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Physiological Approaches to Improving the Ecological Fitness of Fungal Bicontrol Agents

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Introduction

There have been numerous developments of microbial inoculants for the effective control of plant diseases, pests and weeds (Baker, 1987; Burge, 1988; Cook, 1993). In these areas of research, the efficacy of the biocontrol agent (BCA) has been intricately linked with the potential for the production of inocula by either liquid or solid substrate fermentation systems. While such studies have been numerous, most have concentrated on optimizing the quantity of propagules or mycelial fragments produced, with practically no consideration given to quality of inocula. Few, if any, studies have considered the very real practical problem of effective establishment of prospective BCAs in the natural environment, be it for control of pests, diseases or weeds. This can be a crucial bottleneck, limiting the consistency of control under field conditions and the widespread commercialization of BCAs. Fluctuating abiotic factors, particularly water availability, temperature, length of dew periods, microclimate, canopy type and rainfall events, all have an impact on the prospective BCA. Tolerance to such abiotic fluctuations are a prerequisite for the successful development of ecologically competent BCAs for use in the field. Unfortunately, the area of improving the ecological fitness of inocula has received very little attention, although some elegant studies on desiccation tolerance of BCAs have been published (Jin *et al.*, 1991; Jackson *et al.*, 1997). Effective environmental-stress tolerance of inocula may improve establishment, which could contribute significantly to improving the efficacy of BCAs.

There are thus four key questions that need to be addressed: (i) can one manipulate the physiology of non-xerophilic/tolerant fungi to accumulate useful endogenous reserves into inocula for improved environmental-stress tolerance? (ii) would this result in improved germination/growth under environmental stress? (iii) can this improve the establishment of inocula and conserve biocontrol potential in the field? and (iv) does ecophysiological manipulation have a role in improving the production and quality of inocula? This chapter will address these issues and present examples to demonstrate

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(eds T.M. Butt, C. Jackson and N. Magan)

that studies on the quality of inocula may be a valuable component in and approach to improving the formulation and delivery of ecologically competent inocula for field use.

Abiotic Stress Tolerance of Biocontrol Agents – Background

It is well known that xerotolerant and xerophilic fungi are able to tolerate a very wide range of water availability, with *Penicillium, Aspergillus* and *Eurotium* species able to grow at 0.85 water activity (a_w) (= 85% equilibrium relative humidity (ERH)) and 0.70 a_w (= 70% ERH) (Magan, 1997). This is much wider than most BCAs used for fungal plant disease and pest control, which germinate and grow very slowly below about 0.95–0.93 a_w.

Under water-stress conditions xerophilic/xerotolerant fungi are able to synthesize compatible solutes, particularly the low-molecular-weight sugar alcohols glycerol and

