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Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability

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The insect pathogens *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* can be effective biocontrol agents when relative humidity (RH) is close to 100%. At reduced water availability, germination of propagules, and therefore host infection, cannot occur. Cultures of *B. bassiana*, *M. anisopliae* and *P. farinosus* were grown under different conditions to obtain conidia with a modified polyol and trehalose content. Conidia with higher intracellular concentrations of glycerol and erythritol germinated both more quickly and at lower water activity (a_w) than those from other treatments. In contrast, conidia containing up to 235·7 mg trehalose g^{-1} germinated significantly ($P < 0·05$) more slowly than those with an equivalent polyol content but less trehalose, regardless of water availability. Conidia from control treatments did not germinate below 0·951–0·935 a_w (≡ 95·1–93·5% RH). In contrast, conidia containing up to 164·6 mg glycerol plus erythritol g^{-1} germinated down to 0·887 a_w (≡ 88·7% RH). These conidia germinated below the water availability at which mycelial growth ceases (0·930–0·920 a_w). Germ tube extension rates reflected the percentage germination of conidia, so the most rapid germ tube growth occurred after treatments which produced conidia containing the most glycerol and erythritol. This study shows for the first time that manipulating polyol content can extend the range of water availability over which fungal propagules can germinate. Physiological manipulation of conidia may improve biological control of insect pests in the field.

Keywords: conidia, germination, entomopathogenic fungi, polyols, water availability

INTRODUCTION

Although fungal pathogens have been used to control insect pests for more than 100 years (Ferron, 1985), pest control has been inadequate because high water availability is required for fungal germination (Walstad *et al.*, 1970; Doberski, 1981; Magan & Lacey, 1984; Gillespie & Crawford, 1986; Chandler *et al.*, 1994). There has been an ongoing research effort to optimize spore yield and improve formulation of inocula (Barnes *et al.*, 1975; Campbell *et al.*, 1978; Campbell *et al.*, 1983; Inch & Trinci, 1987; Im *et al.*, 1988; Humphreys *et al.*, 1989; Dorta *et al.*, 1990; Bateman *et al.*, 1993; Jenkins & Prior, 1993; Feng *et al.*, 1994). However, there has been little interest in the manipulation of spore physiology in order to enhance germination and growth at low water availability. Two recent studies were carried out to optimize polyol and trehalose accumulation in conidia (Hallsworth & Magan, 1994a, b), but germination of these propagules was not investigated.

The insect-pathogenic fungi (entomopathogens) *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* are potentially useful biological control agents. However, conidia of these species cannot normally germinate below a water activity (a_w) of 0·950 [≡ 95·0% relative humidity (RH)] (Gillespie & Crawford, 1986). Although germination, germ tube growth and therefore host penetration depend on high water availability, fungal growth within the host occurs independently of RH (Ferron, 1977). Periods when RH is close to 100% may only last for several hours in the field. As a result, successful host

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Abbreviations: a_w, water activity; RH, relative humidity; SDA, Sabouraud dextrose agar.

penetration is dependent on germination rate. Hassan *et al.* (1989) have shown that acceleration of germination enhances pathogenicity of entomopathogens towards the insect host.

Glycerol, erythritol, arabitol and mannitol accumulate in fungal cells at low a_w (Beever & Laracy, 1986; Hocking, 1986; Ellis *et al.*, 1991; Kelly & Budd, 1991; Van Eck *et al.*, 1993). Intracellular accumulation of these polyols reduces cytoplasmic a_w and yet does not disrupt enzyme structure and function, thus allowing metabolic activity to continue during periods of low water availability (Brown, 1978). Al-Hamdani & Cooke (1987) found that polyols accumulated in sclerotia (mycelial propagules) of the plant pathogen *Sclerotinia sclerotiorum*, when cultures were grown at reduced a_w. However, germination of modified sclerotia was not assessed on media of reduced water availability. Accumulations of glycerol and/or erythritol occurred in conidia of *B. bassiana*, *M. anisopliae* and *P. farinosus* if cultures were grown at $< 0{\cdot}970$ a_w or supplied with a high concentration of glycerol (Hallsworth & Magan, 1994a, b). Initial studies suggested that conidia modified in this way are more pathogenic at low RH than those containing only trace amounts of glycerol and erythritol (Hallsworth & Magan, 1994c).

