20 h, night 20 to 6 h GMT). No humidity data.

	Temperature [C°]				
	min	max	**mean**	day	night
Mean	**19.4**	**27.7**	**23.5**	**24.7**	**21.3**
SD	1.6	3.2	1.2	1.8	1.4
Min	22.0	35.0	25.3	27.7	24.2
Max	16.0	23.0	21.2	21.3	19.3

A-Tab. 30: Production and AMF colonization at final harvest in pea pot experiment with Ferric Luvisol from Portel (PP 97 c, d, and e)

Iso-Line	N	P	Grain dm [g]	Shoot dm [g]	Root dm [g]	Plant dm [g]	Shoot/root ratio [%]	Harvest Index [%]	AMF colonization [%] TB	AP
Experiment **PP 97c**										
Fri	N450	P100 fun.	**0.96** ±0.75 a	**2.46** ±1.56 a	**0.21** ±0.09 a	**2.67** ±1.65 a	**10.6** ±2.6 a	**33** ±12 a	**48** ±16 a	**66** ±14 a
Fri	N450	P100	**0.50** ±0.15 a	**1.39** ±0.30 a	**0.14** ±0.03 a	**1.54** ±0.32 a	**10.1** ±2.2 a	**35** ± 4 a	**42** ±15 a	**48** ±20 a
P2	N450	P100 fun.	**0.20** ±0.16 a	**1.02** ±0.37 a	**0.15** ±0.01 a	**1.18** ±0.38 a	**6.7** ±2.7 a	**16** ±10 a	**0**	n.d.
P2	N450	P100	**0.35** ±0.15 a	**1.20** ±0.25 a	**0.13** ±0.02 a	**1.33** ±0.26 a	**9.0** ±0.8 a	**28** ± 8 a	**0**	n.d.
Experiment **PP 97d**					ln	ln				
Fri	N150	P100	**2.69** ±1.44 bcd	**4.86** ±2.20 bc	**0.36** ±0.15 ab	**5.22** ±2.34 b	**13.3** ±1.5 bcd	**54** ± 4 ab	**56** ±15 a	**53** ±18 a
Fri	N150	P300	**7.10** ±0.95 a	**13.41** ±3.43 a	**0.83** ±0.26 a	**14.23** ±3.69 a	**16.5** ±1.2 ab	**55** ± 8 ab	**27** ±19 ab	**37** ±24 a
Fri	N450	P100	**0.50** ±0.15 cd	**1.39** ±0.30 c	**0.14** ±0.03 b	**1.54** ±0.32 c	**10.1** ±2.2 cde	**35** ± 4 cd	**42** ±15 ab	**48** ±20 a
Fri	N450	P300	**3.82** ±1.29 abc	**7.76** ±2.05 ab	**0.49** ±0.16 a	**8.25** ±2.20 ab	**16.2** ±2.6 ab	**48** ± 5 abc	**14** ± 1 b	**19** ± 3 a
P2	N150	P100	**0.47** ±0.27 cd	**1.21** ±0.51 c	**0.16** ±0.06 b	**1.37** ±0.56 c	**7.4** ±1.2 e	**36** ± 7 cd	**0**	n.d.
P2	N150	P300	**5.71** ±2.50 ab	**9.16** ±4.00 ab	**0.49** ±0.23 a	**9.65** ±4.21 ab	**19.4** ±3.5 a	**62** ± 2 a	**0**	n.d.
P2	N450	P100	**0.35** ±0.15 d	**1.20** ±0.25 c	**0.13** ±0.02 b	**1.33** ±0.26 c	**9.0** ±0.8 de	**28** ± 8 d	**0**	n.d.
P2	N450	P300	**2.40** ±1.16 bcd	**5.16** ±2.31 bc	**0.36** ±0.17 ab	**5.53** ±2.48 b	**14.4** ±1.4 abc	**46** ± 5 bc	**0**	n.d.
Significant F-value					ln	ln				
		I	6	8	10	16	5	4	-	-
		N	19	12	10	13	n.s.	26	n.s.	n.s.
		P	55	51	47	99	76	35	13	5
		I x N	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-	-
		I x P	n.s.	n.s.	n.s.	n.s.	8	10	-	-
		P x N	4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		I x N x P	n.s.	n.s.	n.s.	n.s.	10	n.s.	-	-
Experiment **PP 97e**										
Fri	N150	P100	**7.41** ±1.24 a	**23.23** ±2.07 ab	**2.20** ±0.30 a	**25.44** ±2.30 ab	**10.7** ±1.0 c	**32** ±4 a	**77** ±4 a	n.d.
Fri	N150	P300	**7.41** ±1.48 a	**26.99** ±3.31 a	**2.54** ±0.64 a	**29.53** ±3.90 a	**11.3** ±1.8 bc	**28** ±7 a	**48** ±6 b	n.d.
P2	N150	P100	**1.52** ±0.31 b	**4.34** ±1.03 c	**0.29** ±0.10 c	**4.63** ±1.10 c	**15.8** ±2.8 b	**36** ±3 a	**0**	n.d.
P2	N150	P300	**7.91** ±0.45 a	**19.05** ±0.98 b	**0.84** ±0.08 b	**19.89** ±1.07 b	**22.8** ±1.2 a	**42** ±9 a	**0**	n.d.
Significant F-value			ln		ln	ln				
		I	63	125	114	114	50	7	n.s.	
		P	77	59	17	17	9	n.s.	64	
		I x P	78	21	12	12	6	n.s.	n.s.	

A-Tab. 31: P and N concentrations and contents in pea plants of pot experiments (PP 95, PP 97 d + e)
The inoculum tratment was with the AMF *Glomus manihot* and with *Rhizobium leguminosarum strain PS10*. Letters within double line indicate significant differences according to Tukey's HSD, m.s. were mixed samples, WHC waterholding capacity.