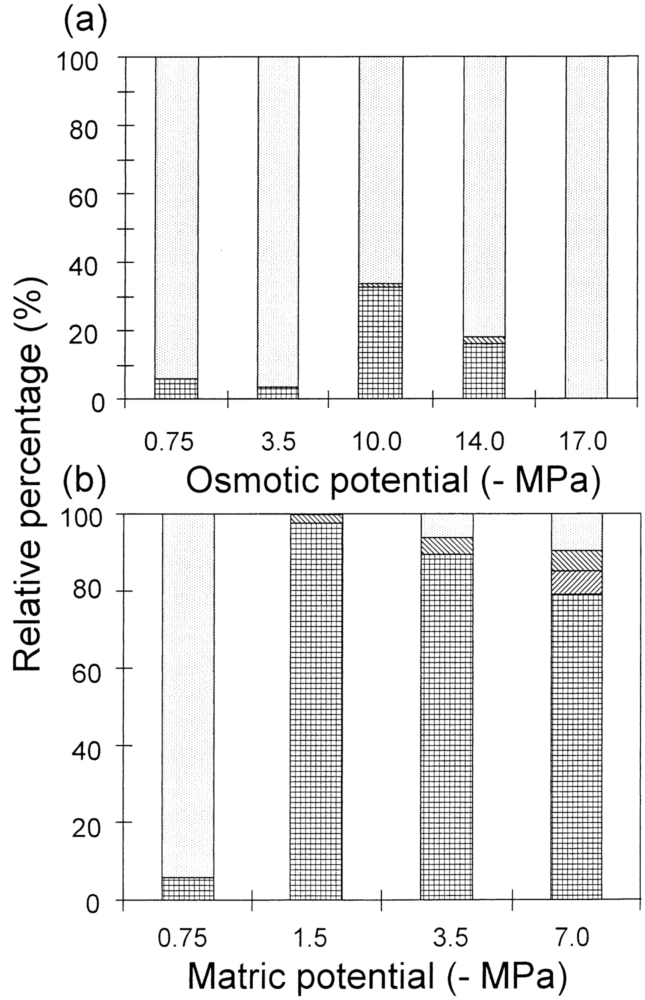

Fig. 9.1. Effect of water stress on modification of the ratio of polyols in conidia of the xerotolerant spoilage fungus *Aspergillus ochraceus.* (Adapted from Ramos *et al.*, 1999.)

erythritol (polyols), which enable their enzyme systems to continue functioning under extreme conditions of environmental stress/shock. For example, *Aspergillus ochraceus*, when grown under osmotic water stress, accumulates significantly elevated amounts of glycerol in its asexual conidia when compared with those produced under conditions with freely available water (Fig. 9.1; Ramos *et al.*, 1999). This certainly suggests that physiological manipulation of growth conditions can significantly modify the endogenous compounds synthesized and channelled into the propagules of fungi.

Interestingly, widening the environmental range for effective growth and biocontrol was also demonstrated to be possible by using low-a_w ultraviolet (UV)-mutant strains of *Metarhizium anisopliae* and *Paecilomyces farinosus* (Matawele *et al.*, 1994). This study demonstrated that the low-a_w mutant strains germinated (minima of 0.957 a_w) better and were more virulent at controlling green leafhoppers than the original wild-type strains (germination minima of 0.975 a_w). Unfortunately, while this work implied that the improved water-stress tolerance of the mutants may have been responsible, no studies were carried out to quantify the endogenous content of the propagules, although it was implied that the sugar alcohol content may have been modified.

Studies on the physiological manipulation of C:N ratios for the production of blastospores of entomogenous fungi also showed that increasing the glycogen content modified and improved the virulence against some pest species (Lane *et al.*, 1991a, b), although other studies suggest that glycogen is accumulated in the absence of stress, with polyol accumulation being more important under C and N stress (van Laere, 1989). While the stability of the low-a_w mutants was not examined, this work certainly pointed to the potential that existed for improving the quality of inocula and perhaps tolerance of a wider range of water availability and improved biocontrol.

It is also surprising that studies in relation to the control of field pests with entomogenous fungi have seldom examined the endogenous reserves present in conidia sporulating on killed insects. It is well known that conidia directly isolated from the body of the dead insect are often more virulent against pests than those obtained from repeated laboratory culture on rich artificial media. Recently, T.M. Butt and N. Magan

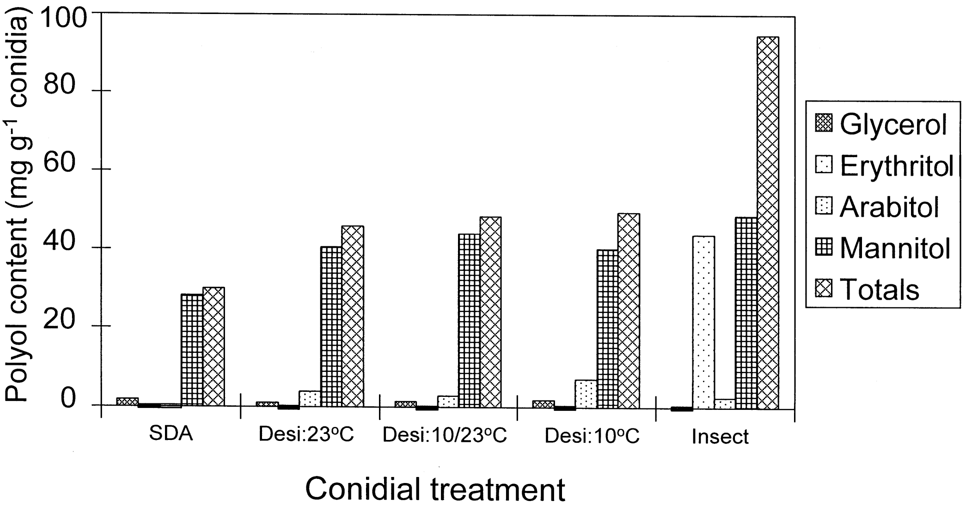

Fig. 9.2. Comparison of component and total polyols in conidia of *Metarhizium anisopliae* isolated directly from an insect, those produced on SDA and those stored under different temperature/desiccation regimes. (T.M. Butt and N. Magan, unpublished data.)