The disaccharide trehalose protects membrane and protein structure during dehydration and upon rehydration (Crowe *et al.*, 1984; Carpenter & Crowe, 1988; Roser, 1991; Colaco *et al.*, 1992; Leslie *et al.*, 1994). Intracellular accumulation of trehalose has been associated with prolonged storage life of conidia of *Trichoderma harzianum* (Harman *et al.*, 1991), *Aspergillus japonicus* (Gornova *et al.*, 1992), *B. bassiana*, *M. anisopliae* and *P. farinosus* (Hallsworth & Magan, 1994c). Conidia of entomopathogens can contain > 200 mg trehalose g^{-1} when cultures are supplied with a high concentration of trehalose (Hallsworth & Magan, 1994b). However, the effect of trehalose accumulation on germination of fungal propagules at reduced a_w has not, to our knowledge, been determined.

This study was carried out to assess the germination of *B. bassiana*, *M. anisopliae* and *P. farinosus* conidia that contained increased concentrations of glycerol, erythritol or trehalose. Germination and germ tube extension were studied over a range of water availability as an indication of the biocontrol potential of modified conidia at reduced a_w.

METHODS

Production of conidia. The isolates of *B. bassiana*, *M. anisopliae* and *P. farinosus* that were used in this study have been described previously (Hallsworth & Magan, 1994b). Cultures were grown on Sabouraud dextrose agar (SDA), SDA + 114·8 g KCl l^{-1}, and media based on SDA but containing 141·4 g glycerol l^{-1} instead of glucose, and 378 g trehalose l^{-1} or trehalose + 54 g KCl l^{-1} instead of glucose; the water availabilities of these media were 0·998, 0·930, 0·952, 0·972 and 0·955 a_w respectively. KCl was added to the trehalose medium used to culture *M. anisopliae* because conidia of this species did not accumulate trehalose if cultures were grown at higher a_w (Hallsworth & Magan, 1994b). All media contained 21·3 g MES l^{-1} and the pH was adjusted by addition of 2·5 M NaOH, to give a final value of $5{\cdot}8 \pm 0{\cdot}3$. A pH stick (Gallenkamp) was used to measure the surface pH of media and the water availability was determined using a Novasina humidistat IC II. Petri plates were sealed with Parafilm and incubated for 30 d at 25 °C, without light.

Conidia were wet harvested (Hallsworth & Magan, 1994b) using either sterile AnalaR water (Merck), and then lyophilized prior to HPLC analysis, or sterile solutions of PEG, and then used for germination assessment. Polyol and trehalose analyses were carried out on conidia that were wet and dry harvested (Hallsworth & Magan, 1994b). The polyol and trehalose content of wet harvested conidia was not significantly different ($P > 0{\cdot}05$) from that of dry-harvested conidia. This suggests that samples were not contaminated with components of the media and that polyols and trehalose did not leak out of conidia during harvesting. Hassan *et al.* (1989) showed that exposure to water (1 a_w) prior to germination affects the germination rate of entomopathogenic spores. However, Matewele *et al.* (1994) harvested and washed *M. anisopliae* and *P. farinosus* blastospores in distilled water in preparation for germination assessment over a range of a_w and bioassays that were carried out at different RH values. The results of these germination and bioassay studies may therefore have been inaccurate. To eliminate this source of error, conidia were harvested in solutions of PEG at the same concentration as the medium on which the germination test was carried out. Bottles containing PEG solutions were cooled in a sterile cabinet without lids and care was taken to obtain the same water availability ($\pm 0{\cdot}001$ a_w at 25 °C) as that of the respective germination medium.

Determination of polyols and trehalose. Dry conidia (5 mg) were sonicated in AnalaR water, boiled and then filtered to extract polyols and trehalose (Hallsworth & Magan, 1994b). Extracts were injected onto a Dionex series 4500 HPLC fitted with a CarboPac PA1 column as described previously (Hallsworth & Magan, 1994b). Glycerol, erythritol, arabitol, mannitol and trehalose were quantified by comparing peak areas of sample profiles with those from standard solutions of known concentration. The limits of detection were 1·6 μg ml^{-1} for polyols and 2·8 μg ml^{-1} for trehalose. The analyses were carried out in triplicate.

