

SESSION 5

WORKSHOP III – TRADE WITH BENEFICIALS AND USEFUL ORGANISMS: A RISK?

Chairman: Dr F Feldmann
 DPG, Braunschweig, Germany

Reasons for and effects of changes in the parasitoid complex of the horse chestnut leaf miner (*Cameraria ohridella*)

G Grabenweger¹, M Stolz

BOKU Vienna, Inst. of Plant Protection, Peter Jordan Straße 82, A-1190 Vienna, Austria

¹present address: *Pflanzenschutzamt Berlin, Mohriner Allee 137, D-12347 Berlin, Germany*

Email: giselher@fjh-berlin.de

S Augustin, S Guichard

INRA Orleans, Zoologie Forestiere, Av. de la Pomme de Pin, F-45166 Ardon, France

N Avtzis

TEI Kavala, Department of Forestry, Proastio, GR-66100 Drama, Greece

S Bacher, P Kehrli²

UNI Bern, Institute of Zoology, Baltzerstraße 3, CH-3012 Bern, Switzerland

²present address: *Lincoln University, Bio-Protection and Ecology, Canterbury, New Zealand*

J Freise³, W Heitland

TU Munich, Dep. of Animal Ecology, Am Hochanger 13, D-85354 Freising, Germany

³present address: *LAVES Niedersachsen, Birkenweg 1, D-26127 Oldenburg, Germany*

S Girardoz, M Kenis

CABI Bioscience, Rue des Grillons, CH-2800 Delémont, Switzerland

C Lethmayer

AGES Vienna, Spargelfeldstraße 191, A-1226 Vienna, Austria

R Tomov

University of Forestry, Bul. Kliment Ohridski 10, BG-1756 Sofia, Bulgaria

L Volter

University of West Bohemia, Klatovská 51, CZ-30619 Plzen, Czech Republic

ABSTRACT

Parasitism of the horse chestnut leaf miner (HCLM) (*Cameraria ohridella*) has remained at a low level in Europe for many years. As a consequence, horse chestnut trees in regions with more than 20 years of HCLM infestation still suffer from heavy leaf miner attack. However, results of our investigations show changes in the parasitoid complex over the years, reflecting three different strategies exemplified by three parasitoid species. (1) The abundance of the most common parasitoid, *Minotetrastichus frontalis*, seems to remain at the same low level, indicating that the parasitoid does not actively search for special leaf miner hosts but rather encounters them by chance; the relative impact of these species decreases as other parasitoids gain importance. (2) Other species, such as *Pediobius saulius*, seem to undergo a slow process of adaptation; their relative

importance in the parasitoid complex increases at many locations infested for a long time. In the long run, parasitism of these species may reach levels exhibiting a controlling effect on HCLM populations. (3) A third group of species seems to follow the spread of its new host and invade new regions. Starting in the year 2000, the originally eastern-European species *Cirrospilus talitzkii* was recorded from HCLM in countries where it has never been found before on any other leaf miner. Meanwhile, the species can be found as a regular element of the parasitoid complex of HCLM in many south and central European countries.

To date, these changes in the parasitoid complex have no significant impact on the overall parasitism of HCLM. However, there are measurable effects, such as an increasing rate of pupal parasitism, owing to the action of *P. saulius*. In the long run, changes in the parasitoid spectrum of HCLM may increase the impact of parasitism and contribute substantially in future to the control of this invasive species.

INTRODUCTION

The spread of the horse chestnut leaf miner (HCLM) (*Cameraria ohridella*) (Lepidoptera: Gracillariidae) in Central Europe started in Austria, probably as a consequence of accidental introduction, and led to mass outbreaks of this pest in the following years. Owing to the epidemic population densities of the leaf miner, and the strong public concern for the health of the popular horse chestnut (*Aesculus hippocastanum*) trees in urban environments, plant protection measures were started immediately throughout Austria and some neighbouring countries. One argument for these measures, including chemical treatments, was the fact that the control of HCLM by natural enemies was very poor. Indeed, mass outbreaks of the pest never decreased extensively at any place in Europe by means of natural control mechanisms (Grabenweger, 2004). In particular, chalcidoid parasitoids, which are often responsible for a substantial part of the leaf miner's mortality, have no impact on the population dynamics of HCLM.

Several reasons for the failure of parasitoids to control the leaf miner have been proposed. There is evidence that such parasitoids search for suitable host trees rather than for particular host insects (Askew & Shaw, 1974). Since white-flowering horse chestnuts are a pontomediterranean tree species, they are poor in herbivorous insects and, therefore, parasitoids may not be attracted to the trees in general. Furthermore, the toxic substances of host leaves consumed by the leaf miner larvae may protect them against attack by natural enemies. In addition, the phenology of the parasitoids is not synchronised with the development of the leaf miner in springtime (Grabenweger, 2004), which hampers parasitism in the first generation.

Further measures for the protection of horse chestnut trees depend on the ability of native natural enemies such as parasitoids to increase their impact on the invasive leaf miner. Up to now, distinct trends towards a more effective parasitism have not been proved. The preliminary results of this study show that there are measurable changes in the parasitoid complex of HCLM. These changes reflect host selection strategies, searching behaviour and spread of certain parasitoid species. In the long run, some of these changes may have

stimulating effects on the parasitism of HCLM and increase the impact of parasitoids in its control.

MATERIALS AND METHODS

Infested leaves of horse chestnut were collected at more than 70 locations all over Europe, for a period of three years. To guarantee comparability of the samples, leaves were picked when the majority of HCLMs were in the pupal stage. Three samples a year were collected following the moth's trivoltine development. Mined leaves were randomly picked from the lower branches of the trees, and the contents of an average of 500 mines were examined under a dissecting microscope for signs of parasitism. Mines containing parasitoids were isolated, and the juvenile parasitoids were kept in glass vials until they completed their development.

Mines with dead leaf-mining stages and emerged or dead parasitoids were counted as parasitized. The percentage of parasitized mines in relation to the total number of mines dissected at each location was chosen as a measure of the parasitism level. To measure a density-dependent response of the parasitoids to their host populations, we estimated the infestation levels of the leaf miners on horse chestnut trees by visual rating as outlined in Gilbert & Gregoire (2003).

Monitoring the development of HCLM parasitism on a large timescale was not feasible within a four-year project. To measure changes in parasitism as a function of time, we compared locations where HCLM has arrived only recently with locations infested earlier. This gave us the opportunity to investigate changes within a time period of at least 15 years.

RESULTS

Minotetrastichus frontalis was the most abundant parasitoid of larval stages of HCLM in most of the examined locations. However, parasitism of this species was negatively correlated with leaf miner abundance on horse chestnut trees, showing a negative response of the parasitoid species to its host's densities (Figure 1).

In addition, larval parasitism did not increase with time (explanatory value of linear regression model $r^2 = 0.005$, $P = 0.612$). *Pediobius saulius* is the main pupal parasitoid in the leaf miner's parasitoid complex. It was the most abundant or at least a dominant species at many places where HCLM was already present for years. A correlation of parasitism through this species and leaf miner abundance showed neither a negative nor a positive trend (Figure 2).

In contrast to the above-mentioned results, pupal parasitism showed a weak, but still measurable, trend to increase with time, mainly due to *P. saulius* (explanatory value of linear regression model $r^2 = 0.107$, $p = 0.012$).

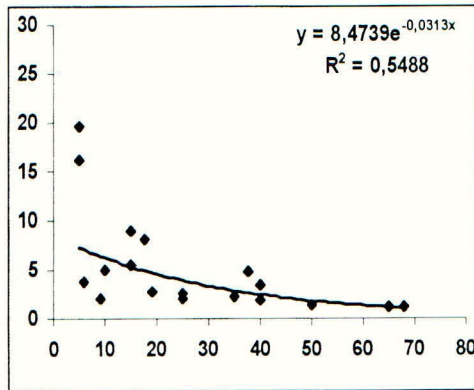


Figure 1. Parasitism rate of *M. frontalis* correlated to leaf miner host density (abscissae = mean percentage of infested leaf-area; ordinatae = percentage parasitism rate)

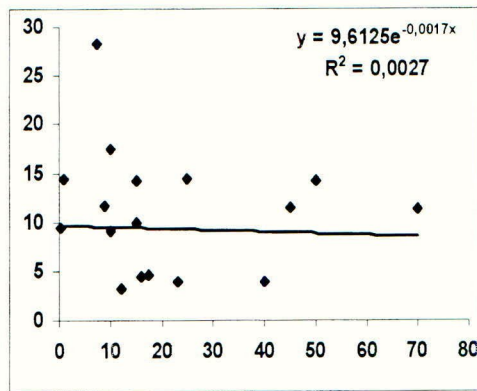


Figure 2. Parasitism rate of *P. saulius* correlated to leaf miner host density (abscissae = mean percentage of infested leaf-area; ordinatae = percentage parasitism rate)

Cirrospilus talitzkii was first found on HCLM in Bulgaria in 2001. In 2002, the species was found on HCLM in Greece and at the same time recorded by Radeghieri *et al.* (2002) from Italy. In 2003, it was already found in Austria and in the south of France. In the last examination of the parasitoid complex of HCLM in Vienna (spring 2004), *C. talitzkii* already

accounted for 1–3% of all species collected, ranging among the five most common parasitoid species in the complex of HCLM.

DISCUSSION

M. frontalis is one of the most common and polyphagous parasitoids of various groups of leaf miners. The probability of this extremely polyphagous species to encounter a suitable host just by chance is high. Therefore, *M. frontalis* is able quickly to integrate new insects into its host spectrum; in fact, this species was among the first parasitoids to be reared from HCLM in Europe. On the other hand, *M. frontalis* will not focus its searching behaviour on a particular host species, and it does not show a positive density-dependent response on the leaf miner's increasing populations. This lack of adaptation will probably inhibit an increase of the parasitoid's relative impact on the pest insect as a function of time.