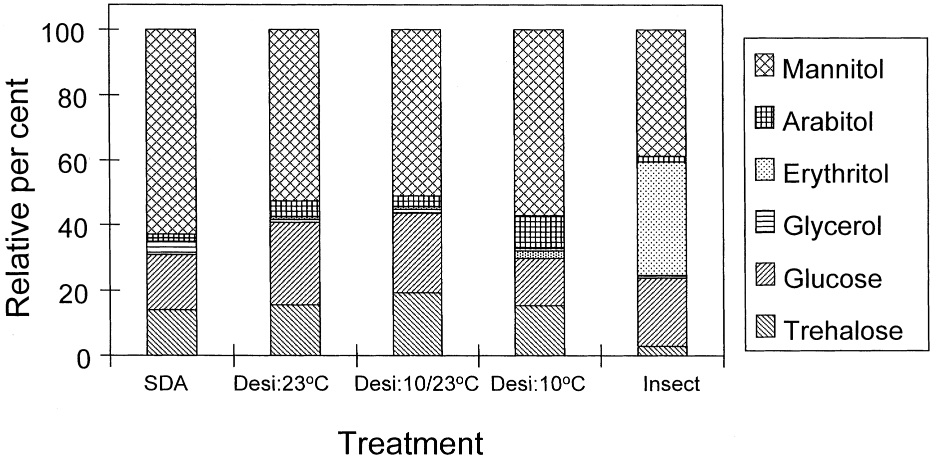

Fig. 9.3. Comparison of the ratio of sugars/polyols present in conidia of *Metarhizium anisopliae* isolated directly from insects, those produced on SDA medium and those stored under different temperature/desiccation regimes. (T.A. Butt and N. Magan, unpublished data.)

(unpublished data) examined this for the first time. They compared the quantities and profiles of sugars and sugar alcohols found in such conidia of *M. anisopliae* with those obtained from artificial media and stored under different desiccation temperatures. The quantities and ratio of endogenous sugars and sugar alcohols differed significantly in the treatments (Figs 9.2 and 9.3). The high amounts of erythritol, mannitol and glucose, with low amounts of trehalose, in conidia from killed insects were very different from those of conidia grown on richer artificial media, where mannitol, glucose and trehalose were the major reserves. This clearly demonstrates that the endogenous profiles of conidia grown on rich artificial media are significantly different from those in conidia obtained directly from insects and is indicative that the nutritional status of artificial media commonly used may not be best for the production of these BCAs. Direct comparisons now need to be made, with bioassays to correlate the relationship between such characterized inocula and the ability to kill insects. Hallsworth and Magan (1994b), in experiments with *Galleria* larvae, did find significant improvements in lethal effects over a wider humidity range. This type of information is very useful, as it suggested that ecophysiological systems could be developed to grow the BCAs under conditions that may be conducive to the synthesis and accumulation of useful compounds in the inocula/conidia in the laboratory, which can then be screened for both environmental-stress tolerance and biocontrol capability. This led to extensive research in the Applied Mycology Group, Cranfield Biotechnology Centre, in collaboration with a number of research groups, on a range of fungal BCAs, in order to understand the impact that physiological manipulations have on the synthesis and accumulation of sugars and sugar alcohols in a number of BCAs on their ecological competence; some of the results obtained in these studies, using the entomogenous fungi *Epicoccum nigrum* (for control of *Monilinia laxa* on peach twigs), *Gliocladium roseum* (for control of *Botrytis cinerea* on a variety of crops), *Candida sake* (for control of *Penicillium expansum* on apples) and *Ulocladium atrum* (for control of *B. cinerea* on a range of crops) as examples, will be described.