Assessment of germination. Germination media were based on SDA, but with 30% the normal nutrient concentration, 21·3 g MES l^{-1} and (per l): 35·5 g PEG 600; 221 g PEG 600; 266 g PEG 600; 266 g PEG 600 + 29 g PEG 200; or 266 g PEG 600 + 90 g PEG 200; their pH values were adjusted as described above, and the water availabilities were 0·989, 0·951, 0·935, 0·923 and 0·887 a_w, respectively. Lids were not placed on the Petri plates until the surface water had evaporated from the media, and a_w was determined after this. Gillespie & Crawford (1986) and Chandler *et al.* (1994) incorporated glycerol into germination media to reduce the water availability. Glycerol can act as an exogenous source of compatible solute that enters the cell and reduces the physiological impact of low water availability. In studies of bacterial spore germination, Anagnostopoulos & Sidhu (1981) and Botha & Holzapfel (1988) found that glycerol favoured germination at reduced a_w relative to NaCl. This suggests that exogenous glycerol can enhance germination of microbial propagules at reduced a_w. In the present study, the water availability of germination media was modified with PEG in order to avoid this problem.

Germination media were inoculated with conidia from each treatment by spreading 350 μl conidial suspension over the surface using a glass rod. Petri plates were sealed with Parafilm before incubation (25 °C, without light). Two 9 mm discs of medium were removed per replicate after 11, 14, 18, 20, 25, 30,

35, 44, 52, 61, 73, 84, 102, 120, 150, 193 and 240 h, stained with lactophenol cotton blue and percentage germination and germ tube length were assessed under the light microscope (Magan, 1988). Conidia with germ tubes longer than their width or diameter were considered to have germinated. To estimate the percentage germination, a total of 300 conidia were examined from each treatment (100 per replicate), and 25 germinated conidia from each replicate plate were randomly chosen for measurement of germ tube length. Experiments were carried out in triplicate. The sample time was identified when germination of conidia from the most vigorous treatment had reached its maximum value. At this time the percentage germination and germ tube length of conidia from each of the four treatments was recorded.

RESULTS AND DISCUSSION

Germination of conidia with enhanced glycerol and erythritol content

The water availability of the control medium (SDA) was higher than that of all other growth media. Conidia from SDA + KCl, glycerol, trehalose and trehalose + KCl treatments contained more glycerol and erythritol (but not mannitol) than those from control treatments (Table 1). Although mannitol is a compatible solute, only glycerol and erythritol accumulate in significant amounts in conidia produced at reduced a_w (Hallsworth & Magan, 1994a, b). The lower M_r polyols glycerol and erythritol can be more effective than mannitol in osmotic adjustment. The water availability of saturated solutions of glycerol or erythritol is lower ($\leqslant$ 0·914 a_w at 25 °C) than that of mannitol (0·978 a_w at 25 °C) (Hallsworth & Magan, 1994b). Even a saturated solution of mannitol, therefore, would not reduce intracellular water availability to < 0·978 a_w (unless other compounds that significantly reduce a_w were present).

Conidia from glycerol treatments contained more glycerol and erythritol than those from all other treatments, and conidia from trehalose treatments contained more trehalose than those from other treatments. These results are similar to those obtained previously (Hallsworth & Magan, 1994b). Conidia of each species from SDA + KCl and glycerol treatments contained significantly ($P < 0·05$) more glycerol and erythritol than those from control treatments. These conidia were associated with high germination rates and rapid germ tube growth relative to those from controls, both at high a_w (0·989 a_w; *B. bassiana* and *M. anisopliae*) and reduced a_w (0·887–0·951; all three species) (Table 2). Conidia from control treatments (containing < 6·5 mg glycerol + erythritol g^{-1}) did not germinate below 0·935 (*M. anisopliae*) or 0·951 a_w (*B. bassiana* and *P. farinosus*). In contrast, conidia with an increased glycerol plus erythritol content (up to 164·6 mg g^{-1}) germinated on media as dry as 0·887 a_w (*M. anisopliae*), 0·923 a_w (*P. farinosus*) and 0·935 a_w (*B. bassiana*). At 0·887 a_w, 19·7% of *M. anisopliae* conidia had germi-

Table 1. Polyol and trehalose content of conidia

Values are means of triplicate HPLC analyses. Cultures were grown on SDA, SDA + 114·8 g KCl l^{-1}, and media based on SDA but containing 141·4 g glycerol l^{-1} instead of glucose, and 378 g trehalose l^{-1} or trehalose + 54 g KCl l^{-1} instead of glucose, and incubated for 30 d. The water availabilities of the media were 0·998, 0·930, 0·952, 0·972 and 0·955 a_w, respectively. Least significant differences (LSD) ($P < 0·05$) between values from different treatments are shown for each species.