Although *P. saulius* is present in the whole area invaded by HCLM, it is recorded mainly from regions where the leaf miner has been present for approximately 10 years. After this comparably long time for adaptation, however, the species gains quickly in relative importance, and soon dominates the parasitoid complex. There is slight, yet significant, evidence that parasitism through this species increases with time. As the relative impact of the species is growing, it may have a controlling effect on its host's populations in the future.

At first sight, records of *C. talitzkii* on HCLM in Europe are somewhat similar to those of *P. saulius*. However, the distribution of the former does not reflect a slow adaptation process but a recent spread of the species as a consequence of the host's invasion. Once established in a new area, it is most likely that the generalist *C. talitzkii* will move from HCLM to other hosts, too. At present, its impact on HCLM, as well as on other naturally occurring hosts in the newly colonized regions, is unclear.

ACKNOWLEDGEMENT

This study was funded by the European Commission as part of the FP5 project CONTROCAM, QLK5-CT-2000-01684.

REFERENCES

- Askew R R; Shaw M R (1974). An account of the Chalcidoidea parasiting leaf mining insects of deciduous trees in Britain. *Biological Journal of the Linnean Society* **6**, 289-335.
- Gilbert M; Grégoire J-C (2003). Visual, semi-quantitative assessments allow accurate estimates of leafminer population densities : An example comparing image processing and visual evaluation of damage by the horse-chestnut leafminer *Cameraria ohridella*. *Journal of Applied Entomology* **127**, 354-359.
- Grabenweger G (2004). Poor control of the horse chestnut leafminer, *Cameraria ohridella* (Lepidoptera: Gracillariidae), by native European parasitoids: a synchronisation problem. *European Journal of Entomology* **101**, 189-192.

Radeghieri P; Santi F; Maini S (2002). New record species for the Italian fauna: *Cirrospilus talitzkii* (Hymenoptera Eulophidae), a new parasitoid of *Cameraria ohridella* (Lepidoptera Gracillariidae) (Preliminary note). *Bulletin of Insectology* 55, 63–64.

Trade with beneficial mycorrhizal fungi in Europe: suitable quality management avoids distribution of unwanted organisms in mycorrhizal inoculum

C Grotkass, I Hutter

INOQ GmbH, Solkau 2, D-29465 Schnega, Germany

Email: grotkass@inoq.de

F Feldmann

BBA, Messeweg 11-12, D-38104 Braunschweig, Germany

ABSTRACT

Mycorrhiza form the most important mutual symbiosis between plant roots and soil-borne fungi. An established symbiosis in the plant roots guarantees the adaptation even under unsuitable soil or climate conditions. The advantages have led to an increasing demand for mycorrhiza products in recent years in Europe. As a consequence, large amounts of mycorrhizal inoculum are transported through the 'borderless' Europe and are even imported from North America, South America and Asia. Because international regulations on the trade with beneficial microorganisms are failing, serious producers of mycorrhizal inoculum founded national and international organisations that are recently voluntarily working out quality-control procedures to avoid the spread of unwanted organisms accompanying mycorrhizal inoculum.

INTRODUCTION

Mycorrhiza form the most important mutual symbiosis between plant roots and fungi. More than 90% of all plants live in a community with soil-borne fungi. The advantages of a functioning symbiosis are manifold: the mycelia of the fungi enlarge the function of the root system and increase water and nutrient uptake of the plants. The symbiosis also results in a higher drought tolerance, the plants become more resistant to diseases, the soil structure is stabilised and the soil aeration, water permeability and aggregation is improved through the mycelia of the mycorrhizal fungi. The advantages of the symbiosis are evident in stress situations such as poor soil or climatic conditions or transplanting shock: the symbiosis guarantees the adaptation to the conditions and a faster growing of mycorrhizal plants.

Consequently, wherever potential hosts of mycorrhizal fungi are produced, companies are interested to test the new mycorrhizal technology in practice. In 2003, the German Federal Research Centre for Agriculture and Forestry found that the world-wide production and trade of mycorrhiza products increased immensely during recent years (Figure 1). The USA and Germany are recently the largest producers and retailers of mycorrhizal inoculum, but a considerable number of companies all over the world are preparing for the market and have already started to spread the inoculum throughout Europe.

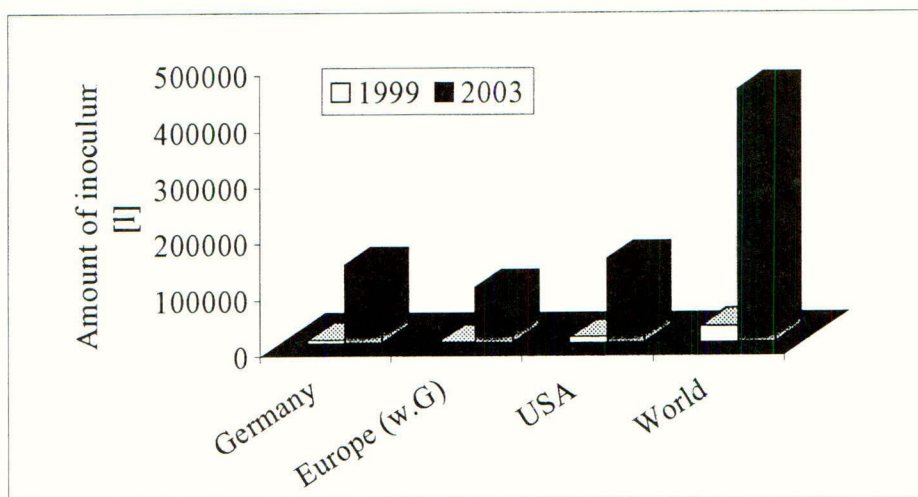


Figure 1. Increasing production of mycorrhiza products show the necessity for a standardised quality management in Europe (w.G. = without Germany). Source: Feldmann (2003).

Plant species of all growth-form types establish a symbiosis with arbuscular mycorrhiza. Others, such as most of the needle trees, need ectotrophic mycorrhiza for their growth. Ericaceae grow together with ericoid mycorrhiza. These major groups of mycorrhiza are produced with specific production methods, defined by specific demands of the fungi produced: the arbuscular mycorrhizal fungi (AMF) are obligatory biotrophic fungi, the others are facultative saprophytes. The first can be produced only on living plants; the second could be produced on sterile nutrient media. However, caused by resulting inefficiency, culturing media are recently recognised to be less and less suitable for the production of ectomycorrhizal fungi, which leads to biotrophic production of these fungi as well. Overall, mycorrhiza products differ in the fungi contained and their carrier material, but the majority are produced on living plants. The inoculum sold, therefore, is a mixture of substrate in which the multiplying plant already grew, mycorrhizal hyphae and spores, and accompanying organisms potentially including saprophytic, hyperparasitic and pathogenic fungi, phytophagous faunistic elements or weeds (Feldmann, 2003). While the risk of damage on useful plants, detrimental influences to environment and biodiversity caused by mycorrhizal fungi are recently estimated to be low (Feldmann, 2003), the risk of the spread of unwanted alien, or even invasive, accompanying organisms is known to be much higher. Suitable quality-control procedures, therefore, need to be applied.

VOLUNTARY COMMITMENT FOR QUALITY CONTROL IN EUROPE

In Germany, trade of mycorrhiza products is regulated by the fertiliser act, which classifies them as soil supplements. Supplements are defined as substances other than fertilisers,

manufactured, sold or represented for use in the improvement of the physical condition of the soil or to aid plant growth or crop yields. Only some physical parameters have to be declared, so that they do not have any relevance for the quality description of mycorrhizal inoculum. All serious German inoculum manufacturers, unified in the German lobby 'Application of mycorrhizal fungi in practice' (for more information see info@mykorrhiza.de), therefore, agreed on a standardised voluntary quality control in 1997 to increase the quality of products even without legal preconditions (Backhaus & Feldmann, 1997). Today, the main biological and physical parameters cited below are controlled in the different companies of the German lobby. Nevertheless, it became obvious that, already, slight differences between procedures and protocols applied could lead to significantly different results. The German lobby therefore induced the process to install voluntary quality-control procedures on a European level (on the basis of COST Action 838) to discuss the problems on an international level and to include European companies as well. One important result was a joined publication of the largest European inoculum producers as a basis for a joined Europe-wide voluntary quality control (von Alten *et al.*, 2002). Another result was the foundation of the 'Federation of European Mycorrhizal Fungi Producers' which started to work out a methodology for a standardised quality control for the EU since May 2003 (www.femfip.com). The aim is to define reproducible, valuable parameters for later external quality control carried out by independent institutions. A seal for 'good inoculum quality' will be developed, which guarantees minimum requirements for certified products. The Federation also works on the transfer of scientific results to practical application, for the benefit of customers. Already, today, high-quality inoculum is on the market for a broad range of applications.

SPECTRUM OF UNWANTED ACCOMPANYING ORGANISMS FOUND IN COMMERCIAL MYCORRHIZAL INOCULUM WITHOUT QUALITY-CONTROL DECLARATION

For the production of AMF, living host plants are necessary because of their obligate biotrophy. The host plants (e.g. *Zea mays*) can be planted in pots or ground beds in the greenhouse. They are inoculated with starter inoculum in this unsterile production system. Peat, expanded clay, lava or vermiculite with different grain sizes are used as carrier materials, depending on the later application methods. After cultivation for at least three months the host plant is removed; the remaining carrier material now contains spores of the fungus and also mycelia and infected roots. The sterile production of AMF is also possible, but products are not competitive on the market at the moment. The production of ectotrophic and ericoid mycorrhiza is possible sterile and also unsterile. The fungal mycelia is propagated on nutrient media in the laboratory for a sterile product that is often mixed with a carrier material, e.g. vermiculite. The mycelium overgrows the carrier material and can be mixed with substrates during planting or, for example, tree restorations. However, investigations show that fungi can stop establishing a symbiosis with their natural hosts after some time in sterile culture. Therefore, inoculated trees grow in nurseries for unsterile production. After some months the substrates can be used as inoculum. It is important to know that the inoculum normally contains variable mixtures of symbiotic fungal populations. The production in such an open system in the greenhouse can lead to contamination of the inoculum with several organisms (Table 1).