Ecophysiological Manipulation of Endogenous Reserves in Inocula

Water stress and temporal accumulation of sugars and polyols in inocula

Modifications of the carbon concentration of the media used can also have a significant influence on the water stress imposed on the fungus. This close interaction has seldom been recognized and in most cases has been ignored (Hallsworth and Magan, 1994a, 1995). Studies of entomogenous fungi using trehalose, glycerol, glucose or starch as a major carbon source have shown that both C concentration and time have a significant effect on the accumulation of both sugars and polyols in conidia. The time at which optimum quantities of low-molecular-weight polyols accumulated varied from 7 to 21 days. Trehalose content increased during the first 5 days and then decreased again. However, these studies were all carried out at one steady-state temperature, 25°C.

Other studies with *G. roseum*, used for the control of a range of soil-borne and foliar diseases, including *B. cinerea*, showed that the total sugars and glycerol accumulated in conidia varies with temperature and level of water stress (Fig. 9.4). Furthermore, by using either glycerol, glucose or trehalose as the major carbon source, the relative proportions of sugars and both high- and low-molecular-weight sugar alcohols varied significantly (Fig. 9.5). Studies with the BCAs *E. nigrum, C. sake* and *U. atrum* have all recently demonstrated that the endogenous contents of sugars and sugar alcohols can all be significantly modified using this approach (Pascual *et al.*, 1996; Frey and Magan, 1998; Teixido *et al.*, 1998a).

Improvements in growth and viability of modified inocula

The next key question is whether it is indeed possible for the modifications demonstrated above to be translated into improved germination capacity over a wider range

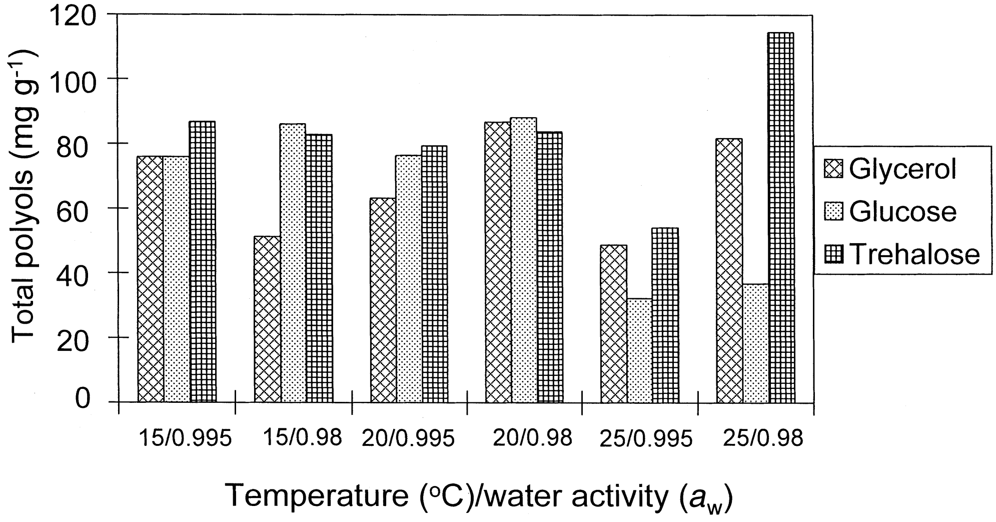

Fig. 9.4. Effect of temperature of incubation on the sugars/polyols in conidia of *Gliocladium roseum* grown on different media. (N. Magan, unpublished data.)