Isoline	Inoculum	Irrigation WHC [%]	N fertilization [µg g^{-1}]	P fertilization [µg g^{-1}]	NITROGEN Concentration Grain [%]	NITROGEN Concentration Straw [%]	NITROGEN Content shoot plant^{-1} [mg]	PHOSPHORUS Concentration Grain [mg g^{-1}]	PHOSPHORUS Concentration Straw [mg g^{-1}]	PHOSPHORUS Content shoot plant^{-1} [mg]
Exp. PP 95: sterilized Vertic Luvisol from Évora in climate chamber (contents ln transformed for ANOVA)										
Frisson	AMF & *Rhizobium*	80	346	50	**4.71** ±0.14 bc	**2.20** ±0.40 d	**351** ± 93 c	**3.07**	**0.58**	**20.1** ± 6.1 b
Frisson	AMF & *Rhizobium*	40	346	50	**5.11** ±0.24 abc	**2.74** ±0.16 cd	**308** ± 34 c	**2.95**	**0.77**	**13.7** ± 1.3 b
P2	AMF & *Rhizobium*	80	346	50	**4.64** ±0.30 c	**3.41** ±0.16 abc	**1006** ±268 a	**2.88**	**0.95**	**51.7** ±13.2 a
P2	AMF & *Rhizobium*	40	346	50	**4.96** ±0.21 abc	**3.88** ±0.15 ab	**277** ± 54 c	**2.86**	**0.95**	**12.9** ± 2.7 b
Frisson	-	80	346	50	**4.63** ±0.27 c	**2.39** ±0.46 d	**872** ±215 a	**3.36**	**0.76**	**52.8** ±13.8 a
Frisson	-	40	346	50	**5.43** ±0.18 a	**2.90** ±0.26 bcd	**456** ± 61 bc	**2.70**	**0.68**	**16.7** ± 2.0 b
P2	-	80	346	50	**4.92** ±0.34 abc	**3.49** ±0.85 abc	**856** ±300 ab	**3.66**	**0.96**	**56.2** ±29.9 a
P2	-	40	346	50	**5.25** ±0.15 ab	**4.10** ±0.57 a	**383** ±112 c	**2.72**	**0.72**	**15.6** ± 6.4 b
Exp. PP 97d: Ferric Luvisol from Portel in greenhouse										
Frisson	-	*ad lib.*	68	20	**4.78**	**3.72** ±0.81 c	**203** ± 75 abcd	**2.39**	**0.70**	**7.9** ± 4.0 cd
Frisson	-	*ad lib.*	68	59	**3.75**	**2.10** ±0.21 d	**395** ± 72 a	**3.45**	**1.13**	**31.7** ± 5.8 a
Frisson	-	*ad lib.*	205	20	**6.98**	**7.30** ±0.34 a	**100** ± 19 bcd	**2.18**	**0.40**	**1.4** ± 0.4 d
Frisson	-	*ad lib.*	205	59	**6.00**	**5.15** ±0.50 b	**429** ±107 a	**3.43**	**0.67**	**15.7** ± 4.9 bc
P2	-	*ad lib.*	68	20	**6.40**	**5.48** ±0.58 b	**69** ± 27 d	**1.94**	**0.30**	**1.1** ± 0.6 d
P2	-	*ad lib.*	68	59	**4.43**	**2.19** ±0.45 d	**324** ±134 ab	**3.64**	**0.42**	**22.3** ± 9.7 ab
P2	-	*ad lib.*	205	20	**7.06**	**6.20** ±0.51 ab	**78** ± 20 cd	**2.29**	**0.36**	**1.1** ± 0.4 d
P2	-	*ad lib.*	205	59	**6.23**	**5.67** ±0.42 b	**301** ±122 abc	**2.97**	**0.52**	**8.6** ± 4.0 cd
Exp. PP 97e: Ferric Luvisol from Portel in greenhouse										
Frisson	-	80	55	16	**3.11** ±0.19 c	**1.21** ±0.19 b	**418** ± 16 a	**3.00** ±0.06 ab	**0.82** ±0.10 a	**35.3** ± 3.9 a
Frisson	-	80	55	48	**3.42** ±0.58 bc	**1.17** ±0.33 b	**490** ±128 a	**3.73** ±0.17 a	**0.68** ±0.07 ab	**40.6** ± 3.0 a
P2	-	80	55	16	**4.96** ±0.23 a	**3.30** ±0.18 a	**166** ± 31 b	**1.70** m.s.	**0.61** ±0.05 b	**4.3** ± 1.1 c
P2	-	80	55	48	**4.09** ±0.20 b	**1.68** ±0.14 b	**509** ± 17 a	**2.20** ±0.59 b	**0.76** ±0.04 ab	**25.9** ± 4.7 b

mixed sample

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Plant materiel used

Plant	Scientific name	Variety	Line	Kindly provided by:
Pea	*Pisum sativum* L.	FRISSON	parental	AGRI OBTENTIONS Guyancourt, F
			mutant P2	Dr. Gérard Duc, INRA Dijon, F
		SPARKLE	parental	Dr. Chuck Simon, Washington State Univ., USA
				Dr. Mike Ambrose John Innes Centre, GB
			mutant E135	Dr. Tom LaRue
			mutant R25	Cornell University, USA
Chickpea	*Cicer arietinum* L.	ELVAR	parental	Eng. Tavares de Sosa ENMP, P
Safflower	*Carthamus tinctorius* L.	WARAMIN	parental	Dr. Sabine Eger University of Hohenheim, D

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