Table 1. Unwanted accompanying organisms in commercial mycorrhizal inoculum, with and without quality control declared on the packing.

| Contaminants | Inoculum Number | | | | | |
|--------------------------------|-------------------------|---|---|----------------------------|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| | with quality control | | | without quality control | | |
| Fungi | + | + | - | + | + | + |
| Potential phytopathogens | - | + | + | + | - | + |
| Hyperparasites | + | - | - | - | - | - |
| Saprophytes | + | + | - | + | + | + |
| Phytophagus faunistic elements | | | | | | |
| Diptera (larvae) | - | - | - | + | - | - |
| Coleoptera (larvae) | - | - | - | - | + | - |
| Collembola | - | - | - | + | + | - |
| Acari | - | - | - | + | + | + |
| Nematoda | - | - | - | - | - | + |
| Gastropoda | - | - | - | - | - | - |
| Plants/seeds | | | | | | |
| Algae | + | + | - | + | + | + |
| Weeds | - | - | - | - | + | + |

CONCURRENT QUALITY CONTROL DURING INOCULUM PRODUCTION

Most of the cases of contamination of mycorrhizal inoculum could be avoided by concurrent quality-control procedures (Table 1). The protocol for the achievement of high-quality inoculum without contamination covered the following relevant aspects:

- Certification of substrate quality necessary for inoculum production: avoidance of pre-contamination (of special importance in case of peat substrates);
- Suitable preparation of the substrate with special reference to porosity: guarantee of good aeration for best root development;
- Moderate irrigation to prevent the substrate from water logging and to prevent the establishment of algae and moss;
- Control of water quality (risk of contamination is high if water from a well is used)
- Low, but frequent fertilisation to optimise the forming of the symbiosis and to prevent luxuriant growth and excessive root exudation of host plants;
- Frequent investigation of host plants in order to detect diseases and pests;
- Adequate phytosanitary action to eradicate disease, pests or weeds;
- In case of disease or pest epidemics, application of adequate plant protection measures;
- Frequent measuring of propagule development of the target organism to cut the inoculum production time to as short as possible;

- Drought period at the end of the production, without substrate disturbance, followed by nearly complete disappearance of microfaunistic elements;
- Removal of host plant parts and rapid drought of the substrate, to reduce saprophytic activity.

POST-HARVEST QUALITY CONTROL

The post-harvest inoculum control investigates the outcome of inoculum production after drying and mixing the substrate, but before packing it. The post-harvest quality control includes several physical properties of the inoculum, biological measures to describe quantity and effectivity of the mycorrhizal fungus multiplied (for details see von Alten *et al.*, 2002), and examination for accompanying organisms (as per Table 1). Of principal importance is the focus on species- and/or genus-specific detection of numerous plant pathogens, as well as beneficial fungi (Table 2). This test could be out-sourced, because it is recently offered as service by an independent company (www.DNA-SCAN.de).

Table 2. Spectrum of microorganisms checked in mycorrhizal inoculum after harvest

| | | |
|-------------------------------------|-------------------------------------|--------------------------------|
| <i>Athelia (Sclerotium) rolfsii</i> | <i>P. citricola</i> | <i>P. sylvaticum</i> |
| <i>Botrytis cinerea</i> | <i>P. cryptogea</i> | <i>P. ultimum</i> |
| <i>Colletotrichum acutatum</i> | <i>P. drechsleri</i> | other <i>Pythium</i> spp. |
| <i>C. coccodes</i> | <i>P. fragariae</i> | <i>Ralstonia solanacearum</i> |
| <i>C. fragariae</i> | <i>P. infestans</i> | <i>Rhizobium radiobacter</i> |
| <i>C. gloeosporioides</i> | <i>P. nicotianae</i> | <i>Rhizoctonia solani</i> |
| other <i>Colletotrichum</i> spp. | <i>P. ramorum</i> | <i>Sclerotinia minor</i> |
| <i>Cylindrocarpon destructans</i> | other <i>Phytophthora</i> spp. | <i>S. sclerotiorum</i> |
| <i>Cylindrocladium</i> spp. | <i>Plectosphaerella cucumerina</i> | other <i>Sclerotinia</i> spp. |
| <i>Didymella</i> spp. | <i>Pseudomonas cichorii</i> | <i>Sclerotinia trifoliorum</i> |
| <i>Fusarium oxysporum</i> | <i>P. marginalis</i> | <i>Thielaviopsis basicola</i> |
| <i>F. solani</i> | <i>P. syringae</i> | <i>Trichoderma asperellum</i> |
| other <i>Fusarium</i> spp. | <i>P. syringae</i> pv. <i>porri</i> | <i>T. hamatum</i> |
| <i>Gnomonia comari</i> | <i>P. viridiflava</i> | <i>T. harzianum</i> |
| <i>Penicillium</i> spp. | <i>Pyrenochaeta lycopersici</i> | other <i>Trichoderma</i> spp. |
| <i>Phoma destructiva</i> | <i>Pythium aphanidermatum</i> | <i>Verticillium albo-atrum</i> |
| <i>Phytophthora cactorum</i> | <i>P. dissotocum</i> | <i>V. dahliae</i> |
| <i>P. capsici</i> | <i>P. irregulare</i> | other <i>Verticillium</i> spp. |
| <i>P. cinnamomi</i> | <i>P. polymastum</i> | <i>Xanthomonas fragariae</i> |

IS THE CONTROL OF TRADE WITH MYCORRHIZAL INOCULUM ADVANTAGEOUS?

Trade with mycorrhizal inoculum is a reality all over Europe. Inoculum is transported between East and West, and sometimes is crossing five borders without the knowledge of the authorities. Very often, the carrier material of the inoculum itself is declared but not the

microorganisms bound to it. Would the duty to declare mycorrhizal inoculum handicap the trade of such products? We, as producers of inoculum, together with consultants of national authorities involved in questions of unwanted organisms, demonstrated over several years that we are prepared to carry responsibility for the consequences resulting from our products. Therefore, we developed high standards of quality control which limit the potential risk of mycorrhizal inoculum already in the pre-competitive phase of the broad introduction of mycorrhizal technology. Declaration at country borders would support our efforts, to offer our customers high-quality goods with reliable characteristics instead of low-quality goods without any certification.

The producers of mycorrhizal inoculum unified in the Federation of European Mycorrhizal Fungi Producers (FEMFiP) are supporting companies and other entities involved in the production and/or developing products based on mycorrhizal fungi across Europe. As already stated, their particular aims are to achieve and maintain the highest standards of inoculum quality. This will be done by educational and training programmes aimed at promoting the use of mycorrhizal fungi as a technology suitable for sustainable agriculture, forestry, horticulture, landscaping etc. Methods for evaluating the quality of inocula will be standardised and certification programme for producers will be introduced. The procedures for compliance will be developed in collaboration with its members and administered through the offices of an independent laboratory. FEMFiP aims to participate in discussions for the establishment of registration procedures of products based on beneficial rhizospheric microorganisms being prepared by European Committee. The quality-control procedures outlined, and the later certification of the products, will help to close an important gap with regard the spread of unwanted organisms.

REFERENCES

- Backhaus G F; Feldmann F (1997). Anwendung arbuskulärer Mykorrhizapilze im Pflanzenbau. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem* **332**, 85 pp.
- Feldmann F; Hutter I; Grotkass C (2003). Mycorrhizal fungi as factors of integrated plant protection in urban horticulture: the state of the art. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem* **394**, 205-210.
- Feldmann F (2003). Weltweiter Handel mit Inokulum arbuskulärer Mykorrhizapilze – Eine Risikoanalyse. *Schriftenreihe BMVEL, Angewandte Wissenschaft* **498**, 165-175.
- von Alten H; Blal B; Dodd J C; Feldmann F; Vosatka M (2002). Quality control of arbuscular mycorrhizal fungi inoculum in Europe. In: *Mycorrhizal Technology in Agriculture*, eds S Gianinazi, H Schuepp; J M Barea & K Haselwandter, pp. 281-296.

Exotic beneficials in classical biological control of invasive alien weeds: friends or foes?

M-K Seier

CABI Bioscience UK Centre, Silwood Park, Buckhurst Road, Ascot, Berkshire, SL5 7TA, UK

Email: m.seier@cabi.org

ABSTRACT

In many parts of the world classical biological control is commonly incorporated into an integrated management strategy to deal with invasive alien plant species. Countries such as Australia, South Africa, New Zealand and the USA have a long-standing experience in implementing this control method; hence, their national regulatory pathways and legal frameworks are well-established and tested. In contrast, Europe has little experience of this management strategy, which involves the introduction of co-evolved and, thus, highly specific natural enemies (arthropods or pathogens), from the centre of origin of the invasive species into its new exotic range, with the aim of reducing its population below a certain threshold. The potential risks associated with the use of exotic organisms to control invasive alien species are of common concern and microorganisms are often perceived as especially problematic. Based on past biocontrol initiatives, particularly those using co-evolved fungal pathogens to control invasive alien weeds, as well as on experiences gained from two recent projects dealing with two European weeds: giant hogweed (*Heracleum mantegazzianum*) and Japanese knotweed (*Fallopia japonica*), this paper illustrates the stringent risk assessments undertaken for potential biocontrol agents and reviews existing guidelines and legislation regulating the introduction of such exotic beneficials.