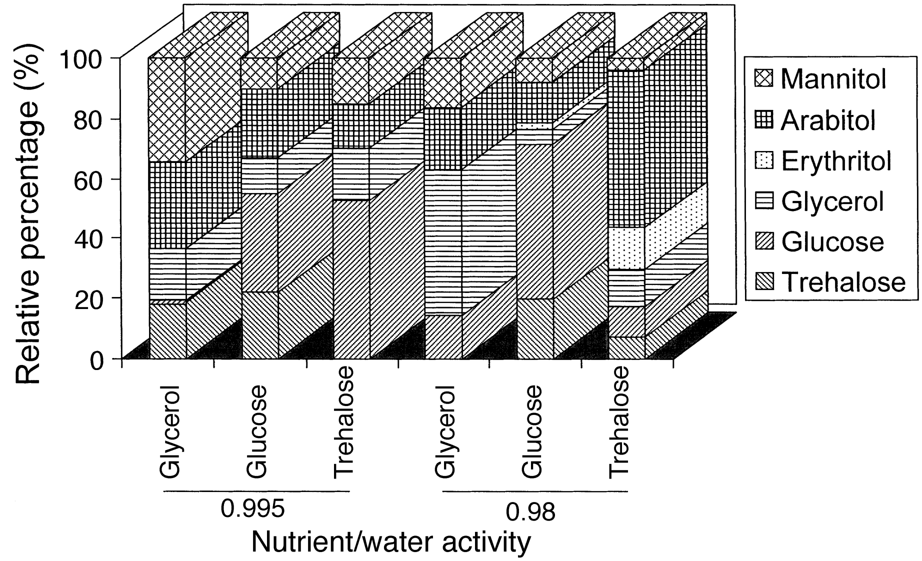

Fig. 9.5. Comparison of relative amounts of sugars/polyols in conidia of *Gliocladium roseum* at 25°C obtained from cultures grown on different media substrates. (N. Magan, unpublished data.)

of environmental stress conditions, and perhaps also more rapidly than unmodified inocula grown on rich unmodified media.

Improved viability, germination and germ-tube extension under water stress

The capacity for withstanding wider water-availability ranges was tested by using weak water-based agar media, modified with polyethylene glycol 200/400 and 600, or mixtures thereof, to avoid using solutes that might be taken up directly by propagules or yeast cells. The inocula were prepared in a diluent of the same a_w as the test agar plates over the range 0.995 (freely available water) to 0.90 or 0.88 a_w (= 90–88% ERH), representing much drier conditions than normally tolerated by these BCAs. In some cases, richer potato-dextrose- or yeast/glucose-based media were also used.

Studies with entomogenous fungi demonstrated that significant improvements in germination could be obtained with characterized modified than with unmodified conidia of *Beauveria bassiana, M. anisopliae* and *P. farinosus* (Table 9.1). It is clear that under marginal conditions for germination the modified inocula were able to germinate, while unmodified inocula could not. Studies with the cells of the yeast *C. sake* used for control of *Penicillium* rot of apples, modified by culture in weak-nutrient yeast broth media modified with either glucose or glycerol to 0.96 a_w, also demonstrated that a greater number of modified yeast cells were viable over a range of water availabilities (0.95–0.93 a_w) than unmodified yeast cells, which were significantly more sensitive (Teixido *et al.*, 1998b).

In contrast, using conidia of dematiaceous BCAs, such as *E. nigrum* and *U. atrum*, where significant physiological modifications of endogenous reserves were possible, no improvement in germination was achieved under water-stress treatment conditions. This suggests that larger, heavily pigmented spores from harsher phyllosphere environments may already, to a large extent, have evolved toleration of environmental stress.

Table 9.1. Mean percentage germination of conidia of *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus* from different treatments over a range of water availability, at 25°C. The germination media contained 13.3 g l^{-1} glucose, 5.0 g l^{-1} mycological peptone and polethylene glycol (PEG) 600 or PEG 600 + PEG 200. (Adapted from Hallsworth and Magan, 1995.)

	Water activity			
Treatment	0.989 (14 h)	0.951 (44 h)	0.935 (61 h)	0.923 (240 h)
B. bassiana				
Control	76.3	5.7	0[a]	0*
SDA + KCl	96.3	59.7	38.0	0*
Glycerol	92.0	87.3	51.0	0*
Trehalose	90.3	16.7	0[a]	0*
LSD ($P < 0.05$)	15.1	13.4	8.2	
M. anisopliae				
Control	0	0	0	0
SDA + KCl	81.3	77.0	49.7	0*
Glycerol	92.7	61.3	51.3	69.0
Trehalose	2.3	0	0	0*
LSD ($P < 0.05$)	7.5	10.7	7.6	
P. farinosus				
Control	47.3	0	0[a]	0[a]
SDA + KCl	43.0	42.7	10.0	0[a]
Glycerol	80.0	86.7	48.7	17.7
Trehalose	4.0	0	0[a]	0[a]
LSD ($P < 0.05$)	22.9	8.0	6.3	

[a] No conidia from these treatments had germinated by 240 h.

SDA, Sabouraud dextrose agar; LSD, least significant difference.