Species and treatment	Intracellular concn (mg g^{-1}) of:				
	Glycerol	Erythritol	Arabitol	Mannitol	Trehalose
B. bassiana					
Control (SDA)	0·93	3·13	0·57	24·70	23·23
SDA + KCl	2·13	12·47	4·27	30·33	1·90
Glycerol	53·43	111·20	3·07	6·47	4·07
Trehalose	4·47	13·63	1·73	19·47	146·33
LSD	6·08	11·82	2·26	4·63	9·44
M. anisopliae					
Control (SDA)	2·33	3·93	0·93	85·87	4·67
SDA + KCl	6·53	21·27	10·93	31·03	0·35
Glycerol	85·77	22·40	3·07	21·80	0·17
Trehalose + KCl	6·07	16·90	3·13	9·27	235·67
LSD	5·92	4·35	1·52	6·43	11·26
P. farinosus					
Control (SDA)	0·67	4·30	2·80	26·33	15·27
SDA + KCl	8·10	24·03	12·30	22·83	5·97
Glycerol	28·27	65·80	5·13	13·33	2·73
Trehalose	5·03	15·67	9·10	15·33	196·67
LSD	3·67	9·47	1·35	3·88	15·54

Table 2. **Germination of conidia over a range of water availability**

Values represent mean percentage germination of conidia from triplicate cultures ($n = 3 \times 100$ conidia). Conidia from different treatments (see Table 1) were incubated on media over a range of water availability. Germination media were based on SDA with 30% nutrient concentration, plus (per l): 35·5 g PEG 600; 221 g PEG 600; 266 g PEG 600; 266 g PEG 600 + 29 g PEG 200; 266 g PEG 600 + 90 g PEG 200. The water availabilities of the media were 0·989, 0·951, 0·935, 0·923 and 0·887 a_w, respectively. Least significant differences (LSD) ($P < 0·05$) between values from different treatments are shown for each species.

Species and treatment		Percentage germination				
	a_w...	0·989	0·951	0·935	0·923	0·887
B. bassiana	**Incubation time (h)...**	**14**	**44**	**61**	**240**	**240**
Control (SDA)		76·33	5·67	0*	0*	0*
SDA + KCl		96·33	59·67	38·00	0*	0*
Glycerol		92·00	87·33	51·00	0*	0*
Trehalose		90·33	16·67	0*	0*	0*
LSD		15·10	13·39	8·24		
M. anisopliae	**Incubation time (h)...**	**14**	**25**	**44**	**120**	**240**
Control (SDA)		0	0	0	0*	0*
SDA + KCl		81·33	77·00	49·67	0*	0*
Glycerol		92·67	61·33	51·33	69·00	19·67
Trehalose + KCl		2·33	0	0	0*	0*
LSD		7·50	10·72	7·58		
P. farinosus	**Incubation time (h)...**	**35**	**61**	**84**	**150**	**240**
Control (SDA)		47·33	0	0*	0*	0*
SDA + KCl		43·00	42·67	10·00	0*	0*
Glycerol		80·00	86·67	48·67	17·67	0*
Trehalose		4·00	0	0*	0*	0*
LSD		22·92	8·01	6·34		

* No conidia from these treatments had germinated by 240 h.

nated by 240 h. Mycelial growth does not occur at such a low a_w, even if glycerol is supplied in the medium (Gillespie & Crawford, 1986). Although germination of *M. anisopliae* conidia at 0·887 a_w did not reach a maximum until after 193 h, conidia that are already metabolically active may grow rapidly during any short period of increased water availability. In contrast, conidia from control treatments may be unable to respond quickly enough to transient increases in water availability in the field (e.g. during dew formation). These conidia will fail to germinate and grow sufficiently to penetrate the host. Several studies have shown that a rapid germination rate correlates with a high pathogenicity (Al-Aidroos & Roberts, 1978; Hassan *et al.*, 1989; Samuels *et al.*, 1989).

Modified sclerotia of *S. sclerotiorum* germinated more quickly than those from control treatments at high a_w (0·990–0·999) (Al-Hamdani & Cooke, 1987). However, it was not possible to distinguish between effects of different polyols because modified sclerotia accumulated both mannitol and lower M_r polyols. In contrast, the conidia of *B. bassiana*, *M. anisopliae* and *P. farinosus* that contained increased amounts of glycerol and erythritol did not contain more mannitol than those from control treatments. As a result, it was possible to identify the effects of the low M_r polyols glycerol and erythritol on germination in the present study.

Generally, germ tube extension was proportional to the percentage germination, regardless of water availability and fungal species (Table 3). Although the germination rate of *B. bassiana* conidia was high in all treatments at 0·989 a_w by 14 h (> 75%), germ tube growth was significantly ($P < 0·05$) greater for conidia from SDA + KCl and glycerol treatments than for those from other treatments. That is, rapid germ tube growth was only associated with treatments where conidia contained high concentrations of low M_r polyols.