INTRODUCTION

Biological invasions, classed second only after habitat loss with respect to their devastating impact on biodiversity worldwide (Vitousek *et al.*, 1997), have become a reality around the globe. Linked to the increase in international trade and transport, the translocation of animals, plants, microorganisms and their products has led to a sharp rise in the threat posed by invasive alien species, often resulting in substantial environmental damages and economic costs (Pimentel, 2002). Whether introduced purposefully or by chance, alien organisms usually arrive in their new ranges freed of the majority, if not all, of their co-evolved natural enemies from their native ranges. In the case of invasive alien plant species, one commonly accepted theory relating the potential impact of neophytes in exotic situations to a reduced regulation through both specialist and generalist natural enemies is proposed by the enemy release hypothesis (Keane & Crawley, 2002; Mitchell & Power, 2003). Based on this hypothesis, classical biological control (CBC) aims to redress this imbalance through the intentional release of co-evolved, highly specific arthropods and pathogens from the centre of origin of the respective alien invasive into its new adventive range. Biological control has been defined as the deliberate use of living organisms to suppress pest species populations to less damaging levels (Waage & Greathead, 1988). With respect to CBC of weeds, co-evolved invertebrates have traditionally been employed as control agents, since the prevailing public

apprehension towards plant pathogens has long hindered the use of such organisms. The exploitation of microorganisms as classical agents against invasive alien weeds has commenced only recently. Long-standing experiences made by countries such as Australia, South Africa, New Zealand and the USA, which integrate CBC routinely into management plans for invasive neophytes, have shown this method to be a successful, environmentally benign and cost-effective control strategy, when undertaken scientifically and in line with well-established national legal frameworks and regulatory pathways (McFadyen, 1998). Europe, however, lacks such experience, having not yet implemented CBC as a tool to control invasive alien weeds. Accordingly, any specific European legislation regulating potential future introductions of classical agents is also lagging behind.

Based on past biological control initiatives, particularly those using co-evolved fungal pathogens to control invasive alien weeds, as well as on experiences gained from two recent research projects dealing with two European weeds, giant hogweed (*Heracleum mantegazzianum*) and Japanese knotweed (*Fallopia japonica*), this paper illustrates the stringent risk assessments undertaken for potential classical agents. The importance of the decision-making process based on such risk assessments is highlighted and current guidelines and legislation regulating potential introductions of exotic beneficials are reviewed.

RISK ASSESSMENT OF CLASSICAL AGENTS

Being exotic organisms themselves, the deliberate release of CBC agents against invasive weeds obviously also carries associated risks. Hoddle (2004) states that "biological control is the fifth invasion route for exotic organisms" following accidental introductions, global trade in exotics, use of exotic species in the hunting and fishing industry, as well as in agriculture. However, in contrast to most other introduction routes of alien species, CBC is based on scientific principles and follows stringent procedures to evaluate the potential of a selected organism as a classical agent, including the likelihood of any potential harmful side-effects before its importation and release. Comprehensive reviews specifying the individual steps followed by a typical weed CBC programme have been published by McFadyen (1998) and Evans *et al.* (2001). Assessing the host specificity of a prospective classical agent constitutes the most critical phase in any CBC initiative and provides crucial data for a risk assessment on which to base all further decisions concerning agent implementation. Besides biota dilution and environmental damage, the attack of non-target species is the most common concern regarding the use of CBC agents. Predicting accurately the potential host range of an exotic arthropod or a plant pathogen in a new environment is, therefore, of vital importance. Agents under evaluation undergo rigorous host-range testing, under greenhouse and/or laboratory conditions, according to the centrifugal phylogenetic method (Wapshere, 1974). Based on the theory that plant species more closely related to the target weed are also more likely to experience attack, the centrifugal phylogenetic testing method, combined with good taxonomic knowledge of both the respective agent and its target weed, has proven to provide reliable results on which to base host-range predictions of CBC agents.

With respect to non-target damage caused by arthropod classical agents, the few cases where there have been unpredicted non-target impacts have been due to shortcomings in the scientific evaluation, most notably inadequate host-range testing. The majority of documented non-target effects caused by invertebrates, particularly those involving native

plant species, had all been anticipated prior to the release of the respective agents since their potential host ranges had been comprehensively established. The European seed weevil *Rhinocyllus conicus*, for example, was known to have a wide host range within the thistle family (Harris & Zwoelfer, 1971), and thus the observed attack of native American *Cirsium* spp. (Turner *et al.*, 1987) following its introduction into the USA to control alien *Carduus* spp. came as no surprise. Cases like this illustrate that the potential impact on native plants of no economic value was previously not viewed as problematic and that, at the time of the decision for importation, the ecological risks posed by some control agents were considered to be acceptable compared to the anticipated economic benefits (McFadyen, 1998).

In contrast to arthropod agents, only 26 fungal pathogens have been used worldwide to date as classical agents against invasive neophytes (Barton, 2004). There have been no unpredicted non-target effects following the release of any of these agents. Attacks on non-target plant species were predicted in the case of three rust pathogens; however, the damage caused was minor and almost exclusively observed during the initial release trials (Barton, 2004). Obviously, many pathogens with biocontrol potential will be rejected during the initial stages of their host-specificity evaluation. For example, Argentinean strains of the facultative pathogen *Cercospora xanthicola* and the powdery mildew *Erysiphe cichoracearum*, assessed for control of the neotropical invasive weed Bathurst burr (*Xanthium spinosum*) in Australia, were also found to attack a number of local sunflower cultivars and were, thus, not considered any further as CBC agents (Morin *et al.*, 2004). On the other hand, additional testing will frequently be required by the decision-making authorities to provide more data on which to base a risk assessment for a specific fungal agent. The Mexican rust *Puccinia abrupta* var. *partheniicola*, a potential control agent for parthenium weed (*Parthenium hysterophorus*) in Australia, was shown to cause resistant or hypersensitive reactions in certain Australian sunflower cultivars. In order to show that this resistance is not lost during 'stress situations', tests were carried out under extreme light, temperature and humidity conditions, as well as following pre-inoculation with the sunflower rust *Puccinia helianthi*, to prove that this was not the case. The rust was subsequently introduced into Australia in 1991 (Parker *et al.*, 1994). However, decisions concerning the release of classical agents are usually not based on results of the host-range testing alone, since host-range expansion of both arthropods and fungal pathogens under artificial greenhouse conditions is a well-documented phenomenon (Evans *et al.*, 2001) and any potential damage caused by a prospective CBC agent must be carefully balanced against the actual and potential impact of the invasive target weed (McFadyen, 1998). For example, evaluation of the Madagascan rust *Marvalia cryptostegiae* as a classical agent for the invasive Madagascan rubbervine weed (*Cryptostegia grandiflora*) in Australia revealed that under greenhouse conditions the pathogen also infects the closely related Australian endemic species *Cryptolepis grayi* (Evans, 2000). However, when the economic and environmental threat posed by Madagascan rubbervine weed to entire ecosystems in northern Queensland was weighed against the potential risk posed by the rust to a single non-target species, the Australian authorities approved import of the pathogen as a classical agent (Evans, 2000; Evans *et al.*, 2001). Released in 1994, *M. cryptostegiae* has so far not been reported from either *C. grayi* nor any other non-target species, whilst its severe impact on Madagascan rubbervine weed fuels hope for the control of this invasive weed to be achieved within 4–6 years (Tomley & Evans, 2004). Finally, decisions about the use of classical agents will also need to be taken in the political and social context of a specific country. In the case of the neotropical rust *Puccinia melampodii*, another control agent for the invasive parthenium

weed, the risk of predicted non-target effects on *Calendula officinalis* and selected sunflower varieties was no hindrance to its release in Australia. Conversely, for India, equally troubled by this invasive weed, but with no protocols for (and no history of) the use of fungal pathogens as classical agents, and with a negative perception of alien organisms, such non-target attacks would not be acceptable, particularly since *C. officinalis* is an important social plant in Indian culture (Evans, 2000).

CBC OF WEEDS IN EUROPE

Whilst in the fortunate position to benefit from the experiences and scientific advances made in weed CBC by nations such as Australia, Europe has been slow to take up this strategy for the control of invasive neophytes. However, two recent European-funded research projects currently evaluating the potential of classical agents to control the invasive alien weeds giant hogweed and Japanese knotweed clearly reflect a change in European policy. During the course of both projects, surveys for co-evolved natural enemies have been conducted in the respective native ranges of the plant species: the western Caucasus mountains in the case of giant hogweed, and Japan for Japanese knotweed. For giant hogweed, none of the pathogens or invertebrates assessed thus far has exhibited sufficient host specificity to be considered as potential CBC agents in the adventive European range of the weed. Under greenhouse conditions, the evaluated fungal agents infected the closely related parsnip (*Pastinaca sativa*) and, to a lesser extent, also coriander (*Coriandrum sativum*) (Seier, 2005). Invertebrate agents predominantly attacked *P. sativa*, as well as the native hogweed species *Heracleum sphondylium* (R Wittenberg, personal communication). For Japanese knotweed preliminary host-range testing is currently under way with a *Puccinia* rust species and a damaging leaf-spot pathogen as the most promising fungal agents. A psyllid and a stem-boring weevil have been prioritized as the arthropod agents (D H Djeddour & R H Shaw, personal communication). Should any of these potential candidates prove to be suitable as CBC agents, based on their biology and the results of full host-range testing, the next challenge will lie in addressing the current European legislation for importation of exotic biocontrol agents. Standards for the 'safe use of biological control' have been developed by EPPO (EPPO, 2001) based on the IIPC Code of Conduct for the import and release of exotic biological control agents (IIPC, 1996). However, neither of these documents is legally binding; the former one even explicitly excludes microorganism. In the UK, applications for the release of exotic arthropods are currently regulated by the *Wildlife and Countryside Act* (1981) and are dealt with by the Department of Environment, Food and Rural Affairs (Defra) supported by the Advisory Committee on Releases into the Environment (ACRE). The potential introduction of fungal pathogens for weed CBC, on the other hand, is covered by the EU directive 91/414 (1991), assigning them the status of a plant protection product and dealt with by the Pesticide Safety Directorate (PSD), potentially, therefore, with immense associated cost. Looking at countries such as Australia, South Africa, New Zealand and the USA, with specific legislation and protocols in place to address the import and release of CBC agents, the need for Europe to standardize procedures and to develop appropriate legal frameworks becomes apparent if the potential of weed CBC is to be exploited.