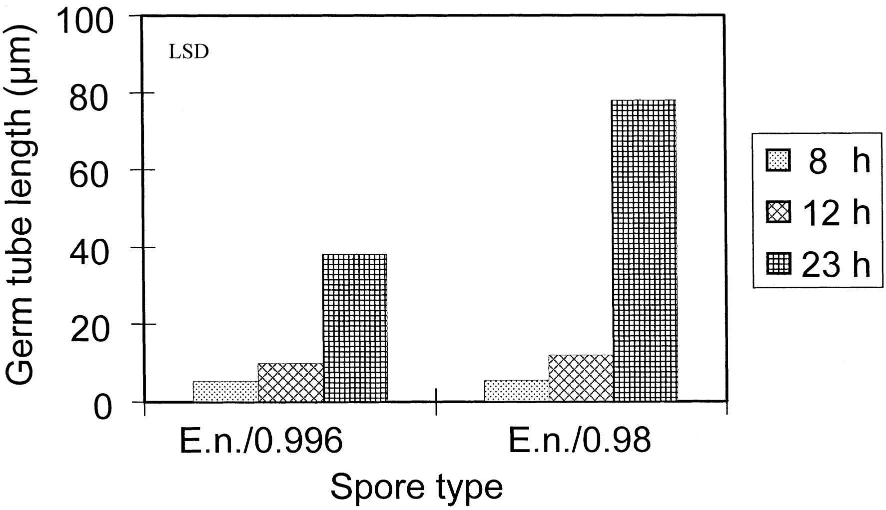

Fig. 9.6. Comparison of the temporal germ-tube extension of characterized spores of *Epicoccum nigrum* produced on unstressed (0.996 water activity) and stressed (0.98 water activity) media and germinated on water agar at 0.935 water activity. (From Pascual *et al.*, 1998.)

After germination, the next important phase is germ-tube extension and establishment, or appressorium formation for some entomogenous species. Detailed studies were made under the different water-stress regimes described previously, which demonstrated that germ-tube extension of modified conidia of *E. nigrum* and *U. atrum* could be significantly improved under water stress. Polyols such as glycerol can be a source of energy and utilized to promote subsequent growth and development. Indeed, in the case of *E. nigrum*, in some weak media, this improvement was maintained during *in vitro* mycelial colony development (Pascual, 1998). Figure 9.6 shows an example of the improved germ-tube extension observed with characterized conidia of *E. nigrum* obtained from colonies grown on low-a_w-stress media (Pascual, 1998).

Trehalose is an important sugar as it prevents damage by replacing water in dehydrated phospholipid membranes in yeasts and filamentous fungi. This inhibits transition of the liquid crystalline phase to the gel phase and in so doing preserves the cell membranes (Crowe *et al.*, 1984). This is critical in desiccation tolerance and maintaining the integrity of cell membranes during wetting and drying cycles. Such tolerance is also an important parameter, especially when BCAs are being formulated as a wettable powder. Studies by Jin *et al.* (1991) with *Trichoderma harzianum* demonstrated that, by manipulating the growth conditions with polyethylene glycol (PEG), the trehalose content of the conidia could be significantly increased and this enabled the inocula to survive desiccation better than control unmodified conidia. Although the PEG molecular weight and concentrations used were not detailed, this study pointed to the importance of considering this approach where desiccation tolerance is of critical importance. Trehalose may be more important in some groups of fungi than in others. For example, the non-xerotolerant BCA yeast *C. sake* accumulated trehalose rapidly (Teixido *et al.*, 1998a, b) and was implicated in the improved viability of cells at lowered a_w. Other elegant studies, by Jackson and Bothast (1990) and Schisler *et al.* (1991) showed that the C:N ratio influenced the relative accumulation of protein and lipid reserves in *Colletotrichum truncatum* spores, affecting biocontrol efficacy and the rate of germination. However, the C:N ratio did not affect *C. truncatum* desiccation tolerance. Jackson and Schisler (1992) and Jackson *et al.* (1997) have also pointed to variation in C:N ratios and limitation as a means of improving the desiccation tolerance of mycoherbicides (*C. truncatum*) and entomogenous species (*P. farinosus*). It needs to be recognized that the effect of ecophysiological manipulation on the endogenous accumulation of reserves varies with fungal species and groups, and that the type of modification must be appropriate for the needs. For example, if increased glycerol is required, solute stress modifications may be important, while C:N limitation and ratios may affect and increase trehalose accumulations in inocula.