Table 3. Germ tube extension over a range of water availability

Values represent mean germ tube length (μm) of conidia from triplicate cultures ($n = 3 \times 25$ germ tubes). Conidia from different treatments (see Table 1) were incubated on media over a range of water availability. Germination media were based on SDA with 30% nutrient concentration, plus PEG 600 or PEG 600 + PEG 200 to reduce the a_w (see Table 2). The water availabilities of the media were 0·989, 0·951, 0·935, 0·923 and 0·887 a_w, respectively. Least significant differences (LSD) ($P < 0·05$) between values from different treatments are shown for each species.

Species and treatment		Germ tube length (μm)				
	a_w...	0·989	0·951	0·935	0·923	0·887
B. bassiana	**Incubation time (h)...**	**14**	**44**	**61**	**240**	**240**
Control (SDA)		24·92	8·28	0*	0*	0*
SDA + KCl		82·24	51·70	10·56	0*	0*
Glycerol		61·20	60·48	15·96	0*	0*
Trehalose		35·32	12·80	0*	0*	0*
LSD		15·43	9·11	4·60		
M. anisopliae	**Incubation time (h)...**	**14**	**25**	**44**	**120**	**240**
Control (SDA)		0	0	0	0*	0*
SDA + KCl		44·37	48·30	33·73	0*	0*
Glycerol		41·24	38·30	18·56	38·92	6·64
Trehalose + KCl		21·44	0	0	0*	0*
LSD		16·15	15·10	9·85		
P. farinosus	**Incubation time (h)...**	**35**	**61**	**84**	**150**	**240**
Control (SDA)		31·28	0	0*	0*	0*
SDA + KCl		17·32	69·08	9·48	0*	0*
Glycerol		102·52	78·12	23·68	19·48	0*
Trehalose		8·92	0	0*	0*	0*
LSD		13·60	21·77	7·88		

* No conidia from these treatments had germinated by 240 h.

Germination of conidia with enhanced trehalose content

The water availability of media containing trehalose (0·972 a_w) or trehalose + KCl (0·955 a_w) was lower than that of SDA (0·998 a_w). Conidia from the trehalose and trehalose + KCl treatments contained significantly more ($P < 0·05$) glycerol and erythritol than those from SDA, significantly less ($P < 0·05$) than those from glycerol, but similar quantities to those from SDA + KCl treatments. Despite this, germination and germ tube extension of conidia from trehalose or trehalose + KCl treatments was slower than that of conidia from both control and SDA + KCl treatments. Furthermore, conidia from trehalose or trehalose + KCl treatments failed to germinate at such low a_w as those from SDA + KCl treatments. Substantial quantities of trehalose in conidia appeared to reduce the beneficial effect of low M_r polyols during germination at reduced a_w. It may therefore be better to maintain viability and improve shelf life of inocula by using low temperature storage and oil formulation (Bateman *et al.*, 1993) than by increasing the trehalose content of conidia. Germination of conidia formulated in oil and stored for more than one year is slower and less vigorous than that of conidia before storage (Moore *et al.*, 1995). Manipulation of intracellular polyols to accelerate germination may therefore be important when considering formulation, mode of storage and shelf life of inocula.

Conclusions

Conditions under which spore production is optimal (Barnes *et al.*, 1975; Campbell *et al.*, 1978, 1983; Inch & Trinci, 1987; Im *et al.*, 1988; Humphreys *et al.*, 1989; Dorta *et al.*, 1990; Jenkins & Prior, 1993) usually differ from those that result in a desirable polyol or trehalose content (Hallsworth & Magan, 1994a, b). The emphasis on maximization of yield might have resulted in the production of poor quality inoculum in the past. Previous

studies have shown that (i) fungal control of insect pests has been inadequate because propagules cannot normally germinate and grow at low RH (e.g. Doberski, 1981; Chandler *et al.*, 1994), and (ii) acceleration of germination enhances pathogenicity (e.g. Hassan *et al.*, 1989). Furthermore, laboratory studies suggest that conidia containing high concentrations of glycerol and erythritol cause insect mortality more rapidly and at lower RH than normal propagules (Hallsworth & Magan, 1994c). Manipulation of intracellular polyols to accelerate germination and allow germination at low water availability may therefore improve the pathogenicity of fungal biocontrol agents in the field. Intracellular accumulation of specific polyols and trehalose might also enhance the survival of fungal propagules during cryopreservation.

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