CONCLUSIONS

Exotic beneficials used in weed CBC have the potential to become either 'friends or foes', since any deliberate introduction of alien species into a new environment involves a risk (Evans, 2000). It is, therefore, paramount that decisions on the development of classical agents are based on good scientific data and on a comprehensive risk assessment, as well as being supported by appropriate legislation. Addressing the concern of potential damage to non-target plant species, the current host-specificity testing protocol has proven to give reliable predictions about the anticipated field host-range of potential agents, although the extent of test species lists may prove debatable. The ecological impact of introduced species is harder to assess and this issue has been flagged by many critics (Louda & Stiling, 2004). Decisions about the release of a CBC agent can only rely on the biological data available and can also be subject to popular perceptions. With advancing science and changing public attitudes, especially towards the 'value' of native species, it is inevitable that some decisions taken in the past would now be frowned upon, such is the case of the aforementioned 'thistle saga' (Strong, 1997) in the USA.

ACKNOWLEDGEMENTS

I thank Harry Evans for his constructive comments on the earlier draft of the manuscript. The research project on giant hogweed was supported by European Union funding under the 5th Framework programme; project number EVK2-2001-00125.

REFERENCES

- Barton J (née Fröhlich) (2004). How good are we at predicting the field host-range of fungal pathogens used for classical biological control of weeds? *Biological Control* **31**, 99-122.
- EPPO (2001). EPPO Standards: Safe use of biological control. Import and release of exotic biological control agents. *EPPO Bulletin* **31**, 29-35.
- Evans H C (2000). Evaluating plant pathogens for biological control of weeds: an alternative view of pest risk assessment. *Australasian Plant Pathology* **29**, 1-14.
- Evans H C; Fröhlich J; Shamoun S F (2001). Biological control of weeds. In: *Bio-Exploitation of Filamentous Fungi*, eds S B Pointing & K D Hyde, pp. 349-401. Fungal Diversity Press: Hong Kong.
- Harris P; Zwoelfer H (1971). Biological control programmes against insects and weeds in Canada, 1959-1968. *CIBC, Technical Communication* **4**, 76-78
- Hodde M S (2004). Restoring Balance: Using exotic species to control invasive exotic species. *Conservation Biology* **18**, 38-49.
- IPPC (1996). Code of Conduct for the import and release of exotic biological control agents. *International Standards for Phytosanitary Measures Publication* **3**. FAO: Rome, Italy.
- Keane R M; Crawley M J (2002). Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology and Evolution* **17**, 164-170.
- Louda S M; Stiling P (2004). The double-edged sword of biological control in conservation and restoration. *Conservation Biology* **18**, 50-53.

- McFadyen R E Crutwell (1998). Biological control of weeds. *Annual Review of Entomology* **43**, 369-393.
- Mitchell C E; Power A G (2003). Release of invasive plants from fungal and viral pathogens. *Nature* **421**, 625-627.
- Morin L; Auld B; Evans H; Ellison C; Reeder R; Anderson F; Pettit B (2004). Argentinian fungi for Bathurst burr fail preliminary host-specificity tests. In: *Proceedings of the XI International Symposium on Biological Control of Weeds*, eds J M Cullen; D T Briese; D J Kriticos; W M Lonsdale, L Morin & J K Scott, p. 351. CSIRO Entomology: Canberra.
- Parker A; Holden A N G; Tomley A J (1994). Host specificity testing and assessment of the pathogenicity of the rust, *Puccinia abrupta* var. *partheniicola*, as a biological control agent of Parthenium weed (*Parthenium hysterophorus*). *Plant Pathology* **43**, 1-16.
- Pimentel D (ed.) (2002). *Biological invasions: economic and environmental costs of alien plant, animal, and microbe species*. CRC Press: Boca Raton, Florida.
- Seier M K (2005). Fungal pathogens as classical biological control agents for invasive alien weeds – are they a viable concept for Europe? In: *Biological invasions: challenges for science, Neobiota 6*, ed. W Nentwig (in press).
- Strong D R (1997). Fear no weevil? *Science* **277**, 1058-1059.
- Tomley A J; Evans H C (2004). Establishment of, and preliminary impact studies on, the rust, *Maravalia cryptostegiae*, of the invasive alien weed, *Cryptostegia grandiflora* in Queensland, Australia. *Plant Pathology* **53**, 475-484.
- Turner C E; Pemberton R W; Rosenthal S S (1987). Host utilization of native *Cirsium* thistles (Asteraceae) by the introduced weevil *Rhinocyllus conicus* (Coleoptera: Curculionidae) in California. *Environmental Entomology* **16**, 111-115.
- Vitousek P M; D'Antonio C M; Loope L L; Rejmanek M; Westbrooks R (1997). Introduced species: A significant component of human-caused global change. *New Zealand Journal of Ecology* **21**, 1-16.
- Waage J K; Greathead D J (1988). Biological control: Challenges and opportunities. *Philosophical Transactions of the Royal Society of London* **B 318**, 111-128.
- Wapshere A J (1974). A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology* **77**, 201-211.

Are arbuscular mycorrhizal fungal species invasive – derived from our knowledge about their distribution in different ecosystems?

E Sieverding

University of Hohenheim, Garbenstrasse 13, D-70593 Stuttgart, Germany

Email: sieverdinge@aol.com

F Oehl

University of Basel, Inst. of Botany, Hebelstr. 1, CH-5057 Basel, Switzerland

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are soil borne. They are obligate symbionts and they require a root of a higher plant to grow and to reproduce. Thus, they occur only under vegetation or where plants grew. Some of the so far 190 species described can be considered to be generalists, e.g. *Glomus mosseae*, *G. geosporum*, *G. etunicatum*, *G. constrictum*, *G. diaphanum*, *G. fasciculatum* and *Scutellospora calospora* because they have been found in diverse ecosystems. These, thus, must be considered to have invasive capabilities. Others are non-generalists, occurring only under, for example, grassland vegetation. Extremely little is known about the natural invasion process of these fungi into an ecosystem. Newly formed pioneer vegetation in areas without former plant cover appear to become slowly colonized. Long-distance transport of propagules of AMF can be by wind and water, and potentially also by agricultural goods as carrier. Once established in an area the speed of local distribution is slow, often slower than root growth. Some species of AMF can persist for several years in the soil, even without a host plant. Successional changes in the composition of the native species composition of AMF are known from agro-ecosystems, and these changes are strongly influenced by agricultural practices. Some practices dramatically decrease the biodiversity of the species community of AMF. The invasion of AMF into areas of low propagule density is seen to be positive for plant growth. It is speculated that the introduction of a 'foreign' species does not have a negative impact on the ecology of an existing ecosystem with its existing species community of AMF.

INTRODUCTION

Fungal species involved in the formation of arbuscular mycorrhiza with higher plants are distributed world wide on all continents and in all terrestrial ecosystems where plants grow. It was estimated that more than 60% of the species of the plant kingdom forms this kind of symbiosis, and that the structures of the arbuscular mycorrhizal fungi (AMF) in soils represent world wide the biggest components (mycelium and spores) of the microbial biomass in all soils. Although so widely spread in soils and ecosystems, relatively little is known about the ecology of single species of the 190 species of AMF so far described. Also, information is scarce on how these fungi spread locally. No research has been done as yet to identify how AMF move geographically. So, the answer to the question whether

species of AMF are invasive has to come from other sources, and we can potentially obtain some information to answer the question from the knowledge we have about their natural distribution in ecosystems.

Over the past few decades we and others have studied the ecology of AMF in different tropical and subtropical agro-ecosystems of Africa and South America (Sieverding, 1991), North America (e.g. Landis *et al.*, 2004) and in different European agro-ecosystems (Jansa *et al.*, 2002; Oehl *et al.*, 2003, 2004, 2005). Often, natural ecosystems were compared with agro-ecosystems; or different utilization intensities were compared for the bio-diversity of AMF. Analysing the known information on the biodiversity of AMF in agro-ecosystems, and also considering some of our as yet unpublished work, we aim to answer the following questions to identify whether specific AMF can be considered to be 'invasive':

- What do we know about the natural distribution of AMF?
- What do we know about the spread of AMF, and can they invade a region or an ecosystem where it was not occurring before?
- Can an alien AMF become dominant and thus take over the natural community of AMF?
- How persistent are AMF?
- Is the 'invasion' of AMF a risk for an ecosystem?

METHODS

AMF are taxonomically organized within the phylum Glomeromycota, in the single class of the Glomeromycetes. They can either be identified by the morphology of their spores or by PCR-based molecular methods. Both methods have some intrinsic weaknesses (Sanders, 2004). To use the morphological method, the AMF must have sporulated, and sporulation may be affected by environmental conditions. Also, spores from field samples are difficult to identify. Molecular methods also may have problems, firstly because a DNA sequence of a particular gene must be known and correctly be identified. The biggest problem, however, is that the genetic variation within a fungal species is high, which may be related to the fact that spores of AMF can contain a few hundreds of nuclei. Morphological characteristics are less variable and, thus, we used the morphological aspects to identify species of AMF in agro-ecosystems. To overcome the problem of 'non-sporulating' periods, spore sampling from ecosystems was repeated over time and trap or bait pot cultures with soil from the agro-ecosystems were established and monitored for the occurrence of spores of specific AMF over a period of up to three years (F Oehl, unpublished). As an indicator for fungal dominance we used the number of spores of AMF per unit of soil – we see currently no other possibility to use a different method as molecular methods are not yet available to quantify species of AMF in soils. We believe that the spore number of individual species of AMF certainly gives an indication on the fitness of the species in each ecosystem.