Recent studies by Wilson and Lindow (1994a, b) with bacterial BCAs have also suggested that the competitiveness of individual strains could be estimated with the *in vitro* niche overlap index (NOI), derived from *in vitro* carbon utilization profiles (NOI is the proportion of the C compounds utilized by one species that is also utilized by the competing species). They hypothesized that the effectiveness of a strain as a preemptive biocontrol agent of epiphytic phytopathogenic bacteria was proportional to the ecological similarity between the BCA and the target pathogen in the phyllosphere of the host plant. They suggested that NOIs > 0.9 indicated that two competing species competed for and occupied the same niche, while those with < 0.9 occupied separate niches. NOIs have been used in studies to understand the interactions between mycotoxigenic *Fusarium* causing ear rot of maize grain and other mycoflora. Interestingly, it was found that the NOIs varied depending on both water availability and temperature (Marin *et al.*, 1998). However, practically no studies have examined whether manipulation of endogenous reserves might affect the NOIs of potential BCAs in relation to environmental factors. The first such study was conducted recently by Pascual (1998), who compared the NOIs of the peach pathogen *M. laxa* with those of the BCA *E. nigrum* grown on unstressed (0.996 a_w) and stressed media (0.98 a_w). The NOIs changed with water stress, suggesting that the relative niche occupation by the antagonist and the pathogen may vary and that the potential for niche exclusion will be influenced by the quality of the inoculum (the water-stress-tolerant and the unmodified strain). This suggests that endogenous modifications of the polyols and sugars can also modify competitiveness based on the NOI system suggested by Wilson and Lindow (1994b).

Improvements in Biocontrol in the Field

The critical component of this strategy is whether biocontrol can be either conserved or improved under a range of environmental factors. To this end, a series of studies were carried out to test the low-a_w-stress inocula described previously. With *B. bassiana* and *M. anisopliae*, tests were carried out using *Galleria* larvae in bioassay systems over a range of humidity regimes and temperatures not commonly used in the laboratory. Table 9.2 shows that at low relative humidity levels, regardless of temperature, the death of the larvae was improved by the modified inocula when compared with the efficacy of unmodified conidia produced on rich-nutrient media. The modified spore inocula were produced on low-water-stress media modified with glycerol, and com-

Table 9.2. Mean percentage mycosis of *Galleria mellonella* larvae over a range of equilibrium relative humidities (ERH), at 25°C. Larvae were inoculated with different characterized treatments. Cultures were grown on either SDA (control, unmodified treatment) or SDA modified with glycerol to 0.96 water activity with glycerol (glycerol treatment) at 25°C for 14 days. Least significant differences between treatments were: 9 days: 18.9, 24.5 and 24.1; 15 days: 22.8, 22.3 and 7.9 for *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus,* respectively. (Adapted from Hallsworth and Magan, 1994b.)

Treatment	Time (days)	100% ERH	86.5% ERH	78.2% ERH
B. bassiana				
Control	9	56.7	11.6	3.9
Glycerol	9	80.0	34.3	0
Control	15	100	11.6	3.9
Glycerol	15	96.9	58.6	0
M. anisopliae				
Control	9	66.7	23.4	0
Glycerol	9	96.5	89.5	70.0
Control	15	100	35.0	4.2
Glycerol	15	96.5	96.9	100
P. farinosus				
Control	9	76.7	48.7	0
Glycerol	9	90	89.5	83.4
Control	15	100	100	0
Glycerol	15	100	100	100