RESULTS & DISCUSSION

Distribution of AMF in ecosystems

AMF regularly occur in communities in soils under vegetation cover; a single species population is seldom found. Only a few natural ecosystems have been investigated for species richness, so far, and almost all were natural grass land ecosystems or forest ecosystems (Landis *et al.*, 2004; Oehl *et al.*, 2003). Some more information is available from different agro-ecosystems (Sieverding, 1991; Oehl *et al.*, 2003, 2004, 2005). The number of different species of AMF found ranged from 15 to 26 at specific locations (Oehl *et al.*, 2003), the number can reach up to 60-70 species for a rather small region such as the Upper Rhine Valley in the three-country corner of Germany, France and Switzerland (F Oehl, unpublished data). All information currently available indicated that *Glomus* spp. are far more frequent than any of the other genera forming AM. Whereas *Acaulospora* spp. are also regularly found, *Entrophospora* spp., *Pacispora* spp. and *Scutellospora* spp. are less frequent. The first reports on a *Gigaspora* spp. from European agro-systems are quite recent (Jansa *et al.*, 2002; Blaszkowski, personal communication). In tropical agro-ecosystems, *Acaulospora* spp., *Glomus* spp. and *Scutellospora* spp. can have the same high frequency, and *Entrophospora* spp. and *Gigaspora* spp. are also often observed, whereas *Pacispora* spp. are less frequent.

All ecological studies indicate that the frequency of species of AMF decreases with increasing land use intensity (Oehl *et al.*, 2003). This may be related to the fact that through increasing land use the richness of plant species decreases and that the phosphorus nutrient status of the soil increases. Both can decrease the bio-diversity of species of AMF (Landis *et al.*, 2004; Oehl *et al.*, 2004) though other studies from the tropics indicate that the species richness of AMF in a monoculture such as sugarcane grown in fertile soils (Molisols of Colombia) can be high (17 species: Sieverding, 1991).

Oehl *et al.* (2003) could actually group the fungal species into ecological groups. They identified so-called 'generalist' AMF (which occur under a wide range of land-use systems) and others which are 'specialists' (which occur only under, for example, permanent grasslands). Some of the generalists are *G. mosseae*, *G. geosporum*, *G. etunicatum*, *G. constrictum*, *G. diaphanum*, *G. fasciculatum* and *S. calospora*. 'Specialists' (in the sense of their occurrence in specific ecosystems such as permanent grasslands) are the sporocarp-forming *Glomus* spp. e.g. *G. aureum*, *G. mortonii*, *G. simosum* and *G. rubiforme*. Some species occur only under specific acid (*Entrophospora colombiana*) or more neutral to alkaline soil conditions (*G. mosseae*, *G. badium* nom. ined.), as indicated by Sieverding (1991) and Oehl *et al.* (submitted for publication). It is obvious from the mycorrhiza literature that some of these generalists are reported from all parts of the world, i.e. from tropical as well as from temperate zones, dry and wet climates, infertile and fertile soils, low pH and higher pH, sandy and clay soils, temporary flooded and none flooded soils, from natural ecosystems and intensively managed ecosystems. This indicates that at least these so-called 'generalists' were able to invade most of the existing ecosystems and could establish there permanently. Other species (non-generalists) need specific ecological conditions to survive and it appears that many species of AMF have a low fitness in intensively managed agro-ecosystems.

Long-distance transport of AMF

We have only a few research results about potential global movement mechanisms for AMF. AMF are estimated to have been on earth for more than 400 million years. So, there was time enough for specific fungal species to move naturally around the globe. One mechanism of long-distance transport can be wind (Warner *et al.*, 1984). Although the study measured spread by wind only over a distance of 55 m, it is very likely that spores of AMF, with their relative small diameter and weight (most are in the range of 50-300 μm , and about 1 million spores have 1 g dry weight), can move over long distances with soil and dust in the stratosphere. A prerequisite for dust to contain spores is that it came from vegetated areas. Dust with spores of AMF can fall-out at any site, somewhere on the globe. It is said that by that way about 1 million tonnes of soil is moved from Africa to the Amazon basin, for example. Transport of propagules of AMF by water is also likely to occur, as our own experience showed that trap plants sown in sand taken from rivers can become mycorrhizal. No information, however, is available as to whether there are differences between infective propagules and between species of AMF in their wind or water transportability.

Another way of long-distance movement, today, is certainly possible with soil and plant roots which are moved in the frame of agricultural goods transport from one continent to another. This may apply for ornamentals or horticultural materials, and transport of the inoculum of AMF may also fall into this category. Speculatively, grain which is shipped in enormous quantities between continents, may a vector for global spread of AMF. Unintended transport of soils with spores of AMF by tourists could be another way of long-distance transport. In conclusion, it is urgently necessary to identify with scientific methods the ways of long-distance transports of AMF.

Local invasion and spread of AMF / speed of invasion

Some more information is available about the ways of local spread of AMF. It appears that grasshoppers, ants and small mammals are important vectors for short distance regional transport of spores (Warner *et al.*, 1984). Earthworms, ants and other invertebrates can transport propagules locally (Gange & Brown, 2002). It appears that small animals have preference for sporocarpic AMF, but the distribution of other infective propagules with digested and excreted roots is speculative. The speed of invasion of new land areas, never previously colonized with AMF and where plant species establish themselves (e.g. volcanic ashes, mine deposits), is slow. Research data on the mycorrhizal propagule density in soils in front of a retreating glacier in the Alps show that even 10 years after the ice has retreated the mycorrhizal infection potential of the soil is very low.

Whether or not a fungal propagule can invade a vegetated and functioning plant ecosystem after, for example, fall-out with dust after long-distance transport, is completely unknown. The chances of survival of an alien propagule in a new environment cannot currently be estimated – it is assumed that they are not very high in practice, considering the problems mycorrhizologists have when they try to establish a single-spore pot culture under controlled conditions. The germination and infection process of a propagule requires often very specific soil, plant and climatic conditions. While this appears true for some AMF which have seldom been established in pure pot cultures (e.g. *Entrophospora infrequens* or *Pacispora* spp.: Oehl & Sieverding, 2004) others (e.g. the so-called 'generalists', see

above) were frequently grown, starting with a single spore – although always in conditions where the growing fungus did not have to compete with many other microorganisms.

The in-situ distribution of AMF with roots appears to be also very slow. The speed of growth of runner hyphae of AMF was defined to be 1–3 mm per day (Olsson *et al.*, 2002). Thus, distal growth of hyphae can be much slower than the growth of roots.

Dominance and persistence of AMF

It has often been shown that an individual species of AMF can become dominant in an agro-ecosystem when specific agronomic practices are applied. Dominance (in terms of spore production) can be a successional phenomenon, and it can take some years until a permanent composition of a species community is established (F Oehl, unpublished data). Dominance of one or two species is often induced by specific agronomic practices (Sieverding, 1991) and often related to the elimination of some species from the species community (Oehl *et al.*, 2004). Dominance has also something to do with the survival capacity and the persistence capacity of fungal species under unfavourable conditions, e.g. absence of a host. When oilseed rape was grown over three years, some species of AMF survived and their number of spores did not decrease (Table 1). In particular, *G. mosseae* and *G. intraradices* appear to be ‘survivors’ and can be quite persistent.

Table 1. Effect of years of cultivation of none host crop (oilseed rape) and host crop (thyme) on persistence of spores of AMF (number of spores/16 ml of soil). Crops grown side by side. (Sieverding & Johne, unpublished)

| AMF | Oilseed rape | | Thyme |
|-----------------------------|--------------|---------|---------|
| | 2 years | 3 years | 3 years |
| <i>Glomus mosseae</i> | 3 | 2 | 3 |
| <i>Glomus tortuosum</i> | 24 | 2 | 2 |
| <i>Glomus intraradices</i> | 12 | 16 | 8 |
| <i>Glomus etunicatum</i> | 3 | 0 | 3 |
| <i>Glomus fasciculatum</i> | 6 | 0 | 13 |
| <i>Paraglomus occultum</i> | 0 | 0 | 16 |
| <i>Diversispora spurca</i> | 0 | 0 | 5 |
| <i>Acaulospora paulinae</i> | 0 | 0 | 1 |
| <i>Acaulospora</i> spp. | 1 | 1 | 16 |
| Total nr. spores/16 ml | 49 | 21 | 67 |
| Number of species of AMF | 6 | 4 | >9 |

CONCLUSIONS

AMF have had millions of years to distribute themselves over the globe. Some species actually did, and are found in all ecosystems (the ‘generalists’). We know very little about the potential mechanisms of long-distance transport of propagules. It is possible that some of the ‘generalists’ can naturally establish themselves in ecosystems where they formerly were not present. In areas where the propagule density of AMF is low, such invasion can be seen positively for the ecosystem, because it is likely that through increased species diversity of AMF the productivity of a plant community increases (van der Heyden & Sanders, 2002). Whether the invasion of a species of AMF into a natural ecosystem with its

established fungal species community has any negative effects on the functioning of the ecosystem, is not known. The introduction of 'generalists' into agro-ecosystems, e.g. with inocula of AMF, is seen to be positive when the inoculation does improve the productivity of the system. In agronomic systems many practices are applied which can dramatically decrease species diversity, and which can result in the natural domination of one species of AMF. The introduction of a species by an inoculation is nothing other than establishing a temporary dominance of the inoculated species in the plants rhizosphere. It is likely that such an introduced species of AMF will survive and persist when it is a so-called 'generalist' which, anyway, is often naturally present in agro-ecosystems.

REFERENCES

- Gange A C; Brown V K (2002). Actions and interactions of soil invertebrates and arbuscular mycorrhizal fungi in affecting the structure of plant communities. In: *Mycorrhizal Ecology*, eds M G A van der Heijden & I R Sanders, pp. 321-344. Springer: Berlin.
- Jansa J; Mozafar A; Anken T; Ruh R; Sanders I R; Frossard E (2002). Diversity and structure of AMF communities as affected by tillage in a temperate soil. *Mycorrhiza* **12**, 225-234.
- Landis F C; Gargas A; Givnish T J (2004). Relationship among arbuscular mycorrhizal fungi, vascular plants and environmental conditions in oak savannas. *New Phytologist* **164**, 493-504.
- Oehl F; Sieverding E (2004). *Pacispora*, a new vesicular arbuscular mycorrhizal fungal genus in the Glomeromycetes. *Angewandte Botanik* **78**, 72-82.
- Oehl F; Sieverding E; Ineichen K; Mäder P; Boller T; Wiemken A (2003). Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. *Applied and Environmental Microbiology* **69**, 2816-2824.
- Oehl F; Sieverding E; Mäder P; Dubois D; Ineichen K; Boller T; Wiemken A (2004). Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. *Oecologia* **138**, 574-583.
- Oehl F; Sieverding E; Ineichen K; Ris E-A; Boller T; Wiemken A (2005). Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. *New Phytologist* **165**, 273-283.
- Olsson P A; Jakobson I; Wallander H (2002). Foraging and resource allocation strategies of mycorrhizal fungi in patchy environment. In: *Mycorrhizal Ecology*, eds M G A van der Heijden & I R Sanders, pp. 93-115. Springer: Berlin.
- Sanders I (2004). Plant and arbuscular mycorrhizal fungal diversity – are we looking at the relevant levels of diversity and are we using the right techniques? *New Phytologist* **164**, 415-418.
- Sieverding E (1991). *Vesicular-arbuscular mycorrhiza management in tropical agrosystems*. GTZ, Eschborn & Hartmut Bremer Verlag: Friedland.
- Warner N; MacMahon J A; Allen M F (1984). Dispersal of VA-mycorrhizal fungi in a disturbed semi-arid ecosystem. *Proceedings of 6th NACOM, Bend, Oregon*, p. 292.
- Van der Heijden M G A; Sanders I R (2002). Mycorrhizal Ecology: Synthesis and perspectives. In: *Mycorrhizal Ecology*, eds M G A van der Heijden & I R Sanders, pp. 441-456. Springer: Berlin.

Joined spread of soil microbes and commercially re-established endangered plants in Europe

K Turnau, S Gawronski, S Zubek, T Anielska, A Jurkiewicz

Institute of Botany of the Jagiellonian University, Lubicz 46, 31-512 Krakow, Poland

Email: ubturnau@kinga.cyf-kr.edu.pl

ABSTRACT

The use of beneficial plant-microbe interactions in the rhizosphere is the key factor in protection of a wide range of rare plant species. Arbuscular and orchid mycorrhizas are the most important microbial symbioses formed by commercially re-established endangered plants in Europe. The use of mycorrhizal fungi and rare plants in private/public gardens and in the establishment of roof gardens is also of importance. Xerothermic grasslands that are endangered as whole community are considered as a source of many interesting plants that could be saved by introducing the plant material into new habitats, together with arbuscular fungi. The development of technologies including joined introduction of plant/fungal/bacterial associations can guarantee successful protection of endangered plants.

INTRODUCTION

The rarity of a given plant, that predispose it to become extinct, may be caused by both anthropogenic and natural factors. Among factors increasing the vulnerability of the species to the risk of extinction one can cite: edaphic specialisation, isolation of a population, inbreeding depression, heteromorphic self-incompatibility, requirement of a large area depressed by fragmentation of habitat and specific mutualism, e.g. pollinators, dispersers and mycorrhizal partners (Falk & Holsinger, 1991; Weller, 1994). Although the importance of symbiotic associations has been documented for a wide variety of habitats and the below-ground diversity of mycorrhizal fungi was proposed as a major factor influencing plant biodiversity and ecosystem functioning (van der Heijden *et al.*, 1998), still little attention is paid to the potential application of the soil microbiota in the protection of endangered plants. The sites where the soil was recently graded, or eroded, seriously overused (e.g. by overgrazing) or where either no vegetation was developed for some time or the soil was used for monoculture of non-mycorrhizal plants (e.g. Brassicaceae) are usually poor in microbiological life and easily colonized by invasive plants, often lacking strong demands for microbial symbionts. They easily out-compete the native rare plants that usually need the presence of well-developed microbiota, including mycorrhizal fungi and associated bacteria. The introduction of microbial symbionts simultaneously with or in advance of the plant material can change the situation and result in the formation of communities resembling those that were naturally developed before the anthropogenic impact took place.

IMPORTANCE OF MYCORRHIZAL FUNGI IN PLANT ESTABLISHMENT AND SURVIVAL

Non-woody rare plants that are of importance for commercial multiplication usually form arbuscular or orchid mycorrhiza. At least in the case of arbuscular associations, the individual root systems are inhabited by several fungal species (Merryweather & Fitter, 1998). The same fungi form a network of thread-like mycelium in the soil, providing the plant with water and mineral compounds that, otherwise, would not be available. They improve the development of proper soil structure, as they bind soil particles; they also strengthen the plant, making it more resistant to pathogens (fungi and nematodes) and stress factors such as pollution and drought. Recently, the role of soil microbiota in maintaining ecosystem biodiversity has been stressed (Gianinazzi *et al.*, 2002); the mycelial network in the soil improves the chances of survival of plant species with less-developed root systems, and influences plant growth to a different degree according to a plant species' dependency on the symbiosis. A well-developed network of mycelium (even connecting plants belonging to different species) allows plants to share the pool of mineral compounds. Young plantlets germinating from seeds soon 'plug in' to the existing network, which grants them fast growth and competitiveness against other, more aggressive species. The mycorrhizosphere is responsible not only for soil structuring, plant nutrition and health, but also for ecosystem biodiversity and stability. Previous findings suggest that increasing the species richness of arbuscular mycorrhizal fungi (AMF) leads to increased spread of plant species highly responsive to AMF, at the expense of the relatively unresponsive ones (van der Heijden *et al.*, 1998). Excess of fertilisers, in common with fungicides, kills mycorrhizal fungi, and soon the site is dominated by few single-plant species. A similar effect is due to pollution, removal of soil layers, use of chemicals and mechanisation in agriculture. Natural processes and anthropogenic activities also strongly affect plant populations, their vitality and biodiversity, and may cause the extinction of more vulnerable taxa. Processes such as eutrophication, chemical contamination, changes in land exploitation and excessive collection of particular plant species are widely concerned by this phenomenon of 'jeopardizing ecosystem richness'. Multiplication of the plant material, both for plant protection and for commercial reasons, is of considerable importance. So far, however, the techniques developed do not take into consideration the beneficial mycorrhizosphere organisms, i.e. AMF and plant growth-promoting rhizobia (PGPR). Unfortunately, the knowledge of soil microbiota *in situ* is extremely limited. Even the mycorrhizal status of rare plants is poorly known. This raises the necessity of careful observations carried out in natural communities from which the rare plants originate, such as those carried out in the case of *Pulsatilla patens* (Öpik *et al.*, 2003). Organisms such as AMF and PGPR may strongly influence the restoration processes, allowing for the development of vegetation either with low (or even without) fertiliser input. Both groups of microorganisms may be used in active protection of endangered plant species. In many cases it is possible to promote native soil microbial activity with properly selected management techniques, including the application of appropriate nutrient levels. Inoculation with selected AMF and PGPR may offer an interesting perspective for *in vitro* and *ex situ* multiplication of the plant material, followed by reintroduction of the plant into the natural stand or its establishment in the compensation site.

POTENTIAL OF AMF USE FOR ESTABLISHMENT OF RARE AND ENDANGERED PLANTS IN GARDENS AND IN ROOF VEGETATION

In recent decades we have witnessed an increase of interest in plant protection. This is caused by the increase of ecological consciousness of the importance of preserving plants whose habitats are destroyed due to human activity. Cultivation of native plants in private or public gardens, and including them in compositions established on roofs, became trendy. Although there are already several books that instruct how to select particular plant species for given soil conditions (e.g. Brookes, 2002), there is little information on how to prepare the below-ground microbiota of the site to increase its sustainability and the success of plant establishment. The microbiota can be obtained: i) from commercially available inocula (Gianinazzi *et al.*, 2002); ii) from nurseries that are specialized in the multiplication of the plants and the obtained specimens are already mycorrhizal; iii) from natural stands where the plants exist. The last possibility is the least advisable, as the collection of rare plants or their roots might lead to serious disturbance of the already-endangered plant population. It is also important that plants should not be obtained from uncertified sources. Careful studies in natural stands are certainly important for increasing our knowledge on endangered plant biology, and the recent development of molecular tools gives us a chance to learn which fungi are effective colonizers under given site conditions. Although there is little specificity concerning the capability of AMF species/strain to colonize roots of a diverse range of plants, there are site-related differences in the AM fungal root community composition. The plant has a role in determining fungal species composition (Johnson *et al.*, 1992). Fungi originating from the natural stand of a plant might not be appropriate in a new site, where the plant is being introduced, in a similar way that native fungi are no longer suitable in a disturbed site (Stahl *et al.*, 1988). If the soil disturbance resulted in a drastic decrease in the number of mycorrhizal propagules, the best solution is to use commercial inocula. Fungal strains provided from similar sites in the vicinity may be best adapted to actual soil and climatic conditions; in some cases, introduction of inoculum developed especially for a given area could be successful. If AMF are still present in the soil to be restored, the most valuable technique would rely on the management of the indigenous microbiota, thereby promoting their proliferation. This aim could be achieved by the application of experimentally determined levels of selected fertilizers, the introduction of mycorrhizal and the avoidance of non-mycorrhizal plants which may have a deleterious influence on symbiotic fungi. The use of commercial inocula and mycorrhizal plants from nurseries seem to be the most appropriate in the case of vegetation established on roofs. This is because the substratum (expanded clay or volcanic material) used is devoid of symbiotic organisms.