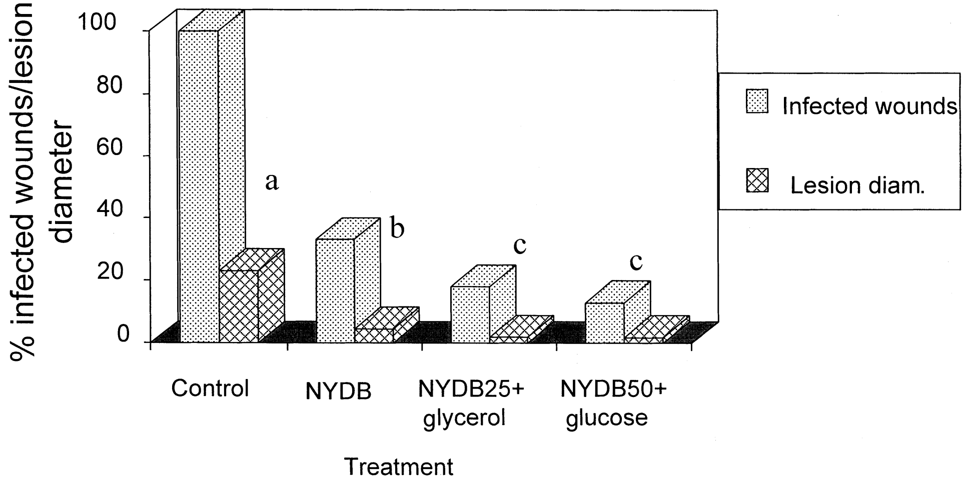

Fig. 9.7. The effect of growth medium on biocontrol capacity of *Candida sake* against postharvest *Penicillium* rot of apples. Key to treatments: NYDB, nutrient yeast dextrose broth; NYDB225+glycerol, 75% diluted medium modified with glycerol to 0.96 water activity; NYDB50+glucose, 50% diluted medium modified with glucose to 0.96 water activity. Treatments with different letters are statistically significant (*P* = 0.05). (From Teixido *et al.*, 1998b.)

pared with spores produced on an unmodified Sabourand dextrose agar (SDA) medium. This work has now been followed up by experiments with *B. bassiana* on leaves of whole plants, where some improvements in efficacy against aphids have been recorded (Anderson, 2000). Further whole-plant studies are needed to examine the potential for improvement in the control of insect pests, particularly under different fluctuating humidities in the leaf microclimate.

More recent studies with field spraying of different inocula of the yeast *C. sake* on apples in orchards prior to harvest and storage have demonstrated two important things. First, low-a_w-tolerant yeast cells grow better on the apple surface and such establishment can give equally good, if not better, postharvest control of *Penicillium* rot (Fig. 9.7) (Teixido *et al.*, 1998a, b, c). This work also suggested that the low-a_w-tolerant inocula could be applied at a lower concentration to obtain the same efficacy. Thus, although in some cases production of modified inocula may result in lower inoculum yields, this can be compensated for by the use of lower application rates. This could be important for the development of economic production, formulation and application systems for BCAs.

Application of *E. nigrum* inocula to peach twigs in the field has also demonstrated that better control of brown rot (*M. laxa*) could be achieved than that obtained with the unmodified inocula of *E. nigrum* or the fungicide captan. This BCA was also examined for postharvest control of *B. cinerea* on cherries under different storage humidity regimes. In this case good control was achieved at different relative humidities, but there were no statistically significant differences between inocula (Pascual *et al.*, 1999). Studies are still in progress with these inocula in field trials for assessing efficacy in the field.

Studies with *U. atrum* for control of *B. cinerea* suggests that manipulation of endogenous reserves can modify the establishment on leaf surfaces and affects the levels of biocontrol achieved (Frey and Magan, 1998). These studies, however, are still in progress and more detailed analyses of the data are needed for accurate interpretation of the results to be carried out.

The above sections have thus demonstrated that the four questions posed can indeed be answered positively, which suggests that potential does exist for this approach. It should be noted, however, that the examples presented are not numerous and practically no other groups have examined this approach for improving the ecological fitness of BCAs in the field.

Future Prospects for Improving the Commercialization of Ecologically Fit Inocula

The key final question that arises is whether this approach will be useful in formulation of BCAs for field use. Many field trials are at present carried out with freshly harvested inocula. This is unrealistic in the long term for commercial exploitation. Thus, both dry and liquid formulations need to be examined for long-term storage and viability. The question of conservation of biocontrol efficacy after long-term storage then also becomes important. A fundamental understanding of the endogenous changes and accumulations of polyols and sugars in inocula of BCAs can also enable information to be obtained on any modifications to internal water and solute potentials. This can be combined with exogenous additions of specific compounds or mixtures of compounds to maintain the concentrations in the inocula for conserving viability and for the long-term stability of formulations that have ecological competence. Studies in my laboratory are now focusing on these aspects, which are critical for the effective exploitation of BCAs.

Acknowledgements

I am very grateful to all my past and present research students and to collaborators for their commitment and support in this area of research: Dr J.E. Hallsworth, Dra S. Pascual, Dra N. Teixido, Dr M. Anderson, Dr S. Sancisi-Frey, Ms M. Abadias, and Dr T.M. Butt, Dr D. Chandler, Dra P. Melgarejo, Dra I. Vinas and Dr J. Usall, respectively.

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