The evaluation of mycorrhiza development could be used as an important indicator of ecosystem changes, and could serve as a tool for biomonitoring soil quality (Haselwandter, 1997).

XEROTHERMIC GRASSLANDS AS ENDANGERED COMMUNITY AND THE SOURCE OF COMMERCIALY VALUABLE PLANTS

Plants originating from xerothermic grasslands can relatively easily establish in disturbed sites characterized by poor physico-chemical structure and long periods of dryness. Most of these plants are perennial and strongly mycorrhizal. The survey of plants collected from

calcareous xerotherms in southern Poland has shown that most of them were strongly mycorrhizal. Three of these plant species, including *Anemone sylvestris*, *Linum flavum* and *L. hirsutum*, grown in sterilized soil inoculated and non-inoculated with AM fungi have shown strongly increased growth in the presence of AMF (Grzyb I & Glanowska B, unpublished results). The seeds of xerothermic plants are usually slowly germinating, and sometimes demand longer dormancy periods or special treatments stimulating germination. Ideally they should be multiplied and transferred from the nurseries as mycorrhizal plantlets. Only a few plant species are available commercially in this way. Among them, the most promising examples concern species of *Gentiana* and orchids (developing orchid mycorrhiza) such as *Orchis militaris*, *O. pallens* and *O. purpurea*. Among rare plants, occurring naturally in xerothermic grasslands, commercially available are: *Adonis vernalis*, *Anemone sylvestris*, *Aster amellus* and *Pulsatilla pratensis*. In natural stands they are usually strongly mycorrhizal. Further, xerothermic plant species are often exchanged between hobby collections; however, not much is known about their mycorrhizal status. Some other plant species from xerothermic grasslands are interesting from the point of view of their introduction into the vegetation established on roofs and for revitalisation of the plant communities. Among them *Allium montanum*, *Anthericum ramosum*, *Inula ensifolia*, *Salvia pratensis*, *Thymus kosteleckyanus*, *T. marchallianus* and *Trifolium rubens* are very promising mycorrhizal plants. The increase of interest in these plants may result in better understanding of these plants' biology. This would be just in time, as xerothermic grasslands are among those that are endangered as whole communities, owing to increased use of fertilizers and to the change of land use (abandoned grazing by herbivores, scything, trampling). The knowledge could be used to protect the biodiversity of plants and associated microorganisms. In some cases, compensation sites will have to be created, especially in places where the creation of infrastructure has caused the fragmentation of the area and dramatically destroyed the surface layer of the soil. Fast restoration of such area is possible only if proper technologies are developed, including storage of the soil containing propagules of microbiota. Storage should not last longer than 2 years (Miller *et al.*, 1985). As anaerobiosis kills AMF, it is important to store the material not in a heap but just as a layer of a depth that will depend on the type of the spoil (Harris *et al.*, 1987).

RARE FOREST-INHABITING PLANTS AS A SOURCE OF COMMERCIALY VALUABLE PLANTS

Although in temperate climate the forests are known as communities dominated by ectomycorrhizal plants, there are still many plants of the herb layer that form arbuscular, ericoid and orchid mycorrhizal symbiosis. Several rare, usually mycorrhizal, plant species are commercially available (owing to wide interest in creating natural communities in private parks or gardens), e.g. *Aquilegia vulgaris*, *Doronicum austriacum*, *Lilium martagon*, *Veratrum lobelianum* and the fern *Matteucia struthiopteris*. Further mycorrhizal species are the subject of hobby-collectors' exchange, e.g. ferns such as *Blechnum spicant*, *Osmunda regalis* and *Polystichum aculeatum*, and orchids such as *Cephalanthera damasonium*, *C. longifolia*, *C. rubra* and *Platanthera bifolia*, that are obligatorily mycorrhizal. In some cases, micro-communities are created in logs (sometimes hollowed out and filled with soil substratum) that keep moisture and allow for the development of abundant plant communities. It resembles plants occurring in natural stands on tree stumps in the forests. Plants developing in such places are extremely dependent on mycorrhizal fungi.

USE OF AMF IN VEGETATIVE PROPAGATION OF RARE PLANTS

In the case of limited plant populations (e.g. a single plant in the given area), vegetative propagation techniques that were developed for agricultural purposes have been recently introduced into protection practices. This method is by now routinely applied in the case of orchids, carnivorous plants and ferns. *In vitro* cultures have been used to multiply ferns such as *Osmunda regalis* and *Phyllitis scolopendrium* (Zenktele, 1993), orchids (Rasmussen, 1998) and monocotyledons such as *Lilium martagon* (Bach & Kedra, 1997) which are under strict protection in most European countries. Non-sterile and sterile micropropagation techniques (Bowes, 1999) are, so far, carried out mostly in the absence of symbiotic organisms. While the *in vitro* plants are cultivated in nutrient-rich artificial substrates, most of the endangered species are known to origin from nutrient-poor environments. The success of the technique concerning the endangered plants might depend therefore on the application of AMF and lowering the availability of the nutrients. The inoculation methods used *in vitro* or during the *post in vitro* stage, depending on the host species, may be adopted from those developed for commercially important plants. The inoculants for each plant species and variety, as well as the time of inoculation and substrate composition, should be chosen carefully. This could strongly increase the chances of plant survival, e.g. during the *post vitro* stage the plants are seriously endangered by pathogens (Mc Rae, 1998). Recently, the first attempts to use cryostorage for the conservation of endangered plants have been achieved (Bowes, 1999). Similar technology could be also used to preserve the diversity of microbiota (Declerck & Angelo Van Coppenolle, 2000).

CONCLUSIONS

Up to 25,000 wild plant species are threatened with extinction. To protect them we need to restore the under-ground communities that should be prepared before the reintroduction of the plants. Although most researchers concentrate on the parameters concerning the root colonisation by the fungus, the formation of an appropriate soil network might be more or at least equally important for the plant establishment and survival. As endangered plant species are usually less competitive, they have to rely on AMF concerning mediation of co-existence within the community. The introduction of mycorrhizal fungi into degraded sites gives the plant more chances, owing to increased nutrient availability, protection against pathogens, and increased water uptake; in addition, mycorrhizal fungi may allow endangered plants to compete with invasive plants that have low demands concerning specific microbiota. The technology of joined introduction of the plant/fungal/bacterial associations should be developed to guarantee the successful establishment of the endangered plant.

ACKNOWLEDGEMENTS

The study was supported by the Polish Scientific Committee PB 2P04G00628 and PB 2P04G00327.

REFERENCES

- Bach A; Kedra M (1997). Somatic embryogenesis of *Lilium martagon* L. seedling culture. *22nd Conference of Embryology. Plants, Animals, Humans. Osieczany. Acta Biologica Cracoviensis* **39** (Supplement 1), 47.
- Bowes B G (1999). *A Colour Atlas of Plant Propagation and Conservation*. Manson Publishing: London.
- Brookes J (2002). *Garden design*. Dorling Kindersley: London.
- Declerck S; Angelo Van Coppenolle M G (2000). Cryopreservation of entrapped monoxenically produced spores of an arbuscular mycorrhizal fungus. *New Phytologist* **148**, 169-176.
- Falk D A; Holsinger K E (1991). *Genetics and conservation of rare plants*. Oxford University Press: New York.
- Gianinazzi S; Schepp H; Barea J M; Haselwandter K (2002). *Mycorrhizal technology in agriculture. From genes to bioproducts*. Birkhauser Verlag: Switzerland.
- Harris J A; Hunter D; Birch P; Short K C (1987). Vesicular-arbuscular mycorrhizal populations in stored topsoil. *Transactions of the British Mycological Society* **89**, 600-603.
- Haselwandter K (1997). Soil micro-organisms, mycorrhiza, and restoration ecology. In: *Restoration ecology and sustainable development*, eds K Urbanska, N R Webb & P J Edwards, pp. 65-80. Cambridge University Press: Cambridge.
- Johnson N C; Tilman D; Wedin D (1992). Plant and soil controls on mycorrhizal fungal communities. *Ecology* **73**, 2034-2042.
- Mc Rae E A (1998). *Lilies*. Timber Press: Portland, Oregon.
- Merryweather J; Fitter A (1998). The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta*. II. Seasonal and spatial patterns of fungal populations. *New Phytologist* **138**, 131-142.
- Miller R M; Carnnes B A; Moorman T B (1985). Factors influencing survival of vesicular-arbuscular mycorrhiza propagules during topsoil storage. *Journal of Applied Ecology* **22**, 259-266.
- Öpik M; Moora M; Liira J; Koljalg U; Zobel M; Sen R (2003). Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. *New Phytologist* **160**, 581-593.
- Rasmussen H N (1998). The underground phae: a special challenge in studies of terrestrial orchid populations. *Botanical Journal of the Linnaean Society* **126**, 49-64.
- Stahl P D; Williams S E; Christensen M (1988). Efficacy of native vesicular-arbuscular mycorrhizal fungi after severe soil disturbance. *New Phytologist* **110**, 347-354.
- Van der Heijden M G A; Boller T; Wiemken A; Sanders I R (1998). Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. *Ecology* **79**, 2082-2091.
- Weller S G (1994). The relationship of rarity to plant reproductive biology. In: *Recovery and Restoration of Endangered Species*, eds M L Bowles & C Whelan, pp. 90-117. Cambridge University Press: Cambridge.
- Zenkter E (1993). Homo- and heterophysis in the *in vitro* reproduction of *Phyllitis scolopendrium* (L.) Newm. *Bulletin of the Polish Academy of Sciences. Biological Sciences* **41**, 254-261.